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Research paper

Innate immune response profiles in pigs injected with vaccine adjuvants polydi(sodium carboxylatoethylphenoxy)phosphazene (PCEP) and Emulsigen

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ABSTRACT

Vaccines are formulated with adjuvants to enhance or direct antigen-specific immune responses against pathogens. However, the mechanisms of action (MOA) of adjuvants are not well understood and are under-investigated in large animal species. We have previously reported that injection of mice induced innate immune responses as indicated by increased cell recruitment and cytokine production at the site of injection with polyphosphazene (PCEP) adjuvant. In the present study, we evaluated whether PCEP induced similar innate immune responses in pigs. Piglets were injected with either PCEP or Emulsigen intradermally (I.D.) and the local cellular infiltration and cytokine production were evaluated at the site of injection and the draining lymph nodes. PCEP induced infiltration of macrophages, T and B cells, leucocytes and necrotic debris at the site of injection as well as PCEP-induced leucocyte infiltration in the draining lymph nodes. Emulsigen induced diffuse infiltration of leucocytes, macrophages, and lymphocytes at the site of injection as well as at the draining lymph nodes. PCEP induced significant production of interleukin IL-1β, and IL-13 at the site of injection and IL-1β, and IL-6 at the draining lymph nodes. Emulsigen promoted the production of IL-1β, IL-6, and IL-12 at the site of injection but not in the draining lymph nodes. No cytokines were detected in blood after injection of either adjuvant. Together, our data indicate that in pigs, the adjuvants PCEP and Emulsigen stimulate early innate immune responses at the injection site by creating an immunocompetent environment that may contribute to increased immunogenicity of the co-administered antigens.

1. Introduction

Effective adjuvants enhance the immunogenicity of highly purified or recombinant antigens but despite being used for decades in vaccines, the mechanisms of action (MOA) of many adjuvants remain unknown. This lack of clarity regarding adjuvant MOA has limited development and approval of new adjuvants for human use (Calabro et al., 2011). Vaccination with antigen alone often triggers little or no specific immune responses unless formulated with an adjuvant that activates the innate immune responses (Calabro et al., 2011; Levitz and Golenbock, 2012; Liang and Loré, 2016; McKee et al., 2009; Pasquale et al., 2015; Schijns, 2002; Wang and Singh, 2011). Studies in mice indicate that vaccine adjuvants enhance immunogenicity of antigen by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection (Gupta and Chaphalkar, 2015; Kanzler et al., 2007; Milligan, 2014).

We previously reported that poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP), a high-molecular weight, water-soluble polymer, enhanced long-lasting immune responses when co-administered with a variety of viral and bacterial antigens in mice, pigs and cattle (Awate et al., 2012; Eng et al., 2010; Magiri et al., 2018a; McNeal et al., 1999; Mutwiri et al., 2008, 2007). We recently reported on PCEP MOA in a series of studies. In mice, PCEP induced recruitment of immune cells to the site of injection and promoted transport of antigen to the draining lymph node (Awate et al., 2014b; Dupuis et al., 2001). The cells recruited following intramuscular (i.m.) injection of PCEP to the site of injection and the draining lymph nodes in mice constituted largely of neutrophils but also macrophages, CD4 + T cells, CD8 + T cells and CD19 + B cells, monocytes and DCs (Awate et al., 2014b). Further, PCEP promoted cytokine and chemokine

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production as well as regulation of a number of 'adjuvant core response genes' at the intramuscular injection site in mice and pigs (Awate et al., 2012; Magiri et al., 2016; Mutwiri et al., 2008). Specifically, studies with mice and pigs revealed species-specific differences in polyphosphazenes induced stimulation of innate immune responses (Awate et al., 2012; Magiri et al., 2016, 2018b). Intramuscular injection of PCEP induced time-dependent changes in the gene expression of many "adjuvant core response genes" (Mosca et al., 2008) such as chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 in mice (Awate et al., 2012) and CCL2 and CXCL10 (but not CCL- 5) in pigs (Magiri et al., 2016). Major transcription factor NF-*k*B gene and the inflammatory cytokine TNF-α genes were up-regulated in response to PCEP in mice (Awate et al., 2012) but not in pigs (Magiri et al., 2016). Additionally, in pigs, PCEP induced IL-6 gene expression but not IL-10, IL-17 or IFN-α (Magiri et al., 2016 PCEP injection in mice increased the expression of TLR4 and TLR9 at the site of injection (Awate et al., 2012) whereas PCEP did not induce significant expression any of the TLR genes in pigs suggesting differences in activation of immune responses in different animal species (Magiri et al., 2016). However, across species, PCEP may modulate antigen-specific immune responses by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection. By promoting the induction of transient innate immune responses, adjuvants may promote antigen-specific immunity.

Very little is known about MOA of adjuvants in pigs. We recently reported that PCEP induced the expression of chemokines and proinflammatory cytokines genes when injected intradermally in pigs (Magiri et al., 2016) suggesting that PCEP may promote recruitment of immune cells at the site of injection. Further unlike what was observed in mice, PCEP did not induce significant expression of any of the TLR genes in pigs suggesting species-specific differences in activation of innate immune responses (Magiri et al., 2016). We hypothesize that PCEP induces an innate immune response at injection sites and thus creates an immune microenvironment to facilitate vaccine triggered adaptive immunity. In the current study, we investigated whether PCEP activates cytokine production and recruitment of immune cells in pigs by evaluating changes at the site of injection and the draining lymph nodes. Emulsigen, a known commercial adjuvant for pig vaccines, was included for comparative purposes.

2. Materials and methods

2.1. Animal experiments

The animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhere to the Canadian Council on Animal Care guidelines for humane animal use. Groups consisted of 2 control groups (naïve, unimmunized animals and pigs injected with PBS) and 2 treatment groups (pigs injected with PCEP or emulsigen).The animals were 3–4 four weeks old commercial cross breed pigs (20 animals per group with n = 5 animals euthanized at 4 specific time points (Days 1, 4, 7 and 14)) were administered intradermal (I.D.) injection at the subcutis on the neck region left and right side (250 µl per side) with sterile phosphate-buffered saline (PBS), 500 µg PCEP, or 20% Emulsigen and an additional group without any injection as naive. The body temperature, and clinical observations & score for local reactions in all groups at both injections sites were taken through out the study period. The local reaction scores were from 0 to 3 with 0 = normal, 1 = minor, 2 = moderate & 3 = severe. Aditionally, markings of injection site were reapplied every day so that they can be visible at all time point. An 8 mm skin punch plug biopsy and the corresponding draining lymph nodes were collected from five animals per group at 1, 4, 7, and 14 days post injection. Biopsies were treated with 10% formalin for histopathology or incubated with RPMI supplemented with 5% FBS for tissue homogenisation and subsequent cytometric bead assay. Fifty microliters of peripheral blood was collected into serum separation tubes (SST) for cytokines cytometric bead assay at each time point in the serum.

2.2. Adjuvants preparation

PCEP was synthesized by the Idaho National Laboratory (Idaho Falls, ID, USA) using methods previously described in (Andrianov et al., 2004; Mutwiri et al., 2007) and its endotoxin level was determined to be less than 0.034 ng/ml as assessed by the Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA). PCEP and Emulsigen an "oil-in-water" formulation (MVP Laboratories, OM, USA) were dissolved in endotoxin-free, sterile PBS, pH 7.4 (Life Technologies, Carlsbad, CA, USA) prior to injection.

2.3. Histopathology preparation

Skin punch biopsy and the draining lymph nodes tissues were prepared for histopathological evaluations as follows: Tissues were fixed with 10% formalin, then incubated for 24 h in 70% ethanol, 100% ethanol and finally 100% xylol. Tissues were then embedded in paraffin block for easier sectioning in the microtome and sliced to 3–5 µm thickness. Slides were stained using standard Haematoxylin-eosin method following the standard operating procedures. Histopathological changes of the skin that were evaluated included granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation. Histopathological changes in the draining lymph nodes included granuloma formation and suppurative inflammation. Scoring was performed by a pathologist blinded to the treatment and the scoring was as follows: A) No pathological changes = 0, B) Patchy pathological changes = 1, C) Moderate pathological changes = 2, D) Severe pathological changes = 3 as described previously by (Magiri et al., 2018a; Mikalsen et al., 2012).

2.4. Cytometric bead assay

Cytokine concentrations were assayed in sera as well as from tissue homogenates from the injection site and the draining lymph nodes. Skin punch biopsy and draining lymph nodes were homogenized in 1 ml PBS with protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) with 2.3 mm Zirconia silica microbeads (Biospec Products Inc., Bartlesville, OK, USA) in a mini-beadbeater™ (Biospec Products, Inc.) via six 10 s pulses interspersed with cooling at 4 °C. The homogenates were centrifuged at 20,000 ×g for 10 min at 4 °C, and supernatants stored at −20 °C before analysis. Cytokine levels were measured by cytometric bead assay (BioRad) according to the manufacturer's recommendations.

Bioplex cytokine assays: Bioplex bead coupling was performed as per the manufacturer's instructions. The reagents used are described in Table 1. BioPlex cytokine assays antibodies against porcine IFN-α, IFN-γ, IL-1-β, IL-6, IL-8, IL-10, IL-12, IL-13, and IL-17 A were coupled to BioRad multiplex assay microsphere beads using the BioRad BioPlex following manufacturer's instructions. Sera and tissue homogenates were tested for IFN-α, IFN-γ, IL-1-β, IL-6, IL-8, IL-10, IL-12, IL-13, and IL-17 A secretion. The multiplexassay was carried out in a 96 well Grener Bio-One Fluotrac 200 96Fblack (VWR, #82050-754), which allows washing and retention of the Luminex beads. The bead sets conjugated with all the cytokine to be analyzed were vortexed for 30 s followed by sonication for another 30 s to ensure total bead dispersal. The bead density was adjusted to 1200 beads per µl in PBSA (1x PBS + 1% BSA (Sigma-Aldrich) + 0.05% sodium azide (Sigma-Aldrich), pH 7.4) and 1 µl of each bead set was added to 49 µl of the PBSA + 1% New Zealand Pig Serum (Sigma-Aldrich P3484) + 0.05% sodium azide (Sigma-Aldrich) which was then added to each well. Plates ware washed using the Bio-Plex Pro II Wash Station (Bio-Rad) to allow soaking for 20 s then washing with 100 µl PBSA. The porcine cytokine protein standards were added to the wells at 50 µl per well at a

Table 1
List of antibodies, bead, detection (secondary antibodies) and suppliers.

Cytokine standards		Standard concentration range
Cytokine	Supplier/ Catalog number	(pg/ml)
rPorc IFN alpha	Genentech	200-1 pg/mL
rPorc IFN gamma	CG	2000-8 pg/mL
rPorc IL-1β	R&D 681PI010 (10 μg)	5000-20 pg/mL
rPorc IL-6	R&D 686PI025 (25 μg)	5000-20 pg/mL
rPorc IL-8	Kingfisher RP0109S-005 (5 μg)	200-1 pg/mL
rPorc IL-10	Invitrogen PSC0104 (10 μg)	5000-20 pg/mL
rPorc IL-12	R&D 912PL025 (25 ug)	5000-20 pg/mL
rPorc IL-13	Kingfisher RP0007S-005 (5 μg)	5000-20 pg/mL
rPorc IL-17A	Kingfisher RP0128S-005 (5 μg)	2000-8 pg/mL
Coating antibodies to Coupled beads		
Cytokine	**Catalog number**	**Bead region (all from BioRad)**
MAb anti IFN alpha	GeneTex GTX11408	45 (BioRad MC10045-01)
MAb anti porc IFN gamma	Fisher PIMP700	43 (BioRad MC10043-01)
MAb anti porc IL-1b/IF2	R&D MAB6811	26 (BioRad MC10026-01)
Goat anti porcine IL-6	R&D AF686	65 (BioRad MC10065-01)
MAb anti sheep IL8 (86.9% homology)	AbD Serotec MCA1660	27 (BioRad MC10027-01)
MAb anti swine IL10	Invitrogen ASC0104	28 (BioRad MC10028-01)
Porc IL-12	MAb anti porc IL12	36 (BioRad MC10036-01)
Goat anti swine IL-13	Kingfisher PB0094S-100	52 (BioRad MC10052-01)
Rabbit anti porcine IL-17A	Kingfisher KP0498S-100	62 (BioRad MC10062-01)
Detection antibodies (all biotinylated)		
Cytokine	**Catalog number**	**Desired starting conc (ug/ml)**
MAb anti pig IFN alpha (biotin in house)	R&D 27105-1	1/5000
Rabbit anti porc IFN gamma (biotin in house)	Fisher PIPP700	1/400
Goat anti porc IL-1b/IF2 biotin	R&D BAF681 (50 μg)	0.5 ug/mL
Goat anti porcine IL-6 biotin	R&D BAF686 (50 μg)	0.5 ug/mL
MAb anti porc CXCL8/IL8 (biotin in house)	R&D MAB5351 (500 μg)	1/500
MAb anti swine IL10 biotin	Invitrogen ASC9109	0.5 ug/mL for cell sups, 1 ug/mL for sera
MAb anti porc IL12/IL23 p40 biotin	R&D BAM9122	0.5 ug/mL
Goat anti swine IL-13 biotin	Kingfisher PBB0096S-050	0.5 ug/mL
Rabbit anti porcine IL-17 A biotin	Kingfisher KPB0499S-050	0.5 ug/mL

final concentration as described in Table 1 below and the serum (prediluted 1:4) and tissue homogenate (injection sites and draining lymph nodes; prediluted 1:2) were added to the wells at 50 μl per well. After sealing the plates with plate sealer (Thermo Fisher Scientific, #232,702), the plates were agitated at 800 rpm for 1 h at room temperature. After 1 h incubation with serum, the plates were washed using the Bio-Plex Pro II Wash Station (Bio-Rad; soak 30 s, wash with 150 μl PBS plus 1% Tween-20). A 50 μl volume of biotin cocktail consisting of biotinylated porcine antibodies (as described in Table 1) was added to each well. The plates were again sealed, covered and agitated at 800 rpm for 30 min at room temperature then washed again as described above. A 50 μl of Streptavidin RPE (Cedarlane PJRS20; diluted to 5 μg/ml) was added to each well. The plate was again sealed, covered and agitated at 800 rpm for 30 min at room temperature and washed as indicated above. A 100 μl of 1xTris-EDTA (TE buffer-10 mM Tris, bring to pH 8.0 with HCl and 1 mM EDTA) was added to each well and then the plate was vortexed for 5 min before reading on the Luminex100 xMAP™ instrument following the manufacturer's instructions). The fluorescence on the beads was read on a BioRad BioPlex 200 reader (60 μl volume, 50 beads per region).

2.5. Data analysis

Statistical analyses were performed using Graph-Pad Prism 7 software (GraphPad Software, San Diego, CA, USA). Differences between groups were identified using a non-parametric Kruskal-Wallis test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between the two adjuvants and PBS control relative to each time point. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$ which are stated in the text.

3. Results

3.1. Histopathological changes of the skin and draining lymph nodes after I.D. Injection with PCEP and Emulsigen

Pigs were injected I.D. with PBS, PCEP or Emulsigen. Body temperature was taken and injection sites were examined for gross changes. In all the animals, body temperatures remained normal throughout the study period suggesting that the adjuvants did not induce a systemic response (data not shown). The local inflammatory reaction did not induce gross ulceration at the site of injection as observed macroscopically (data not shown).

Sites of injection and draining lymph nodes were excised and evaluated for evidence of an inflammatory response and cytokine production. We evaluated changes in histopathology including granuloma formation (microscopic aggregation of macrophages transformed into epithelium-like cells surrounded by a collar of mononuclear leucocytes, principally lymphocytes, and plasma cells), lymphocytes infiltration (infiltration of tissue by T and B lymphocytes), epithelial necrosis (death of cells and living tissues), and suppurative inflammation (forms as a result of the action of polymorphonuclear leucocytes e.g. neutrophils) (Fig. 1). Representative photomicrographs show a typical cellular infiltrate observed in the skin at the site of injection (Fig. 1 left) and the draining lymph nodes (Fig. 1 right) on day 14 post-injection. The top panel shows normal skin (Fig. 1A) or draining lymph nodes (Fig. 1B) after injection with PBS at 200x magnification. In the skin PCEP (Fig. 1C) induced multifocal to coalescing inflammation and a mixture of macrophages (blue arrow), lymphocytes (yellow arrow) and some neutrophils (red arrow) with much necrotic debris in the inflammation. In the draining lymph nodes, PCEP (Fig. 1D) induced multifocal infiltration of macrophages, with multinucleated giant cells (blue arrow) and sparse neutrophils (red arrow). In the skin, Emulsigen (Fig. 1E) induced diffuse infiltration of inflammatory cells, composed of

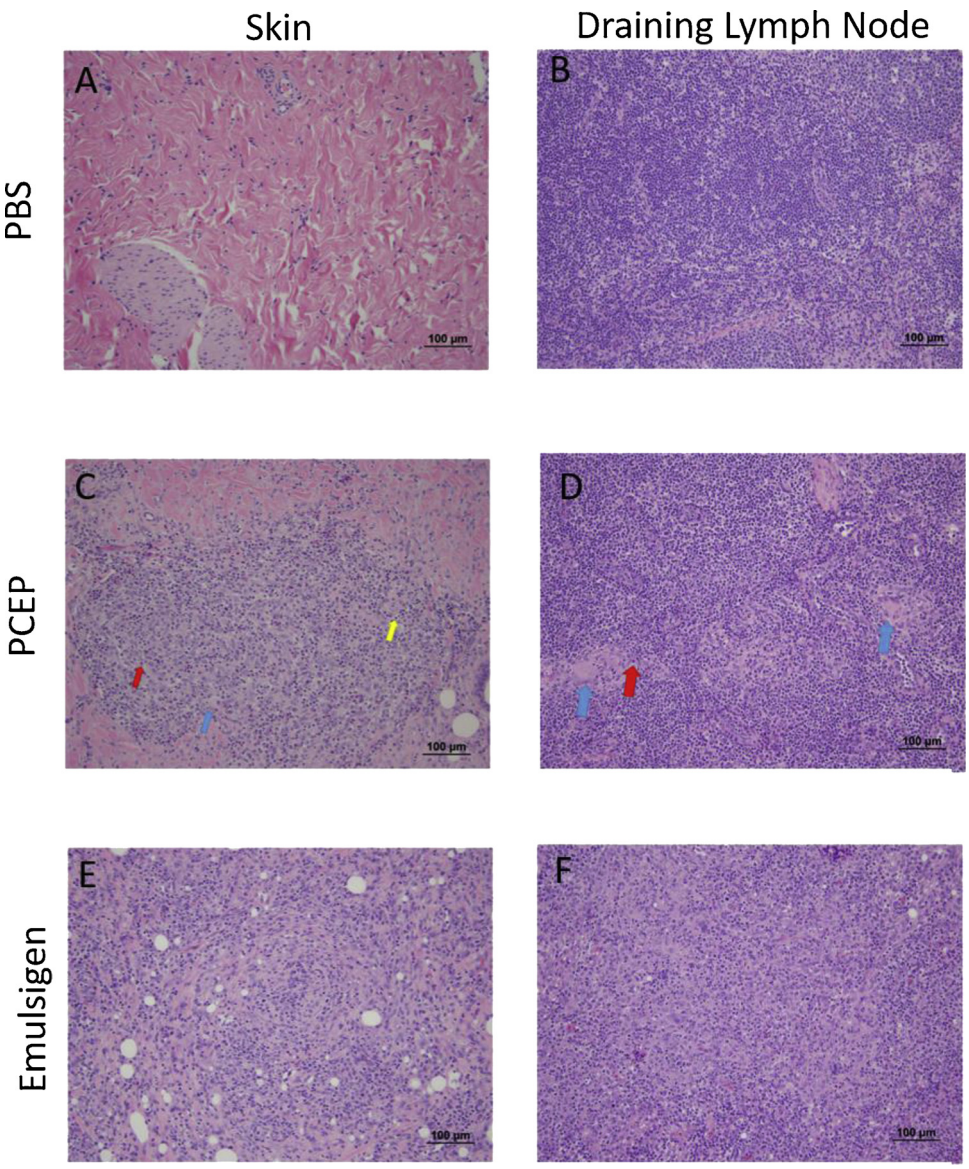

Fig. 1. Representative images of the cellular infiltrate at the injection site of pig skin and draining lymph nodes after PCEP or Emulsigen intradermal injection. Representative skin histology images (200x magnification) are shown after injection with PBS (A), PCEP (C) and Emulsigen (E). PCEP (B) induced multifocal to coalescing inflammation and a mixture of macrophages (blue arrow), lymphocytes (yellow arrow) and some neutrophils (red arrow) with many necrotic debris in the inflammation. Emulsigen (E) induced diffuse infiltration of inflammatory cells, composed of macrophages, lymphocytes and small granuloma. Representative histology images of the draining lymph nodes (200x magnification) are shown after injection with PBS (B), PCEP (D) and Emulsigen (F). PCEP (D) induced multifocal infiltration of macrophages, with multinucleated giant cells (blue arrow) and sparse neutrophils (red arrow) while Emulsigen (F) induced coalescing areas of granulomatous inflammation and inflammation is composed of mostly macrophages. For each site, 5 biological replicates were assessed per group per time point (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

macrophages and lymphocytes and small granuloma. In the draining lymph nodes, Emulsigen (Fig. 1F) induced coalescing areas of granulomatous inflammation and inflammation is composed of mostly macrophages.

At the site of injection, adjuvants induced significant granuloma formation, lymphocytes infiltration, suppurative inflammation and necrosis compared to the skin of pigs injected with PBS as a control. Interestingly, no granulomas were observed until after 14 days with pigs injected with Emulsigen ($p < 0.05$) and PCEP ($p < 0.01$) showing significant granuloma formation at the injection site relative to time-matched PBS control animals (Fig. 2A). Further, pigs injected with PCEP showed significant lymphocytic infiltration 4 ($p < 0.05$) and 14 days ($p < 0.01$) post administration relative to the PBS control pigs (Fig. 2B). Pigs injected with Emulsigen showed significant lymphocytic infiltration 14 days ($p < 0.05$) post administration (Fig. 2B). PCEP and Emulsigen induced significant tissue necrosis 1, 4 and 7 days post administration ($p < 0.01$, $p < 0.01$, $p < 0.01$ for PCEP); $p < 0.05$, $p < 0.05$, $p < 0.01$ for Emulsigen, respectively) at the site of injection relative to time-matched PBS controls (Fig. 2C). However, signs of necrosis were resolved by 14 days suggesting a rapid resolution of the tissue reaction. PCEP induced significant suppurative inflammation with predominant eosinophil and neutrophil infiltration for 1 ($p < 0.01$), 4 ($p < 0.05$), 7 ($p < 0.01$) and 14 ($p < 0.01$) days post injection relative to the PBS controls (Fig. 2D). Emulsigen induced significant suppurative inflammation on days 1 ($p < 0.05$), 4 ($p < 0.01$) and 7 ($p < 0.05$) post-injection (Fig. 2D).

In the draining lymph nodes, Emulsigen induced significant granuloma formation at day 4 ($p < 0.05$) and day 14 ($p < 0.05$) and PCEP induced significant granuloma formation at 7 days ($p < 0.05$) post injection relative to the PBS control animals (Fig. 2E). Finally, PCEP induced significant suppurative inflammation in the draining lymph nodes 1 day post-injection relative to lymph nodes from PBS control animals ($p < 0.05$) but the response was not significantly different from controls at other time points suggesting a rapid resolution of the inflammation (Fig. 2F). PCEP and Emulsigen induced histopathological changes in skin and draining lymph nodes relative to pigs with PBS as a control. Collectively, these data show the ability of adjuvants to promote local inflammation, which may be important for the initiation of the innate and acquired immune response.

3.2. Cytokine profile in the pig skin, draining lymph nodes and peripheral blood after I.D. Injection with PCEP and Emulsigen

Punch biopsies were obtained from the site of intradermal injection

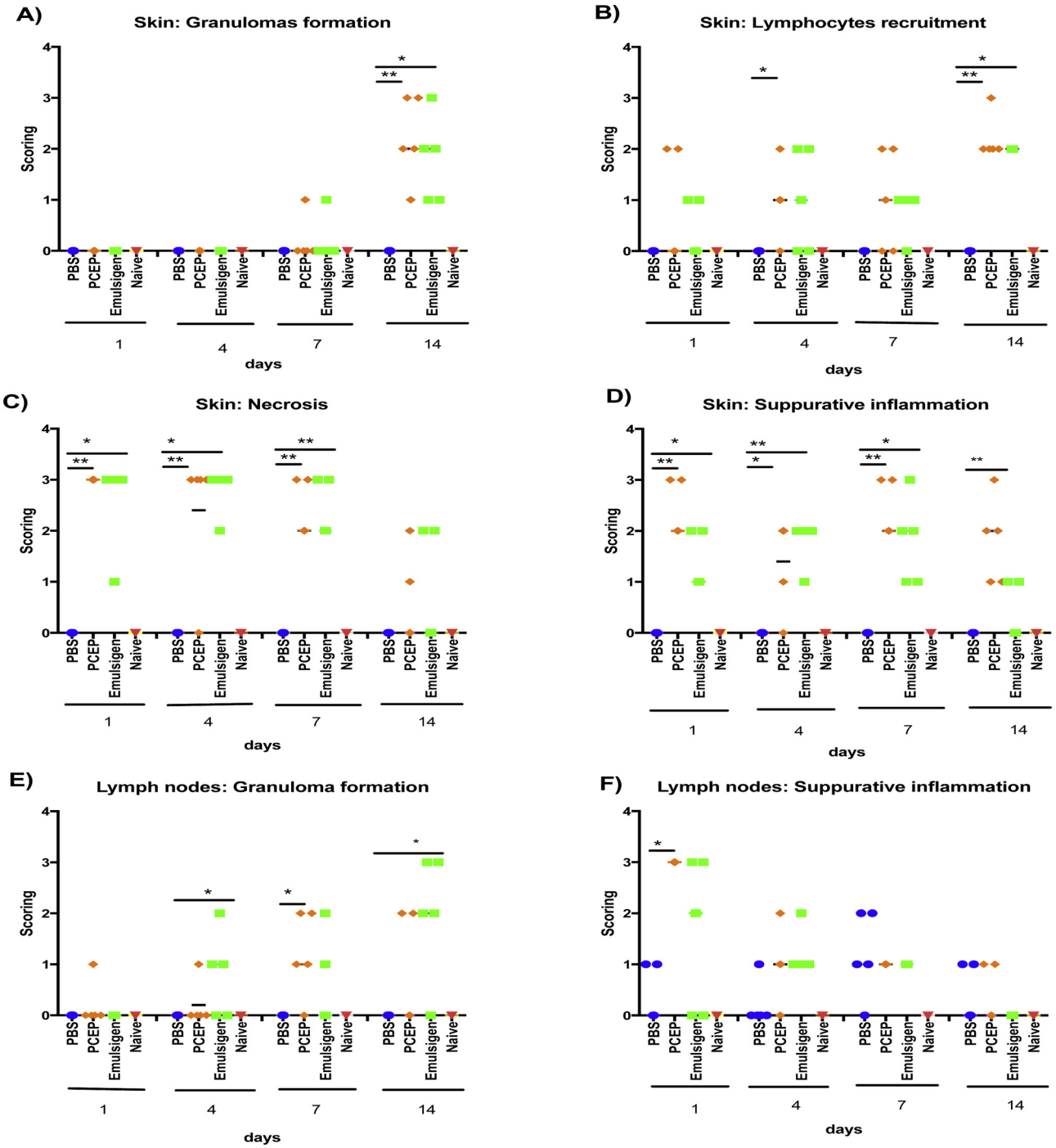

Fig. 2. The histopathological changes of in the skin and draining lymph nodes in response to intradermal injection of PCEP or Emulsigen. The site of injection at the skin and the draining lymph nodes were collected from the draining lymph nodes at 1, 4, 7, and 14 days post injection. The lines indicate the median value of five replicates at each time point. All tissues are compared to time-matched skin or lymph nodes tissues from PBS-injected control animals. Histopathological changes in the skin included assessment of granuloma formation, lymphocyte recruitment, necrosis, and signs of inflammation whereas histopathology of draining lymph nodes included assessment of granuloma formation and suppurative inflammation. Scoring was performed by pathologist blinded to sample identification on 5 biological replicates per site per group.

was collected after 1, 4, 7 and 14 days in pigs inoculated with PBS, PCEP and Emulsigen. Cytometric bead cytokine analysis was performed on homogenates of the biopsies. Emulsigen triggered significantly increased production of IL-6 ($p < 0.05$; Fig. 3D) and IL1β ($p < 0.05$; Fig. 3C) after 4 days and of IL-12 ($p < 0.05$; Fig. 3G) after 7 days. PCEP triggered increased local production of IL1β ($p < 0.01$; Fig. 3C) and IL-13 ($p < 0.01$; Fig. 3H) after 4 days relative to the control skin tissue. These data indicate that despite the evidence of microscopic histopathological evidence at the site of injection in response to PCEP and Emulsigen, only acute inflammatory cytokines IL-6 (Emulsigen only) and IL1β (PCEP and Emulsigen) were transiently induced at the site of injection in pigs 4 days post-injection. Neither of the adjuvants triggered local production of IFN-α (Fig. 3A), IFN-γ (Fig. 3B), IL-8 (Fig. 3E), IL-10 (Fig. 3F) or IL-17 A (Fig. 3I) relative to the biopsies from the PBS injection sites.

When cytokine analysis was assessed at the draining lymph nodes,

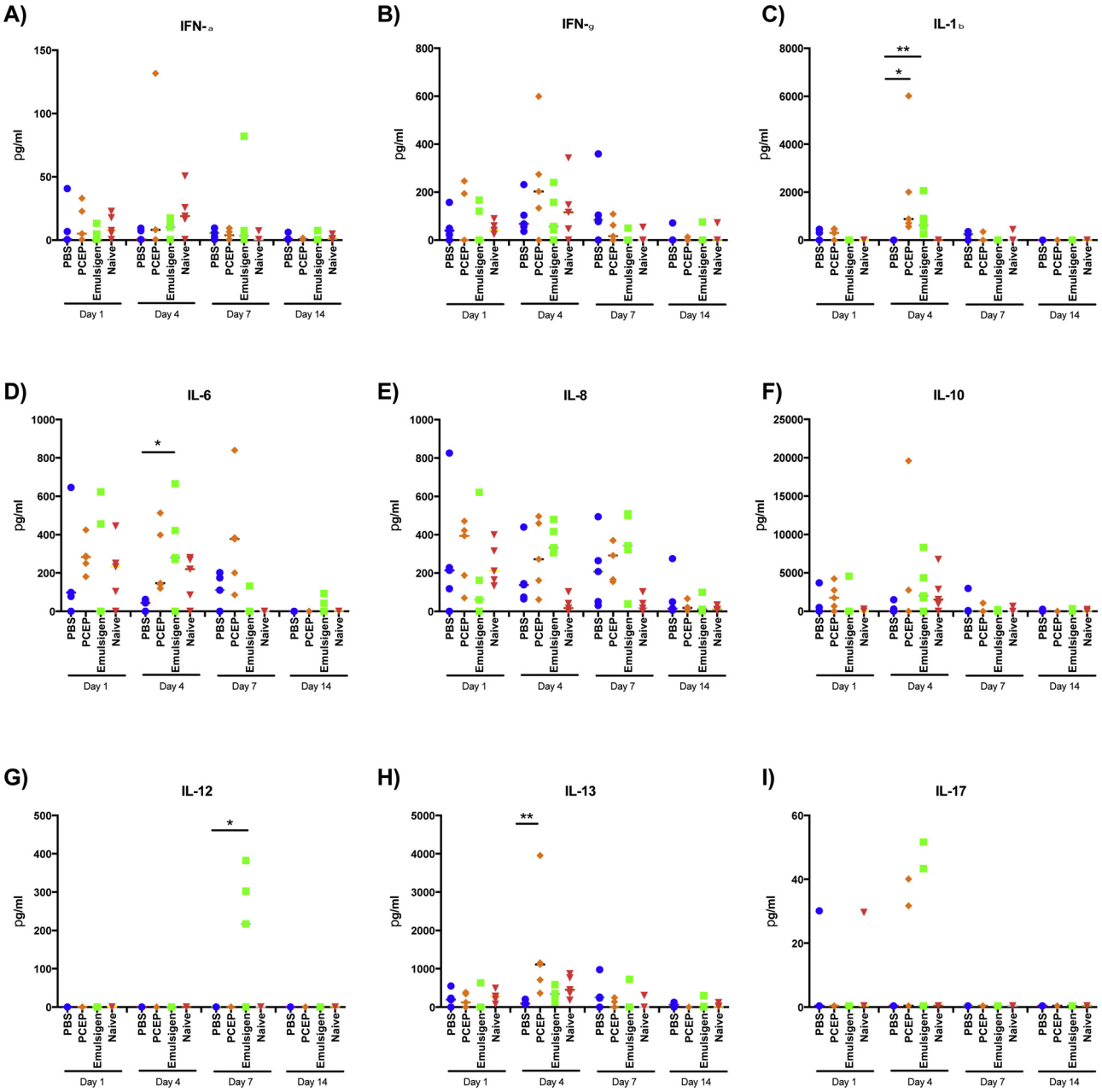

Fig. 3. Cytokine responses in the skin at the injection site. The injection site were collected from one of the sites at 1, 4, 7, and 14 days post injection incubated with RPMI supplemented with 5% FBS for tissue homogenisation in PBS and cytometric bead assay using BioPlex analysis. Results shown are the mean ± SD five biological replicates at each time point compared to PBS.

PCEP induced secretion of interleukin IL-1β ($p < 0.01$; Fig. 4A) and IL-6 ($p < 0.01$; Fig. 4B) on day 1 post-injection but this elevation of these returned to baseline levels by day 4 post-injection (Fig. 4). Further, Emulsigen did not induce secretion of IFN-α, IFN-γ (data not shown), IL-8 (Fig. 4C), IL-10 (Fig. 4D), IL-12 (Fig. 4E), IL-13 (Fig. 4F) or IL-17 A (Fig. 4G) at the draining lymph nodes over time.

Finally, serum cytokine profiles were assessed over time in pigs I.D. injected with PCEP, Emulsigen and PBS. None of the immunostimulants resulted in elevated production of serum IFN-α, IL-8, IFN-γ, IL-17 A, IL-6, IL1β, IL-10, IL-12 or IL-13 (Data not shown) indicating that the effects of the PCEP and Emulsigen as immunostimulants were observed locally but not systemically.

4. Discussion

Vaccination continues to be a very important public health tool in the control of infectious diseases in the world. The main goal of vaccination is to stimulate potent immunological responses which promotes protection against specific pathogens (Kaech et al., 2002). It has been reported that immunostimulatory adjuvants can direct innate and adaptive immune responses by promoting DC maturation and the concomitant release of pro-inflammatory cytokines (Liang and Loré, 2016; Mizumoto et al., 2005). Together, these effects significantly impact the overall efficacy of vaccines when immunostimulatory adjuvants are included (Coler et al., 2011). Our study attempts to elucidate the impact that immunostimulants PCEP and Emulsigen have at sites of injection and draining lymph nodes in pigs over a two-week period when compare to PBS. However, injection of PBS intradermal

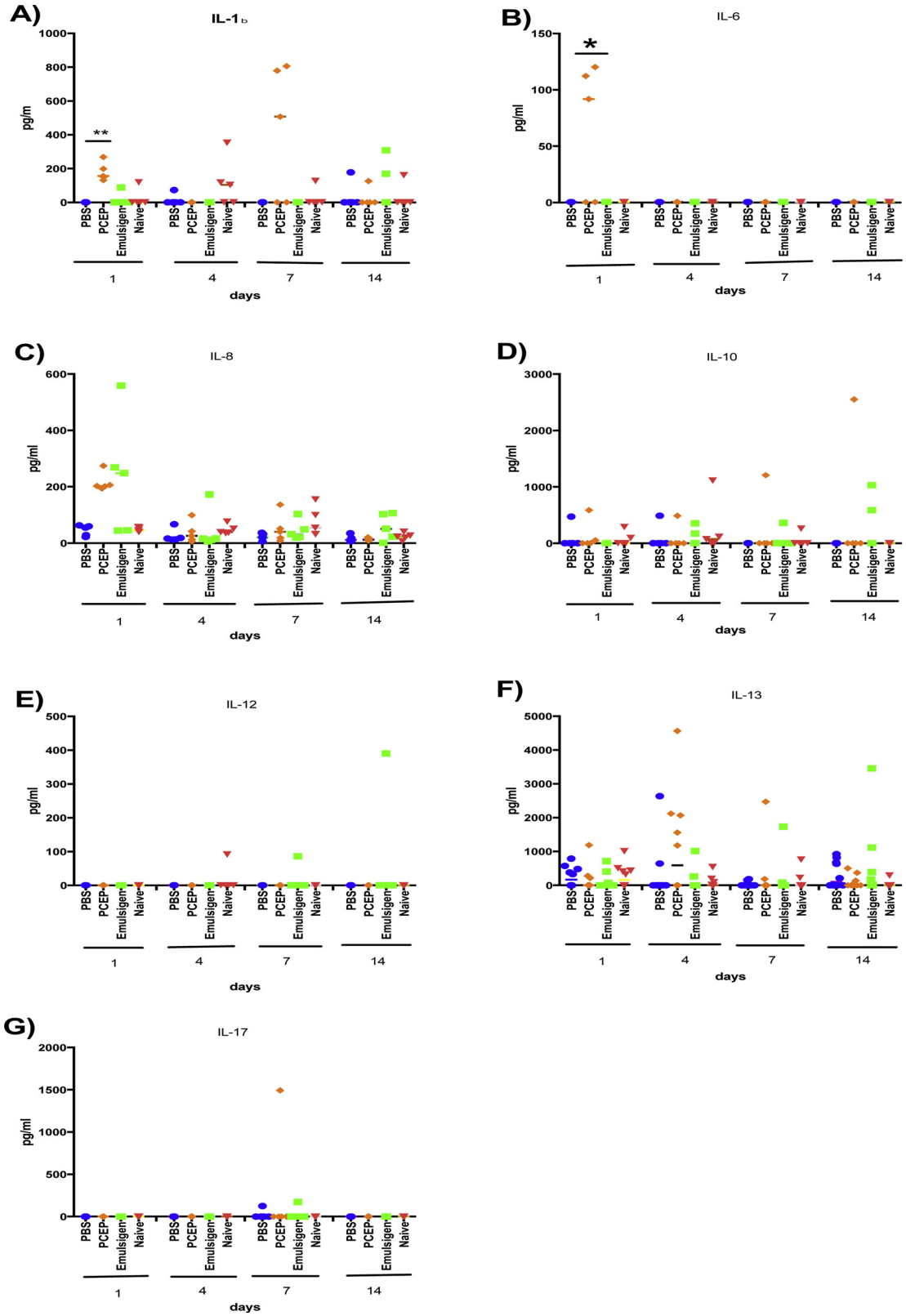

Fig. 4. Cytokine responses in lymph nodes draining the injection site. The lymph nodes were collected from one of the sites at 1, 4, 7, and 14 days post injection incubated with RPMI supplemented with 5% FBS for tissue homogenisation in PBS and cytometric bead assay using BioPlex analysis. Results shown are the mean ± SD five biological replicates at each time point compared to PBS.

and/or the volume injected may be irritating to the skin causing some local tissue response that might mask the immune responses to our injected adjuvants. The impact on the innate immune and inflammatory response is instructive of how the adjuvants will influence the immune response when used as part of a vaccine.

We selected the I.D. route of administration for our analysis because it is a common route for administration of vaccines in livestock (Zhang et al., 2017) and recently, we showed that I.D. administration of a

H1N1 vaccine which included PCEP as adjuvant gave a superior immune responses compared to pigs immunized with the same vaccine but administered via the intramuscular route (Magiri et al., 2018a). Skin-associated lymphoid tissue consists mainly of Langerhans cells and dermal antigen-presenting cells which circulate between the skin and the lymph nodes (DeBenedictis et al., 2001; Ray and Gately, 1996). As detailed in Bertho et al., 2011, skin dendritic cells at a semi-mature stage in pigs spontaneously migrate in lymph without microbial stimulation. These cells can be further activated upon microbial exposure or PAMP stimulation such as inactivated influenza antigen and LPS, respectively(Bertho et al., 2011). Some reports suggest that I.D. vaccines induce superior protective immune responses and required less antigen relative to vaccines administered I.M. or subcutaneously (Herbert et al., 1989; Itzchak et al., 1992; Magiri et al., 2018a). Overall, our results showed that PCEP and Emulsigen induced a strong inflammatory response at the site of injection and the draining lymph node, suggesting that inflammation may be critically important for the initiation of the innate immunity and the promotion of acquired immunity.

We investigated histopathological changes such as granuloma formation with the presence of macrophages, lymphocytes infiltration with T and B cells, epithelial necrosis, and suppurative inflammation (characterized predominantly as neutrophils and eosinophils). Interestingly, no (gross) macroscopic changes were observed for either adjuvant but PCEP induced significant macrophages infiltration, lymphocyte infiltration, leucocyte infiltration and necrotic debris at the site of injection and the draining lymph nodes whereas Emulsigen induced diffuse infiltration of leucocytes, macrophages, and lymphocytes at both sites. PCEP-induced signs of necrosis were resolved after 14 days post-injection of the immunostimulants. These results are consistent with results from other authors who have described adjuvants induce a local microscopic inflammatory reaction at an injection site (Calabro et al., 2011; Schijns, 2000) without gross macroscopic ulcerations (das Dores Moreira et al., 2009). Early stages of inflammation can occur through the activation of pattern-recognition receptors on many cells including lymphocytes, granulocytes, and endothelial cells (Faure et al., 2001; Muzio et al., 2000). Inflammatory responses induced by tissue necrosis at the site of injection by adjuvants was advanced by (Matzinger, 1994) in his “danger signal” hypothesis. Danger signals from damaged cells can trigger activation of the immune system through activation of inflammasome leading to release of pro-inflammatory cytokine such as IL-1β (Kool, Pétrilli et al. 2008).. Molecules associated with tissue damage such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates, and cytokines are released at the injection site due to tissue damage (Shi et al., 2003). These non-infectious damage signals have now been named damage-associated molecular patterns (DAMPs) to distinguish them from pathogen-associated molecular patterns (PAMPs). Particulate adjuvants cause local tissue damage and cell death at the injection site and release of DAMPs thereby activating inflammasome (Kool et al., 2008). In addition, many adjuvants induce the release of pro-inflammatory cytokines at the site of injection including PCEP (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). These damage signals trigger non-specific activation of the innate immune system, subsequently stimulating adaptive immunity. Recently inflammasomes have been one of the most widely investigated topics due to their potential role in adjuvant activity.

The role of neutrophils in adjuvant activity is not completely understood but increased neutrophils at the site of injection may attract other immune cells due to chemokine release ultimately resulting in increased antigen transport to the draining lymph nodes (Calabro et al., 2011; Morel et al., 2011).

In agreement with the current findings, PCEP injection was shown to lead to robust local infiltration by T and B lymphocyte in mice (Awate et al., 2014). Studies in mice have shown that PCEP is a potent inducer of cell recruitment at the injection site with lymphocytes, neutrophils and macrophages being the most abundant cells, followed by monocytes and DCs in mice (Awate et al., 2014b).

Although the adjuvants PCEP and Emulsigen are able to influence the immune response, their mechanism of action that can differentiate the intensity or duration of the required immune response may not be conserved. Thus, we also evaluated the cytokine profile to clarify how early events relate to precise immune response to adjuvants. Many adjuvants induce the release of pro-inflammatory cytokines at the site of injection in mice (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). Other studies have shown the ability of oil-in-water emulsion AS03 to co-localize with antigen to trigger colony-stimulating factor 3 (CSF3) and IL-6, and leukocyte- recruiting chemokines CCL2, CCL3, and CCL5 at the site of injection (Morel et al., 2011) and draining lymph nodes (Morel et al., 2011). Studies in mice and pigs showed that PCEP induced strong expression of adjuvant core response genes coding for multiple cytokines and chemokines and immunomodulatory proteins (Awate et al., 2012; Magiri et al., 2016). In the current study, we observed that PCEP and Emulsigen induced secretion of proinflammatory cytokines acute inflammatory cytokines IL-1β and IL-6 (Emulsigen only). IL-6 is a proinflammatory cytokine that is involved in the initiation of an immune response and it also promotes the differentiation of B cells (Helle et al., 1988a, b; Hilbert et al., 1989; Houssiau et al., 1988). IL1β cytokine is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation and differentiation, and apoptosis. IL1β cytokine is proteolytically processed to its active form by caspase 1 activating multiprotein inflammasomes which assemble in the cytoplasm of cells. Activated caspase-1 cleaves the pro-forms of the interleukin-1 cytokine family members leading to their activation and secretion of the active form of IL1β (Bennouna et al., 2003; Brereton et al., 2011; Gris et al., 2010; Latz, 2010). Intramuscular injection of PCEP induces NLRP3, an inflammasome receptor gene, and inflammatory cytokines, including IL-1β and IL-18, in mouse muscle tissue (Awate et al., 2012). Caspase-1 is required for the processing of pro-forms of IL-1β and IL-18 into their mature forms and is a critical constituent of the NLRP3 inflammasome (Sagulenko et al., 2013). in vitro stimulation of enriched splenic DCs with PCEP led to the secretion of pro-inflammatory cytokines IL-1β and IL-18 in a caspase-1-dependent manner (Awate et al., 2014a). PCEP and Emulsigen-induced inflammasome activation may play an important role in activating innate immunity thus contributing to adjuvant activity in pigs. Results from in vitro experiments with murine cells indicate that PCEP stimulated the significant production of the innate immune response cytokines IL-12 and IFN-γ (Garlapati et al., 2011; Mutwiri et al., 2008). Activated DCs produce cytokines such as IL-12 which stimulates the development of T helper cells that produce IFN-γ and promote cell-mediated immunity (Th1 cells). These results in mice are in contrast to our findings in pigs wherein we observed transient induction of IL-6 and IL-1β at draining lymph nodes. At the skin, PCEP induced transient expression of IL-1β as well as IL-13 a mediator of allergic inflammation and its secreted by many cells including T helper type 2 (Th2) cells (Cocks et al., 1993; Wynn, 2003).

Overall, our data suggest that PCEP and Emulsigen induced cell recruitment which culminated in adjuvant-specific cytokine profiles. These changes may be important in establishing and integrating the immune-competent environment favorable for antigen processing, presentation, and subsequent stimulation of antigen-specific immune responses. However, further studies are still needed to evaluate and identify which cell types are essential to induce the appropriate response for any given pathogen by characterising T and B cells. The mechanism of action of vaccine adjuvants must be fully elucidated in vivo to make clear how different cell types cooperate in establishing an integrated immunocompetent environment (Mosca et al., 2008). This information will be critical for the design of new effective vaccines.

Even so, our data suggest that activation of innate immune responses may be important in the induction of antigen specific immune

response after an immunization. As with Emulsigen, PCEP induced acute but not chronic inflammatory responses at the injection site and the draining lymph nodes when injected into pigs indicating that this adjuvant may be regarded as safe for use in pigs. We speculate that induction of innate immune responses at the site of injection may be an important mechanism through which adjuvant PCEP exert its adjuvant activity.

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References

Andrianov, A.K., Svirkin, Y.Y., LeGolvan, M.P., 2004. Synthesis and biologically relevant properties of polyphosphazene polyacids. Biomacromolecules 5, 1999–2006.
Awate, S., Wilson, H.L., Lai, K., Babiuk, L.A., Mutwiri, G., 2012. Activation of adjuvant core response genes by the novel adjuvant PCEP. Mol. Immunol. 51, 292–303.
Awate, S., Eng, N.F., Gerdts, V., Babiuk, L.A., Mutwiri, G., 2014a. Caspase-1 Dependent IL-1β Secretion and Antigen-Specific T-Cell Activation by the Novel Adjuvant, PCEP. Vaccines 2 (3), 500–514.
Awate, S., Wilson, H.L., Singh, B., Babiuk, L.A., Mutwiri, G., 2014b. The adjuvant PCEP induces recruitment of myeloid and lymphoid cells at the injection site and draining lymph node. Vaccine 32, 2420–2427.
Bennouna, S., Bliss, S.K., Curiel, T.J., Denkers, E.Y., 2003. Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. J. Immunol. 171, 6052–6058.
Bertho, N., Marquet, F., Pascale, F., Kang, C., Bonneau, M., Schwartz-Cornil, I., 2011. Steady state pig dendritic cells migrating in skin draining pseudo-afferent lymph are semi-mature. Vet. Immunol. Immunopathol. 144 (3-4), 430–436.
Brereton, C.F., Sutton, C.E., Ross, P.J., Iwakura, Y., Pizza, M., Rappuoli, R., Lavelle, E.C., Mills, K.H., 2011. Escherichia coli heat-labile enterotoxin promotes protective Th17 responses against infection by driving innate IL-1 and IL-23 production. J. Immunol., 1003789.
Calabro, S., Tortoli, M., Baudner, B.C., Pacitto, A., Cortese, M., O'Hagan, D.T., De Gregorio, E., Seubert, A., Wack, A., 2011. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 29, 1812–1823.
Cocks, B.G., de Waal Malefyt, R., Galizzi, J.P., de Vries, J.E., Aversa, G., 1993. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. Internat Immunol 5, 657–663.
Coler, R.N., Bertholet, S., Moutaftsi, M., Guderian, J.A., Windish, H.P., Baldwin, S.L., Laughlin, E.M., Duthie, M.S., Fox, C.B., Carter, D., 2011. Development and characterization of synthetic glucopyranosyl lipid adjuvant system as a vaccine adjuvant. PLoS One 6, e16333.
das Dores Moreira, N., Giunchetti, R.C., Carneiro, C.M., Vitoriano-Souza, J., Roatt, B.M., Malaquias, L.C.C., Corrêa-Oliveira, R., Reis, A.B., 2009. Histological study of cell migration in the dermis of hamsters after immunisation with two different vaccines against visceral leishmaniasis. Vet. Immunol. Immunopathol. 128, 418–424.
DeBenedictis, C., Joubeh, S., Zhang, G., Barria, M., Ghohestani, R.F., 2001. Immune functions of the skin. Clinic Dermatol 19, 573–585.
Didierlaurent, A.M., Morel, S., Lockman, L., Giannini, S.L., Bisteau, M., Carlsen, H., Kielland, A., Vosters, O., Vanderheyde, N., Schiavetti, F., 2009. AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J. Immunol. 183, 6186–6197.
Dupuis, M., Denis-Mize, K., LaBarbara, A., Peters, W., Charo, I.F., McDonald, D.M., Ott, G., 2001. Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. Eur. J. Immunol. 31, 2910–2918.
Eng, N.F., Garlapati, S., Gerdts, V., Potter, A., Babiuk, L.A., Mutwiri, G.K., 2010. The potential of polyphosphazenes for delivery of vaccine antigens and immunotherapeutic agents. Curr. Drug Deliv. 7, 13–20.
Faure, E., Thomas, L., Xu, H., Medvedev, A.E., Equils, O., Arditi, M., 2001. Bacterial lipopolysaccharide and IFN-γ induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-κB activation. J. Immunol. 166, 2018–2024.
Garlapati, S., Eng, N.F., Kiros, T.G., Kindrachuk, J., Mutwiri, G.K., Hancock, R.E., Halperin, S.A., Potter, A.A., Babiuk, L.A., Gerdts, V., 2011. Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces protective immunity against pertussis. Vaccine 29, 6540–6548.
Gris, D., Ye, Z., Iocca, H.A., Wen, H., Craven, R.R., Gris, P., Huang, M., Schneider, M., Miller, S.D., Ting, J.P.-Y., 2010. NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. J. Immunol., 0904145.
Gupta, A., Chaphalkar, S.R., 2015. Vaccine adjuvants: the current necessity of life. Shiraz E-Medical J 16.
Helle, M., Boeije, L., Aarden, L.A., 1988a. Functional discrimination between interleukin 6 and interleukin 1. Eur. J. Immunol. 18, 1535–1540.
Helle, M., Brakenhoff, J.P., De Groot, E.R., Aarden, L.A., 1988b. Interleukin 6 is involved in interleukin 1-induced activities. Eur. J. Immunol. 18, 957–959.
Herbert, M., Butler, A., Roome, A., Caul, E., 1989. Comparison of intradermal and intramuscular hepatitis B vaccination in university students. Vaccine 7, 395–396.
Hilbert, D.M., Cancro, M.P., Scherle, P.A., Nordan, R.P., Van Snick, J., Gerhard, W., Rudikoff, S., 1989. T cell derived IL-6 is differentially required for antigen-specific antibody secretion by primary and secondary B cells. J. Immunol. 143, 4019–4024.
Houssiau, F.A., Coulie, P.G., Olive, D., Van Snick, J., 1988. Synergistic activation of human T cells by interleukin 1 and interleukin 6. Eur. J. Immunol. 18, 653–656.
Itzchak, S., Jacob, B., Avraham, R., Ben-Ami, P., 1992. Enhancement of the immune response by intradermal vaccination in cattle with enterotoxigenic Escherichia coli (ETEC) vaccine without adjuvant. Vaccine 10, 217–220.
Kaech, S.M., Wherry, E.J., Ahmed, R., 2002. Effector and memory T-cell differentiation: implications for vaccine development. Nat. Rev. Immunol. 2, 251.
Kanzler, H., Barrat, F.J., Hessel, E.M., Coffman, R.L., 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat. Med. 13, 552.
Kool, M., Pétrilli, V., De Smedt, T., Rolaz, A., Hammad, H., Van Nimwegen, M., Bergen, I.M., Castillo, R., Lambrecht, B.N., Tschopp, J., 2008. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J. Immunol. 181, 3755–3759.
Latz, E., 2010. The inflammasomes: mechanisms of activation and function. Curr. Opin. Immunol. 22, 28–33.
Levitz, S.M., Golenbock, D.T., 2012. Beyond empiricism: informing vaccine development through innate immunity research. Cell 148, 1284–1292.
Liang, F., Loré, K., 2016. Local innate immune responses in the vaccine adjuvant-injected muscle. Clin. Transl. Immunol. 5.
Magiri, R., Lai, K., Chaffey, A., Wilson, H., Berry, W., Szafron, M., Mutwiri, G., 2016. Response of immune response genes to adjuvants poly [di (sodium carboxylatoethylphenoxy) phosphazene](PCEP), CpG oligodeoxynucleotide and Emulsigen at intradermal injection site in pigs. Vet. Immunol. Immunopathol. 175, 57–63.
Magiri, R., Lai, K., Chaffey, A., Zhou, Y., Pyo, H.-M., Gerdts, V., Wilson, H.L., Mutwiri, G., 2018a. Intradermal immunization with inactivated swine influenza virus and adjuvant polydi (sodium carboxylatoethylphenoxy) phosphazene (PCEP) induced humoral and cell-mediated immunity and reduced lung viral titres in pigs. Vaccine 36, 1606–1613.
Magiri, R., Mutwiri, G., Wilson, H.L., 2018b. Recent advances in experimental polyphosphazene adjuvants and their mechanisms of action. Cell Tissue Res.
Matzinger, P., 1994. Tolerance, danger, and the extended family. Ann Rev Immunol 12, 991–1045.
McKee, A.S., Munks, M.W., MacLeod, M.K., Fleenor, C.J., Van Rooijen, N., Kappler, J.W., Marrack, P., 2009. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. J. Immunol. 183, 4403–4414.
McNeal, M.M., Rae, M.N., Ward, R.L., 1999. Effects of different adjuvants on rotavirus antibody responses and protection in mice following intramuscular immunization with inactivated rotavirus. Vaccine 17, 1573–1580.
Mikalsen, A.B., Haugland, O., Rode, M., Solbakk, I.T., Evensen, O., 2012. Atlantic salmon reovirus infection causes a CD8 T cell myocarditis in Atlantic salmon (Salmo salar L.). PLoS One 7, e37269.
Milligan, G.N., 2014. Adjuvants: making vaccines immunogenic. Vaccinology 93–108.
Mizumoto, N., Gao, J., Matsushima, H., Ogawa, Y., Tanaka, H., Takashima, A., 2005. Discovery of novel immunostimulants by dendritic-cell–based functional screening. Blood 106, 3082–3089.
Morel, S., Didierlaurent, A., Bourguignon, P., Delhaye, S., Baras, B., Jacob, V., Planty, C., Elouahabi, A., Harvengt, P., Carlsen, H., 2011. Adjuvant System AS03 containing α-tocopherol modulates innate immune response and leads to improved adaptive immunity. Vaccine 29, 2461–2473.
Mosca, F., Tritto, E., Muzzi, A., Monaci, E., Bagnoli, F., Iavarone, C., O'Hagan, D., Rappuoli, R., De Gregorio, E., 2008. Molecular and cellular signatures of human vaccine adjuvants. Proc. Natl. Acad. Sci. U. S. A. 105, 10501–10506.
Mutwiri, G., Benjamin, P., Soita, H., Townsend, H., Yost, R., Roberts, B., Andrianov, A.K., Babiuk, L.A., 2007. Poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) is a potent enhancer of mixed Th1/Th2 immune responses in mice immunized with influenza virus antigens. Vaccine 25, 1204–1213.
Mutwiri, G., Benjamin, P., Soita, H., Babiuk, L.A., 2008. Co-administration of polyphosphazenes with CpG oligodeoxynucleotides strongly enhances immune responses in mice immunized with Hepatitis B virus surface antigen. Vaccine 26, 2680–2688.
Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., 2000. Differential expression and regulation of toll-like receptors (TLR) in human leucocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164, 5998–6004.
Pasquale, A.D., Preiss, S., Silva, F.T.D., Garçon, N., 2015. Vaccine adjuvants: from 1920 to 2015 and beyond. Vaccines 3, 320–343.
Ray, M.C., Gately, L.E., 1996. Basement membrane zone. Clin. Dermatol. 14, 321–330.
Sagulenko, V., Thygesen, S.J., Sester, D.P., Idris, A., Cridland, J.A., Vajjhala, P.R., Silke,

J., 2013. AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. Cell Death Differ. 20 (9), 1149.
Schijns, V.E., 2000. Immunological concepts of vaccine adjuvant activity: commentary. Curr. Opin. Immunol. 12, 456–463.
Schijns, V.E., 2002. Antigen delivery systems and immunostimulation. Vet. Immunol. Immunopathol. 87, 195–198.
Shi, Y., Evans, J.E., Rock, K.L., 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 425, 516.
Wang, W., Singh, M., 2011. Selection of adjuvants for enhanced vaccine potency. World J. Vacc. 1, 33.
Wynn, T.A., 2003. IL-13 effector functions. Annu. Rev. Immunol. 21, 425–456.
Zhang, F., Ekine-Dzivenu, C., Vinsky, M., Basarab, J.A., Aalhus, J.L., Dugan, M.E.R., Li, C., 2017. Phenotypic and genetic relationships of residual feed intake measures and their component traits with fatty acid composition in subcutaneous adipose of beef cattle. J. Anim. Sci. 95 2813-1824.