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Research paper

Strategies to link innate and adaptive immunity when designing vaccine adjuvants

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ABSTRACT

Adjuvants are important components of vaccine formulations. Their functions include the delivery of antigen, recruitment of specific immune cells to the site of immunization, activation of these cells to create an inflammatory microenvironment, and maturation of antigen-presenting cells for enhancement of antigen-uptake and -presentation in secondary lymphoid tissues. Adjuvants include a large family of molecules and substances, many of which were developed empirically and without knowledge of their specific mechanisms of action. The discovery of pattern recognition receptors including Toll-like-, nucleotide-binding oligomerization domain (NOD)- and mannose-receptors, has significantly advanced the field of adjuvant research. It is now clear that effective adjuvants link innate and adaptive immunity by signaling through a combination of pathogen recognition receptors (PRRs). Research in our lab is focused towards the development of novel adjuvants and immunomodulators that can be used to improve neonatal vaccines for humans and animals. Using a neonatal pig model for pertussis, we are currently analyzing the effectiveness of host defence peptides (HDPs), bacterial DNA and polyphosphazenes as vaccine adjuvants.

1. Adjuvants for vaccines

Adjuvants constitute important components of human and animal vaccines. They can be grouped into particle-based delivery systems, such as liposomes, micro- or nanoparticles, and molecules that either directly or indirectly induce the expression of cytokines and chemokines thereby modulating the local microenvironment for activation and stimulation of immune cells. Most of today's adjuvants have been developed empirically and include a wide variety of formulations including cell-wall components, alum, QuilA, carbomers, and oil-in water emulsions to name a few. With the recognition of pathogen recognition receptors (PRRs) such as Toll-like, mannose and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), it has become clear that many of these adjuvants signal through highly specific pathways resulting in increased NF-κB and/or type I interferon (IFN) production, which subsequently leads to an up-regulation of chemokines and cytokines needed for maturation of dendritic cells (DCs) and improved presentation of the antigen. Since invading microorganisms are likely to simultaneously interact with many PRRs, we hypothesize that effective vaccine formulations need to stimulate multiple PRRs to both enhance the magnitude and the quality of immune responses to the vaccine antigens. Here,

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we highlight some of our strategies to enhance immune responses against *Bordetella pertussis*, an important human pathogen responsible for more than 300,000 deaths and 50 million cases in infants and young children worldwide (Crowcroft et al., 2003). We recently demonstrated that newborn piglets are highly susceptible to infection with *B. pertussis* and show severe signs of respiratory distress, weight loss and moderate to mild fever. The pathology following infection is similar to that seen in human infants including a thickening of the alveolar wall, severe influx of macrophages and neutrophils and complete tissue destruction of underlying interstitial tissues (Elahi et al., 2005). Using this model our research is focused on utilizing innate immune modulators such as 'CpG motifs' (CpG ODN), host defence peptides (HDPs) and polyphosphazenes to activate and imprint neonatal DCs towards a Th1 type of response, which ultimately will help to enhance neonatal immunity against infectious diseases such as pertussis. Here, we highlight the potential of some of these immune modulators for use as vaccine adjuvants for neonatal vaccines.

2. Host defence peptides

HDPs, also called cationic antimicrobial peptides, are innate immune molecules found in almost every life form. Their wide spectrum of functions includes direct antimicrobial activities, immunostimulatory functions of both innate and acquired immunity, as well as involvement in wound healing, cell trafficking, vascular growth and both the induction and inhibition of apoptosis (Barlow et al., 2006; Bowdish et al., 2006, 2005; Brown and Hancock, 2006; Lau et al., 2006; Mookherjee et al., 2006b). For example, HDPs have been shown to recruit immature DCs and T-cells, enhance glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells (De et al., 2000; Yang et al., 2004, 1999, 2001). Other HDPs have been demonstrated to neutralize pro-inflammatory cytokine induction and lethality in response to LPS/endotoxin (Barlow et al., 2006; Bowdish et al., 2006, 2004; Davidson et al., 2004; De et al., 2000; Finlay and Hancock, 2004; Lau et al., 2006; Mookherjee et al., 2006a; Scott et al., 2002, 2000). For example, the innate defense-regulator peptide (IDR-1), which targets monocytes and macrophages, provided protection against infection with multi-resistant bacteria in mice, and induced a more balanced or controlled immune response by decreasing pro-inflammatory cytokines such as TNF-α and IL-6 at the site of infection (Scott et al., 2007).

HDPs can be largely grouped structurally into defensins and cathelicidins based on the respective presence of β-sheets and α-helices (McPhee and Hancock, 2005). They are expressed by a wide range of cells including epithelial cells, neutrophils, macrophages and DCs (Brown and Hancock, 2006). Expression is often regulated by the presence of microorganisms (Veldhuizen et al., 2006) and/or stimulation with TLR ligands, such as LPS. HDPs may also act as TLR ligands. For example, murine β-defensin 1 directly stimulated TLR4 expression in immature DCs and lead to the maturation of these cells (Biragyn et al., 2002). Interestingly, some HDPs such as LL-37 were able to modulate the effects of TLR agonists in the presence of LPS by decreasing the amount of NF-κB translocation into the nucleus consequently altering patterns of gene expression (Mookherjee et al., 2006a). Furthermore, HDPs have been demonstrated to also enhance adaptive immune responses, and thus are considered an important link between innate and acquired immunity. For example, the human neutrophil peptides (HNP) 1–3, human β-defensins (HBD) 1 and 2, as well as murine β-defensins were shown to chemoattract immature DCs, lymphocytes, monocytes and macrophages (Biragyn et al., 2001; Soruri et al., 2007; Territo et al., 1989; Yang et al., 2000). Recruitment of immature DCs occurred through signaling via the chemokine receptor 6 (Biragyn et al., 2001; Yang et al., 1999) and other not yet identified receptors (Yang et al., 2000). Maturation of DCs was demonstrated following co-culture of immature DCs with HDPs (Davidson et al., 2004). Moreover, fusion of the murine β-defensin 2 with the gene encoding the human immunodeficiency virus-1 glycoprotein 120 (HIV gp120) resulted in specific mucosal, systemic, and CTL immune responses after immunization (Biragyn et al., 2002, 2001). Ovalbumin (OVA)-specific immune responses were enhanced after intranasal co-administration of ovalbumin and HNP 1–3 in C57/Bl mice (Lillard et al., 1999) and intraperitoneal injection of HNP 1–3 and KLH of B-cell lymphoma idiotype Ag into mice enhanced the resistance to subsequent tumor challenge (Tani et al., 2000). Fusion of β-defensins mBD2 or mBD3 to a B-cell lymphoma epitope sFv38 induced stronger anti-tumor immune responses in mice (Biragyn et al., 2002, 2001). Thus, these examples provide evidence that HDPs have been successfully used as adjuvants to enhance vaccine-specific immunity.

To investigate the potential of HDPs for enhancing the immune response in neonates, we are currently using murine, human and porcine DCs. Screening of HDPs is based on the ability to induce expression of chemokines and cytokines in these cells, as well as the up-regulation of co-stimulatory markers and MHC class II. For example, two subsets of porcine DCs, namely monocyte-derived DCs (moDC) and blood-derived DCs (bDC) are being used the latter of which include both myeloid and lymphoid DCs. MoDC were generated by isolation of $CD14^+$ cells (monocytes) and subsequent culturing in the presence of IL-4 and GM-CSF (Raymond and Wilkie, 2004; Summerfield et al., 2003), whereas bDC were isolated based on their expression of $CD172^+$(Summerfield et al., 2003). Fig. 1 shows an example of the expression of pro-inflammatory cytokines in moDC and bDC following stimulation with HDP. MoDC were stimulated at day 6 of culture with 133 μg/ml of the 12 amino acid peptide HH2 (VQLRIRVAVIRA-NH_2). BDC were isolated and rested for 16 h, after which time they were stimulated in the same manner. Twenty-four hours after stimulation, supernatants were collected from both moDC and BDC for interleukin (IL)-8 analysis by ELISAs. Following an 8 h stimulation of moDC, cells were collected for qPCR analysis. Fig. 1A shows that stimulation with HH2 resulted in enhanced expression of interleukin IL-8 in moDC but not in bDC. Furthermore, 8 h stimulation by peptide HH2 resulted in a 6- and 8-fold respective increase

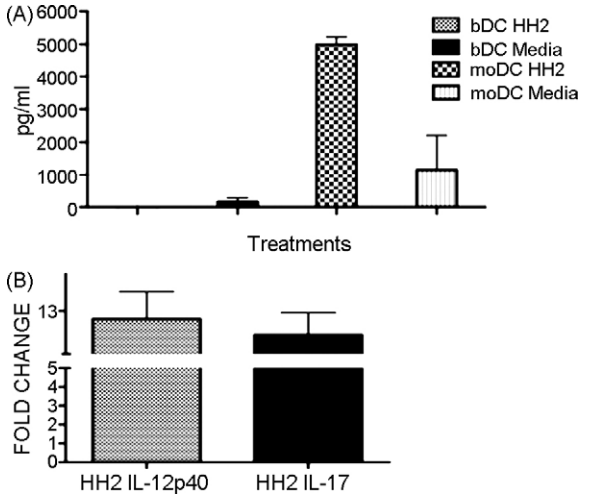

Fig. 1. The effect of peptide stimulation on porcine bDC and moDC. After 24-h stimulation with peptide HH2 (133 μg/ml) IL-8 levels were examined by ELISAs in moDC and BDC (A). Following an 8-h stimulation with HH2, the gene expression of IL-12p40 and IL-17 was examined by qPCR in moDC (B). Results are demonstrated as mean ± S.E.M. ($n = 4$). The following primers were used: IL-17F:ACGTACGTGCTACGT; IL-17R:AGCTGTAACCGGTT; IL-12p40-F:GAAATTCAGTGTCAAAAGCAGCAG; IL-12p40-R: TCCACTCTGTACTT-CTTATACTCCC. The IL-8 was detected by ELISA using the anti-IL-8 antibodies (R&D MAB5531 at 2 μg/ml; R&D BAF 535 at 25 ng/ml), and recombinant cytokine standards (R&D 533-IN, concentration of highest standard 40 ng/ml).

in the expression of IL-12p40 and IL-17 in moDC (Fig. 1B). IL-17 plays a role in the activation of macrophages to kill *B. pertussis* (Higgins et al., 2006), recruitment of neutrophils and in an increase of IL-8 production (Prause et al., 2003). Thus, this example shows that HDPs can induce the expression of cytokines involved in the recruitment and activation of immune cells. Current research is focused on assessing potential synergies between CpG ODN and HDPs to further enhance specific immune responses against *B. pertussis* in newborn pigs.

3. CpG ODN

Bacterial DNA, as well as short oligonucleotides containing CpG ODN, are potent immune modulators in both human and animal species. CpG ODN signal through TLR9, and their immunomodulatory activity, either as 'stand alone'-innate immune treatments or as vaccine adjuvants, has been shown by numerous investigators in a variety of species. Excellent reviews are available to summarize the activity of CpG ODN (Bot and Bona, 2002; Higgins et al., 2007; Klinman, 2006; Krieg, 2006; Wilson et al., 2006). When used as a vaccine adjuvant, CpG ODN promote predominantly Th1 type immune responses in adults, a quality needed for optimal protection against pertussis (Byrne et al., 2004; Conway et al., 2001; Higgins et al., 2006; Knight et al., 2006; Mills, 2001; Sugai et al., 2005).

The strong ability to skew vaccine-induced immune responses towards a Th1 type response make CpG ODN a logical choice to stimulate balanced or Th1-type immune responses in the neonate. To date immunomodulatory activities of CpG ODN that enhance neonatal immune responses have been demonstrated in a variety of species including mice, humans and pigs (Angelone et al., 2006; Brazolot Millan et al., 1998; Butler et al., 2005; Ito et al., 2005, 2004; Linghua et al., 2007, 2006; Nichani et al., 2006; Olafsdottir et al., 2007; Pedras-Vasconcelos et al., 2006; Weeratna et al., 2001a). In the case of a hepatitis B vaccine co-formulated with CpG ODN, these responses were enhanced even in the presence of maternal antibodies (Weeratna et al., 2001b).

To assess the ability of neonates to respond to stimulation with CpG ODN in vitro several studies were performed using either neonatal PBMC or DCs, which were isolated from either human cord blood or the blood of animals. For example, comparable amounts of IFN-α were found in whole blood from adults and neonates following stimulation with CpG both neonatal and adult DCs can elicit Th1 responses (Gold et al., 2006; Sun et al., 2005). However, in this study the response in DCs was down-regulated by IL-10 secretion from $CD5^+$ B-cells in response to systemic inflammation following TLR9 triggering (Sun et al., 2005). It has also been demonstrated that stimulation with CpG ODN induced secretion of IgM, up-regulation of expression of HLA-DR and CD86, induction of MIP-1 α, and proliferation of adult and cord blood B-cells (Tasker and Marshall-Clarke, 2003). Furthermore, similar amounts of IgM were produced by adult and umbilical cord B-cells following stimulation with CpG ODN (Landers and Bondada, 2005). In contrast, the production of IFN-α in response to CpG ODN was dramatically impaired in cord blood plasmacytoid DCs (De Wit et al., 2004) whilst it was also demonstrated that immune responses to tetanus toxoid, co-formulated with CpG ODN, were higher in adults than in newborns (Pihlgren et al., 2003). Similarly, evidence exists that neonatal immune responses to CpG ODN differ from those seen in adults and indeed Th2-responses to allergens were increased following addition of CpG ODN to house dust mite allergens (Prescott et al., 2005). This contradictory evidence highlights the need for further research to understand CpG ODN activity in the neonate and to also assess the long-term consequences of treating neonates with CpG ODN.

More recent evidence to support the use of CpG ODN in the neonate comes from recent observations demonstrating that CpG ODN can stimulate the expression of the BAFF-receptor TACI, a factor needed for survival of activated B-cells and plasmablasts (Bossen et al., 2008). CpG ODN, therefore, might help to extend the lifespan of neonatal plasma cells and induce the earlier development of germinal centres (Siegrist, 2007). Stimulation of B2 and B1 cells with LPS or CpG ODN not only induced MyD88-dependent plasma cell differentiation and intracellular expression of BAFF and APRIL (Chu et al., 2007) but also strongly up-regulated the expression of the BAFF-receptor TACI (Acosta-Rodriguez et al., 2007; Katsenelson et al., 2007) needed for survival of activated B-cells and plasmablasts Thus, in addition to skewing the immune response towards a Th1 type immune response in the neonate, CpG ODN may help to elicit effective cell priming and long-term responses in the neonate.

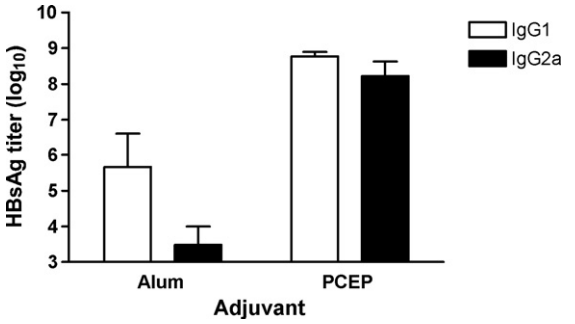

Fig. 2. Adjuvanticity of PCEP. Balb/c mice ($n = 6$) were given a single immunization with 10 μg HBsAg alone or in combination with alum or PCEP. IgG1 and IgG2a serum antibody responses were assessed by ELISA at 12 weeks after immunization.

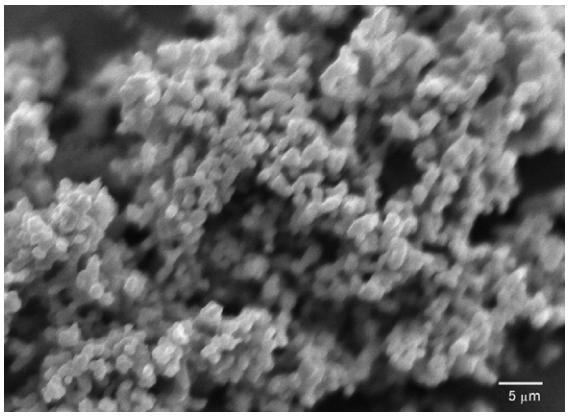

Fig. 3. Formation of PCPP-ovalbumin microparticles. Scanned electron microscopy (SEM) of PCPP-ovalbumin microparticles prepared by coacervation method (1000× magnification). The scale corresponds to 5 μm.

4. Polyphosphazenes

Polyphosphazenes are synthetic, water-soluble and biodegradable polymers that can function both as vaccine adjuvants as well as delivery-vehicles for vaccines when formulated into microspheres. Polyphosphazene polymers have long chain backbones of alternating nitrogen and phosphorous atoms with two side groups attached to each phosphorous (Allcock, 1990). Different side groups can be substituted at these positions to synthesize polymers with different physiochemical properties, such as water solubility and biodegradability, which make them amenable for use as biomedical polymers, membranes, hydrogels, bioactive and biodegradable polymers (Allcock, 1990). Polyphosphazenes have been used extensively for drug and vaccine delivery. For example, poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP) displayed strong adjuvant activity in mice with a variety of viral and bacterial antigens in mice (McNeal et al., 1999; Payne et al., 1998; Wu et al., 2001) and poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) not only enhanced the magnitude but also modulated the quality of immune responses to influenza X:31 antigen towards a Th1 type immune responses, resulting in more balanced immunity (Mutwiri et al., 2007). PCEP similarly induced a balanced Th1/Th2-type immune response with Hepatitis B surface antigen, and the magnitude of antibody responses was much higher than with the conventional adjuvant alum, which induced a predominantly Th2-type response (Fig. 2). Furthermore, polyphosphazenes are very safe to use. Their water-soluble nature reduces the risk of injection site reactions, which is often seen when using conventional adjuvants like mineral oil and Alum (Payne and Andrianov, 1998). Thus, the combined effects of their potent adjuvanticity and negligible toxicity make them potential components for commercial vaccine formulations. We are currently assessing a panel of modified polyphosphazenes for their ability to enhance specific immune responses against *B. pertussis*. Indeed, preliminary experiments already indicate that the co-formulation of polyphosphazenes with pertussis toxoid (PTd) and CpG ODN leads to higher antibody responses and secretion of PTd-specific SIgA into BAL and nasal fluids in mice (data not shown). We expect that these responses can be further enhanced by using polyphosphazenes-based microparticles, which contain antigen, CpG ODN and HDPs (Fig. 3).

5. Microparticle-based delivery

Particle-based delivery of antigens has proven to be highly efficacious for antigen delivery, especially when compared to the delivery of soluble proteins. Microparticles are phagocytosed by a variety of cells including macrophages and DCs (Lutsiak et al., 2002; Newman et al., 2002). Once taken up by these cells, antigen is released and subsequently selected for presentation via MHC II. Interestingly, this process occurs in a phagosome-autonomous manner and is controlled by the presence of TLR ligands (Blander and Medzhitov, 2006). As a result, DCs can distinguish between self and non-self antigens allowing for self/non-self discrimination (Blander and Medzhitov, 2006). Furthermore, by being present in either early or late endosomes, various TLRs can be stimulated, therefore enhancing the overall response to the antigen (Trinchieri and Sher, 2007).

Particulate delivery systems, such as microparticles and nanoparticles, are typically less than 10 μm in size and consist of hydrophobic polymers or polysaccharides with the protein of interest incorporated at incorporation efficiencies of between 70 and 90%. Concerns regarding the use of particle-based delivery systems include inefficient incorporation, stability and integrity of the antigen during the formulation process or storage (Devineni et al., 2007). By creating a depot effect, microparticles help to increase the persistence of antigens for a longer time, which is important for the induction of efficient protective T-cell responses (Storni et al., 2004; Trinchieri and Sher, 2007). Furthermore, by masking the antigen inside the particles, microparticles help overcome interference with maternal antibodies, which is a major challenge for vaccinating the neonate. Microparticles are typically co-formulated to deliver both the antigen and adjuvant to the target cell. Indeed, microparticles and liposomes have been successfully used for delivery of a

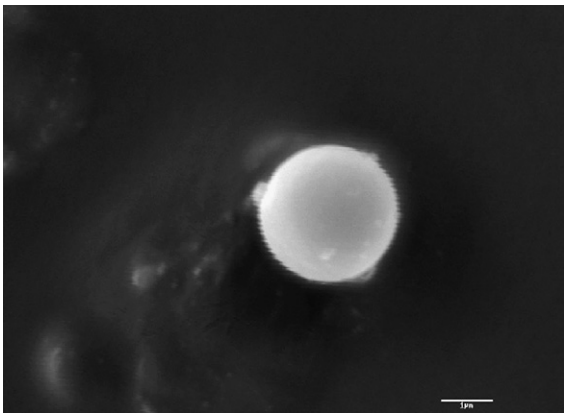

Fig. 4. Formation of a PCPP-microparticle. SEM of the PCPP-ovalbumin microparticles at 10,000× magnification, showing a spherical structure with smooth surface with frequent blebs on the surface.

wide range of antigens and adjuvants including CpG ODN using models for cancer, allergies and infectious diseases (Alcon et al., 2005; Babiuk et al., 2004; de Jong et al., 2007; Diwan et al., 2004; Fu et al., 2006; Kazzaz et al., 2006; Martinez Gomez et al., 2007; Standley et al., 2007; Tafaghodi et al., 2006a, 2006b; Zaks et al., 2006). In primary human plasmacytoid DCs, CpG ODN was delivered by cationized gelatin nanoparticles and this resulted in IFN-α production (Zwiorek et al., 2008). Poly(lactic-co-glycolic) microspheres have also been used for both the delivery of antigen and CpG ODN to APC, and their delivery resulted in the activation of endosomal TLR (Heit et al., 2007). Maturation and cytokine secretion as well as antigen-cross-presentation was observed. Furthermore, in the same study immunization with these microspheres triggered clonal expansion of primary and secondary antigen-specific $CD4^+$ and $CD8^+$ T-cells in vivo.

Many of the currently used microparticles, however, have the disadvantage of exposing antigen during the assembly process to harsh conditions such as high temperature, organic solvents or low pH levels (Andrianov and Payne, 1996). The ability of polyphosphazenes PCPP and PCEP to form microspheres under mild conditions either by using spray drying of polymer–protein mixtures onto $CaCl_2$ solution (Allcock, 1990), coacervation with NaCl and subsequent stabilization of microparticle sized coacervates by cross-linking with Ca^{++} ions (Andrianov et al., 1998), or by ionic complexation of polyphosphazenes with spermine (Andrianov et al., 2006), makes them attractive encapsulation agents. This is particularly useful for encapsulation of biologically labile entities, such as proteins, CpG ODN and/or HDPs. Using the coacervation technique with bovine serum albumin (BSA) and chicken ovalbumin, we observed spherical microparticles in the range of 0.7–3.0 μm in diameter (Figs. 3 and 4). Using FITC labeled OVA and Alexafluor-546 labeled CpG ODN we showed that the incorporation ranged from 70% to >90%, respectively. The integrity of the particles after lyophilization and resuspension appeared to be normal even after storage at room temperature for 2 months. Uptake studies using porcine moDC at a ratio of 5 microparticles per DC

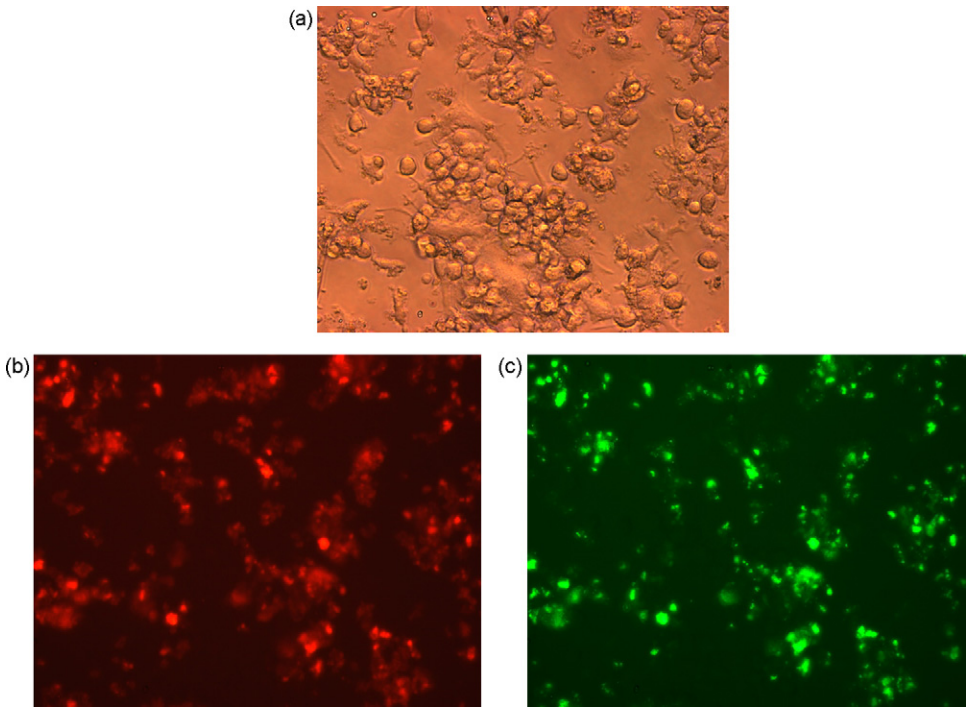

Fig. 5. Uptake of PCPP-ovalbumin microspheres by MoDC. Monocyte-derived porcine DCs were overlaid with PCPP-ovalbumin-CpG microparticles in 5:1 Mps:DCs. The ovalbumin was labeled with FITC and the CpG ODN labeled with Alexafluor-546 Dye. The photomicrographs (40× magnification) were taken using a Zeiss Fluorescent microscope under transmitted light (a), TRITC (b) and FITC (c) filters after 30 min of MP addition. The above results were also confirmed by FACS (results not shown).

confirmed that the particle uptake was apparent at 30 min after addition of microparticles (Fig. 5). Current research in our lab is focused on further improvement of these microparticles using layer-by-layer (LbL) microparticles, which consist of colloid sized core particles onto which oppositely charged molecules are added (Wang et al., 2006). The generation of these particles has several advantages including the potential of adding multiple adjuvants onto the outside layers.

6. Conclusion

Adjuvants are important components of vaccines, both for humans and animals. Here, we have highlighted the potential of CpG ODN, HDPs and polyphosphazenes as adjuvants for neonatal vaccines. CpG ODN, HDPs and polyphosphazenes act via different pathogen recognition receptors and signaling pathways, each of them resulting therefore in slightly different activation of the innate immune system. By combining these immune modulators and thereby providing multiple signals for stimulation of the immune system, we may be able to develop highly effective vaccine formulations for both adults and neonates.

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Conflict of interest

I do not have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled," Strategies to link intake and adaptive immunity when designing vaccine adjuvants".

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