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Short Communication

Carbopol improves the early cellular immune responses induced by the modified-life vaccine Ingelvac PRRS® MLV

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SUMMARY

Adjuvants enhance both the magnitude and duration of immune responses, therefore representing a central component of vaccines. The nature of the adjuvant can determine the particular type of immune response, which may be skewed toward cytotoxic T cell (CTL) responses, antibody responses, or particular classes of T helper (Th) responses and antibody isotypes. Traditionally, adjuvants have been added to intrinsically poor immunogenic vaccines, such as those using whole killed organisms or subunit vaccines. Here, we have compared cellular immune responses induced by the immunogenic modified life-attenuated vaccine Ingelvac PRRS® MLV when administered alone or in combination with carbopol, a widely used adjuvant in veterinary medicine. Using functional readouts (IFN-γ ELISpot and cell proliferation) and analyzing phenotypical hallmarks of CD4T cell differentiation, we show that carbopol improves cellular immunity by inducing early IFN-γ-producing cells and by preferentially driving T cell differentiation to effector phenotypes. Our data suggest that adjuvants may enhance and modulate life-attenuated – not only subunit/inactivated – vaccines.

1. Introduction

Modified life-attenuated vaccines (MLVs) comprise weakened versions of pathogens that often confer long-lasting immunity similar to that seen in natural infection. Nonetheless, adverse events, including the potential to cause disease, may occur (Pulendran and Ahmed, 2011). Increased efforts in current vaccine design lie in the enhancement of the immune responses of weak experimental and commercial killed and subunit vaccines by means of using adjuvants, ingredients that directly or indirectly contribute to vaccine efficacy (Levitz and Golenbock, 2012). Although key components of vaccine formulations, the mechanisms by which adjuvants enhance immunity are not fully understood (Martin-Fontecha et al., 2004). Specifically, whether adjuvants impact cellular immunity triggered by immunogenic MLVs have not been explored in deep.

The porcine reproductive and respiratory syndrome (PRRS) is caused by an enveloped RNA virus-(PRRSV), affecting pigs worldwide and leading to great economic losses in swine industry (Holtkamp et al., 2013). Infections can occur in pigs of all ages, provoking respiratory

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syndromes, cyanosis of extremities as well as reproductive failures in sows (Christianson et al., 1992). Two main PRRSV genotypes, the European (type 1) and the North-american (type 2) (Han et al., 2013) have been described so far.

In this study we investigated the potential of a selected carbomer adjuvant (Carbopol® 971P) to impact the cellular immune response induced by vaccination of pigs with a modified live-attenuated vaccine. The immune response after a single immunization with Ingelvac PRRS® MLV (Boehringer Ingelheim) delivered with or without carbopol was evaluated at different time points. Our results provide evidence to consider carbopol as optimal adjuvant to improve the early cellular immunity elicited by MLVs.

2. Material and methods

2.1. Animals

In total, 24 piglets (crossbreed Landrace, Large White and Pietrain) were obtained at the age of six weeks from a conventional piglet producing farm in Austria. The farm is known to be free of PRRSV, which is tested at distinct intervals by the University Clinic for Swine, University of Veterinary Medicine, Vienna. The animals chosen for the study were all clinically healthy and were negative for PRRSV-specific antibodies (tested by ELISA at study day (SD)-7; PRRS X3, IDEXX, Ludwigsburg, Germany). Experimental procedures were approved by the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz–TVG) and the Federal Ministry for Science and Research (reference number BMWF-68.205/0083-II/3b/2013).

2.2. Immunizations (Table 1)

Animals were immunized once intramuscularly in the neck at SD0 with modified live-attenuated PRRS virus (Ingelvac PRRS® MLV, Boehringer Ingelheim) in the presence or the absence of a 0.1% solution of carbopol (Carbopol® 971P NF Polymer, Lubrizol, Cleveland, OH) in PBS. PBS and carbopol served as negative control groups. All animals received a total volume of 2 mL.

2.3. PBMC

At SD14 and 21, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation as described elsewhere.

Table 1
Study design and animal groups. The four groups of animals used for the study are listed, including details to the immunization procedure. PBS, phosphate buffered saline; MLV, modified live-vaccine Ingelvac® PRRS (Boehringer Ingelheim); $TCID_{50}$, tissue culture infectious dose, at least $10^{4.9}$ live virus per dose, according to Boehringer Ingelheim data sheet.

Treatment	Animals/ Group	Animal code	Dose MLV (TCID50)
PBS	6	#1–6	n/a
Carbopol 0.1%	6	#7–12	n/a
MLV	6	#13–18	$10^{4.9}$ live virus
MLV + carbopol 0.1%	6	#19–24	$10^{4.9}$ live virus

2.4. IFN-γ ELISpot

96 well plates with a polyvinylidene fluoride membrane (Millipore, Billerica, USA) were coated over night with 100 μL/well of a monoclonal antibody specific to porcine IFN-γ (clone pIFN-γ, Mabtech, Nacka Strand, Sweden) at 4 °C, adjusted to 10 μg/mL. After washing, freshly isolated PBMC were added to wells (3×10^5 PBMC and 200 μL final volume per well) in cell culture medium. This consisted of RPMI 1640 with stable glutamine (PAN Biotech, Aidbach, Germany) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS, PAA, Pasching, Austria), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAA). Cells were stimulated with different concentrations of virus (ATCC VR2332; MOI = 1.0, 0.5, 0.25; grown on the MA-104 cell line). As control group, cell culture supernatant of non-infected MA-104 cells (mock control to virus) was added in an equal amount as for virus MOI = 1.0 to PBMC. Additionally, cells without stimulus (medium only) were tested as negative control in parallel. Concanavalin A (ConA, 3 μg/mL, GE Healthcare, Pittsburgh, USA) stimulated cultures where used as a positive control. All samples were analyzed in duplicates. After incubation at 37 °C and 5% CO_2 for 24 h, PBMC were discarded and plates incubated with 100 μL/well of a second IFN-γ-specific biotinylated antibody (clone PAN, Mabtech, 1 μg/mL) for one hour, and for one additional hour with streptavidin alkaline phosphatase (Roche, Mannheim, Germany; 1:2000; 100 μL/well). Finally, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (100 μL/well, Sigma-Aldrich, Vienna, Austria) was added for five minutes. Spot development was stopped by washing plates under tap water and enumerated in a camera-based automated counting system (AID, Straßberg, Germany). Animals with more than 30 spots were arbitrarily considered responders. This number is at least 2.5 times above the average number of spots of all animals in control groups (PBS and carbopol) of both SD14 and SD21 when stimulated with PRRSV (mock-stimulated cells were always below this cut off).

2.5. Cell proliferation assay

PBMC were adjusted to $5–20 \times 10^6$ cells per mL PBS. 1 mL of a 5 μM solution of the Violet Cell Trace dye (Life Technologies, Carlsbad CA, US) was added per mL initial cell solution. After incubation for 10 min at 37 °C, 2 mL FCS (PAA) were added per mL initial cell solution, followed by an incubation at room temperature in the dark for 15 min and washed thoroughly afterwards. For *in vitro* stimulation cells were added to 96-well round-bottom plates at a concentration of 2×10^5 PBMC per well in a final incubation volume of 200 μL and stimulated with life virus (ATCC VR 2332, US strain, MOI = 1.0). In parallel equivalent mock control was carried out as negative control. Additionally, cells cultured in medium only served as additional negative control. After incubation at 37 °C and 5% CO_2 for four days, PBMC were further used in multicolour flow cytometry.

2.6. Flow cytometry (FCM)

The following primary antibodies were used for cell surface staining: PerCP-Cy5.5-conjugated anti-CD3 (IgG2a, clone BB23 8E6 8C8, BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-CD4 (IgG2b, clone 74-12-4, BD Biosciences), PE-conjugated anti-CD8α (IgG2a, clone 76-2-11, BD Biosciences), Alexa488-conjugated anti-CD8β (IgG1, clone PPT23), Alexa647-conjugated anti-CD27 (IgG1, clone b30c7, and biotinylated anti-TCR γδ (IgG2b, clone PPT16). All non-commercial monoclonal antibodies (Saalmüller, 1996) were produced at the Institute of Immunology, Veterinary University, Vienna. These antibodies had been purified and covalently conjugated to fluorochromes (Alexa Fluor Protein Labeling kits, Life Technologies) or Biotin (Sulfo-NHS-LCBiotin, Thermo Scientific, Pierce, Vienna, Austria). Fixable Near-IR Dead Cell Stain Kit (Life Technologies) was used to discriminate between live and dead cells. FCM analyses were performed on FACSCanto II (BD Biosciences). Data of at least 2.5×10^5 lymphocytes per sample were recorded. Data were analyzed with FACSDiva (Version 6.1.3, BD Biosciences) and FlowJo (Version 10.0, Tree Star Inc, Ashland, OR, USA) software.

3. Results

As a surrogate of vaccine efficacy, we first used ELISpot assays to evaluate the frequency of IFN-γ producing cells after *in vitro* re-stimulation with PRRSV. On SD14, all animals in MLV group had a ELISpot count below the cut off value (Fig. 1). In contrast, the carbopol-MLV group included three animals (#19, #21 and #23) with a high number of spots. On SD21, spots were readily detected in three animals of the MLV group (#13, #14 and #18). Furthermore, the same three animals that responded at SD14 in the carbopol-MLV group showed rather unchanged levels of IFN-γ spots. As expected, antigen-specific IFN-γ production detected in all SDs in the control groups (PBS and carbopol alone), as well as in cells stimulated with mock virus or left unstimulated fell under the cut off (not shown).

We next sought to study memory progression within the CD4 T cell population on MLV and carbopol-MLV groups. To this purpose, PBMC were labelled with a fluorescent dye, stimulated with PRRSV or mock virus and analyzed by flow cytometry four days later. Fig. 2 shows the gating strategy to define proliferating, ($CD4^+Violet^{dim}$) and non proliferating ($CD4^+Violet^{high}$) cells (Fig. 2A), and to determine T_{CM} and T_{EM} cells according to CD27 and CD8α expression (Fig. 2B). At SD14, the overall percentage of PRRSV-specific proliferating CD4T helper cells (Fig. 2C), CD8β CTL, γδ T cells, and whole PBMC was similar between MLV and carbopol-MLV groups (not shown). When analyzing surface marker expression by CD4T cells in SD14 samples, we decided to include animals of MLV group that did not produce IFN-γ early on but became responders at SD21 (#13, #14 and #18), and compare them to #19, #21, and #23 of the MLV carbopol group. This allowed us a direct comparison between phenotype and function. The percentage of T_{CM} cells was heterogeneous in the three animals of the MLV group (11.4%, 3.5% and 1.0%) compared to the three responders of the carbopol-MLV group (9.3%, 4.9% and 12.3%) (Fig. 2C). In sharp contrast, a higher proportion of T_{EM} cells was detected in the three responder animals of the carbopol-MLV group (25.7%, 9.6% and 22.7%) respect to the MLV group (5.1%, 3.9% and 4.2%). Remarkably, the proportion of total memory cells ($T_{CM} + T_{EM}$) was higher in carbopol-MLV group (35%, 14.5% and 35%) than in MLV group (16.5%, 7.4%, and 6.1%). Furthermore, the differences between MLV and carbopol-MLV groups detected at SD14 were no longer so at SD21. Altogether, these data suggest that carbopol promotes an early onset of cellular immunity by facilitating T cell differentiation towards effector phenotypes and by efficiently inducing naïve to memory transition.

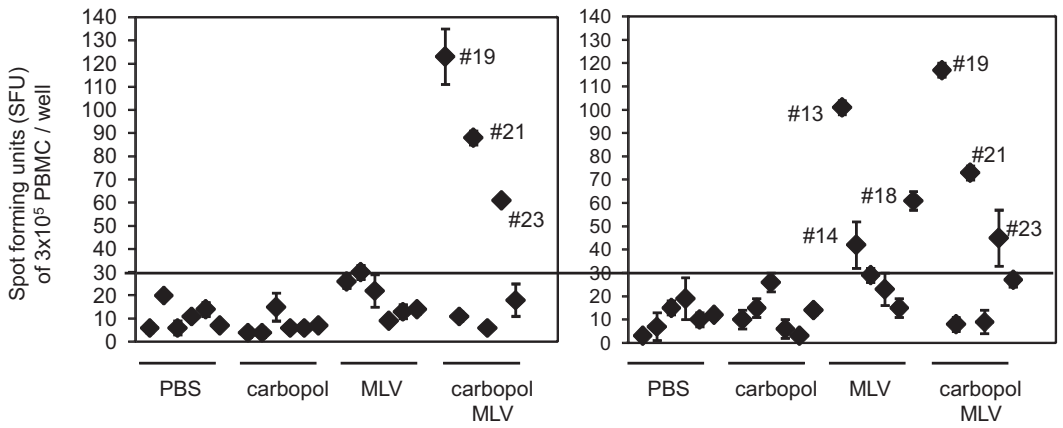
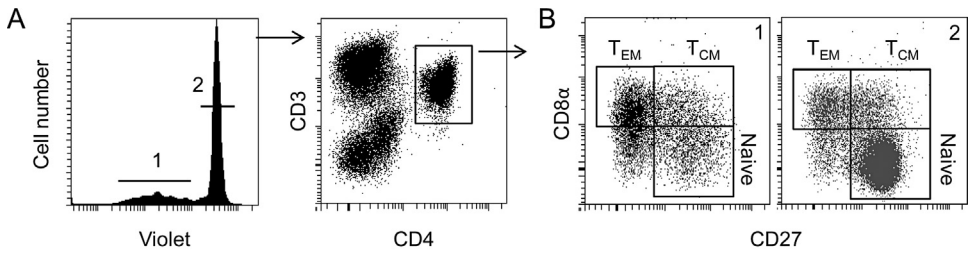


Fig. 1. Frequency of IFN-γ producing lymphocytes analyzed by ELISpot. The frequency of IFN-γ producing cells of individual animals at SD14 (left panel) and SD21 (right panel) is displayed within 3×10^5 PBMC at MOI = 1.0. Results are shown as mean of duplicates, including the standard deviation. Results are shown for the different stimulation groups: PBS, carbopol, MLV, and carbopol-MLV. The line indicates the cut off value. Responder animals have been identified by #code.



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Group	Animal	% prolif. CD4 Th	$\%T_{EM}$	$\%T_{CM}$	Total memory
MLV	#13	20.5	5.1	11.4	16.5
	#14	7	3.9	3.5	7.4
	#18	0.1	4.2	-0.7	3.5
carbopol-MLV	#19	21.1	25.7	9.3	35
	#21	16.5	9.6	4.9	14.5
	#23	0.6	22.7	12.3	35

Fig. 2. Phenotype of *in vitro* activated, virus specific CD4T cells. SD14 PBMC were labelled with Violet Cell Trace and stimulated *in vitro* with PRRSV (example shown is swine #19). (A) Proliferating (1, Violet^{dim}) and non proliferating (2, Violet^{high}) total lymphocytes were gated on CD4T helper cells (CD3$^+$/CD4$^+$). (B) Expression of CD8α and CD27 in proliferating (left panel) and non proliferating (right panel) CD4T helper cells; CD8α$^+$CD27$^-$, T_{EM}; CD8α$^+$CD27$^+$, T_{CM}; CD8α$^-$CD27$^+$, naïve. (C) Frequencies of proliferating CD4T helper cells and CD4T cell subsets as defined in A and B. Total memory = T_{CM} + T_{EM}. All percentages of virus-stimulated cells were corrected by substraction of mock-stimulated cells.

4. Discussion

Due to its low reactivity, non virucidal nature and efficacy in one shot vaccination schedules carbopol, a lightly cross-linked polymer of acrylic acid, has become a widely used adjuvant in the veterinary field (Diamantstein et al., 1971). In spite of wide applications, there is little information available relating to the type and magnitude of adaptive immune response induced by carbopol compared to other well characterized adjuvants. Specifically, the contribution of carbopol to enhance/modulate cellular immunity of swine MLVs was not well studied. Although the precise immune pathways mediated by carbopol remains to be understood, our data suggest that addition of carbopol to MLVs improves cellular responses by inducing early IFN-γ-producing cells and by preferentially driving T cell differentiation to effector phenotypes. The bias towards a Th1 phenotype is supported by human preclinical studies where carbopol generated strong cellular responses to soluble HIV-1 envelope glycoprotein along with high IgG2a antibody titres (Krashias et al., 2010). Th1 responses are associated to increased CD8T cell proliferation (Ekkens et al., 2007), production of opsonizing antibodies (Lefeber et al., 2003) and maximizing macrophage killing activity (Stout et al., 2005), thus providing an optimal milieu for anti-viral immunity. Importantly, the early generation of tissue-tropic effector cells induced by carbopol may counteract the immune escape mechanism evolved by PRRSV such as delayed production of neutralizing antibodies, suppression of interferon type-I IFNs, and up-regulation of IL-10 (Yoo et al., 2010).

The immune response can vary greatly between individuals. Whereas most healthy vaccinated animals develop a quantifiable antibody response, a small percentage of immunocompetent individuals fails to do so. The latter are defined as *non-responders*. Gene set enrichment-based approach has been used to develop predictors of human vaccine outcome, suggesting the genetic background as determinant in inducing immune responses (Tan et al., 2014). Evidence for the impact of genetic variation exists also for a number of livestock diseases (Glass et al., 2012 and Cordes et al., 2012). In addition to histocompatibility genes, microarray analysis showed transcriptional differences in several immune-related genes between high and low responders (Nino-Soto et al., 2008). Genes identified as differentially expressed include several critically involved in triggering immunity such as cytokines and T cell receptor. Also, the frequency of antigen specific cells varies among unexposed/non vaccinated individuals, therefore impacting responsiveness and magnitude of immunity (Jenkins et al., 2012). With this in mind, we would consider all the above as plausible explanations as to why vaccination, not only of the carbopol group but also in the non adjuvanted one, did not result in the induction of the immune responses measured (IFN-γ production quantified by ELISpot and CD4T cell phenotype) in all animals.

The precise mechanisms for the adjuvancy provided by carbopol remain elusive. As far we know there are not available data proving the half-life of carbopol once injected *in vivo*, therefore making difficult to ascribe its benefits to any time point after vaccination. Thus, although the readouts used in the present study suggest that early immune response can be modified in the presence of carbopol (some hypotheses are discussed below), we cannot rule out that carbopol could also impact late responses.

The cross-linked network enables carbopol the entrapment of drugs in the hydrogel domains, thus making carbopol-based formulations optimal for release control of small molecules (Singla et al., 2000). By extrapolation it has

been suggested, although not formally proven, that these features would endow carbopol with adjuvant properties by exerting an antigen *depot* effect for single shot vaccines (Lai et al., 2012). Should this be the case, it would provide arguments to support the differentiation to effector cells observed in our study (Reutner et al., 2013). Indeed, a persistent antigenic stimulation is known to induce a preferential switch to effector over memory T cell differentiation (Lanzavecchia and Sallusto, 2005).

Dendritic cell (DC) activation triggered by the interaction between Toll-like receptors (TLR) expressed on DC and TLR ligands carried by pathogens lead to immunity (Levitz and Golenbock, 2012). Vaccinology is currently exploiting this phenomenon to improve vaccine efficacy, whereby small molecules mimicking the action of TLR agonists are being added to vaccines as adjuvants (Demento et al., 2009). To test the possibility of direct activation of DCs by carbopol, we generated dendritic cells *in vitro* using recombinant porcine GM-CSF according to routine protocols (Muss et al., 2013). After 6 days in culture, immature DCs were exposed to increasing dilutions of carbopol or to LPS and phenotypical and functional responses were evaluated at different time points after stimulation. None of the conditions tried with carbopol induced on DCs the expected signs of activation (changes in the expression of surface markers such as CD40, CD80 and CD86, and in TNF production; not shown), suggesting the absence of *alert* receptors specific for the polymeric structure of carbopol.

Other alternatives may explain the effect of carbopol on lymphocytes and the downstream outcome observed in our *in vivo* study; for example, a local network environment could favour cell-to-cell interactions for optimal cell activation in peripheral tissues and subsequent cell migration to draining lymph nodes. Also, the trapping of soluble immune mediators such as cytokines and chemokines could result in better intercellular signalling and into more efficient leukocyte recruitment to the site of vaccine delivery. Alternatively, carbopol action could rely on non-immune cells, whose role in promoting immunity would be revealed in subsequent time.

The identification of immunological correlates alternative to or complementing serological responses remains a great challenge in modern vaccinology. When antibodies cannot reflect vaccine performance, having additional readouts will be instrumental tools for vaccine design. The main goal of our exploratory study was to identify such correlate candidates in the context of a clinical and economical relevant swine disease. As such, our data represents a first step towards more ambitious validation settings where challenge will follow vaccination.

5. Conclusion

In summary, here we have provided evidence for carbopol to be a cellular enhancer and modulator of immune responses. In settings where MLVs – not only subunit-based or inactivated vaccines – are applied, formulation with this polymer may have beneficial effects in the protection against harmful pathogens.

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