Immune response assessment of inactivated Newcastle disease virus liposomal-based vaccine

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Abstract

Liposomes were evaluated as an alternative carrier to deliver inactivated Newcastle disease virus (NDV). The ability of a liposomal-based NDV vaccine to activate both cell-mediated immunity (CMI) and humoral immunity against NDV was assessed and compared with conventional NDV vaccines. Birds were assigned to 4 groups and received either no vaccine (control), NDV entrapped into liposomes (LN-NDV), NDV with oil adjuvant (oil-NDV), or live attenuated NDV (live-NDV). All birds were stimulated after 40 days of vaccination *in vivo* by an intravenous injection of inactivated NDV crude antigen, which is considered an *in vivo* NDV-specific stimulation of the chicken immune system. After vaccination and *in vivo* stimulation, serum samples were collected for NDV-specific antibody response evaluation by a hemagglutination inhibition test (HI) and an enzyme-linked immune sorbent assay (ELISA). The CMI and humoral immunity were evaluated by a measurement of the chicken interferon gamma and specific antibody response in the serum, respectively. Conventional NDV vaccines were able to stimulate a strong humoral and CMI response. Although the newly tested vaccine induced a weak NDV-specific antibody response after vaccination, the response was highly up-regulated, several folds above the protective level, after *in vivo* stimulation. All NDV vaccine formulas were able to induce a CMI response after vaccination at variable time points. This study revealed that a liposomal NDV-based vaccination in this experimental model tends to induce CMI and can only be beneficial in priming vaccinated birds to promote a strong antibody response to later NDV exposure.

Introduction

Chickens that survive infection from a virulent Newcastle disease virus (NDV) develop a long-lasting immunity to further infection. The basis of this immunity is the presence of circulating antibodies, a secreted antibody on the mucosal surfaces, and cell-mediated immunity (CMI). In the first few days, or weeks, following vaccination with live lentogenic strains such as the LaSota strain, the chickens often exhibit respiratory symptoms (vaccination reaction).[1] The inactivated NDV vaccines usually invoke a strong circulating antibody response.[2] Previous work has demonstrated that the combination of both a live attenuated vaccine and an inactivated adjuvant vaccine was able to activate a high level of antibody production, as well as the CMI, represented by an increment in IFN- levels.[3]

Liposomes are vesicular structures consisting of hydrated bilayers that form spontaneously when phospholipids are dispersed in water.[4] Liposomes can be used to deliver antigens and provide a convenient vehicle where a liposoluble antigen is incorporated in the lipid membrane and hydrosoluble antigens are included in the liposomes' internal cavity.[4] Therefore, this approach results in the induction of immune responses by the simultaneous delivery of the antigen encapsulated in the liposome that can be delivered directly into the cytosolic as well as into the endosomal processing pathways or the compartment of the antigen presenting cells (APCs), thus generating both the CD4+ T helpers and CD8+ cytotoxic T cells.[5]

In this study, NDV was used as a model antigen for the preparation of a liposome-encapsulated vaccine that is thought to have a potentially effective immune modulating ability. The utilization of liposomes as a delivery system for the antigens represents an advanced vaccine technology. In other words, this study explored a new delivery method for inactivated viral vaccine preparation and then evaluated the type of immune response that will be activated following vaccination with NDV as an experimental antigenic model.

Materials and Methods

Conventional Newcastle disease vaccine

The oily and live attenuated NDV vaccines were kindly donated by the Jordan Bio-Industries Center JOVAC, Amman-Jordan. Live-NDV LaSota strains were formulated to be administered by an eye drop (30 μL) at a dose equal to 10^6 EID50/bird. The oily vaccine was prepared according to the manufacturer's recommendation (JOVAC Amman, Jordan). Briefly, the oily vaccine was formulated from a virus titer that was $10^{10.5}$ EID50/mL and inactivated by binary ethylamine to yield a 9 hemagglutination unit after inactivation. Crude inactivated NDV viral antigens were mixed with an oily adjuvant supplied by the manufacturer at a 1:1 ratio. A stable emulsion was obtained as a solid clump in water without dispersion.

The multilamellar liposome-based Newcastle disease vaccine preparation

The phospholipids that were used in the liposome preparation were 1,2-dimeristoyl-sn-glysero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-sn-glysero-3-phosphocholin (DPPC), and 1,2- dipalmitoyl-sn-glysero-3-phosphoglycerol.Na (DPPG.Na) (Genzyme, USA). Cholesterol (CHoL) was also added to the phospholipids mixture to improve stability

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Contributions: MK designed the study, performed the statistical analysis, and wrote the manuscript; SA carried out the liposomal vaccine preparation; WAS conducted the experiments and the laboratory work; MG assisted in interpreting the results and writing the manuscript.

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(Sigma, USA). Phospholipids have a diverse melting temperature (Tc) and charge, where DPPC Tc was 41°C and the net charge was neutral at a pH of 7.4; DPPG.Na Tc was 41°C and had a –1 anionic charge at a pH of 7.4; and DMPE was Tc 50°C and neutrally charged at a pH of 7.4. The phospholipids were mixed with cholesterol at a molar ratio (12: 3: 1.5: 1.5 M) (*i.e.*, DPPC: DPPG.Na: DMPE: CHoL, respectively) to yield a total final concentration of 18 M. The phospholipids and the CHoL were handled under sterile conditions with nitrogen gas being pumped in the surrounding environment to prevent phospholipid oxidation. The phospholipids: the CHoL mixtures were then dissolved in 80 mL of chloroform, and the CHoL mixture in chloroform was placed in a bath sonicator (LANGFORD Electronics Ltd, Birmingham B30 3HY, UK) at 40°C to ensure that the lipid contents completely dispersed into the chloroform. The solution was then placed in a 500 mL round bottomed flask. The chloroform solution was evaporated by a Rotatory Evaporator (Bibby Sterlin Ltd, RE 200B, UK) until a dried thin film layer was obtained. During the time of evaporation, 50 mL of PBS and 50 mL of crude ND antigen were heated in a water bath at 51°C to reach Tm for the phospholipids mixture. The transition temperature of the mixture was chosen to be above the chicken's body temperature (41°C) and below the temperature that may have affected the viral protein stability. This ensured a slow release of viruses from the liposome at the phase transition temperature.

The liposomal vaccine preparation was constituted by incorporating 25 mL of the crude antigen that was heated to 51°C into the lipid phase of the liposome (*i.e.*, LN-NDV group). In order to form the multilamellar liposome, the crude antigen was laid on the top of the dried lipid film, incubated for 1 hour at 51°C, with vigorous shaking of the mixture every 10 minutes. Then, 25 mL of warm PBS was added to the incorporated antigen liposomal preparation to reach to a viral concentration that was similar to the oily adjuvant vaccine.

Liposome encapsulation evaluation

The estimation of antigen encapsulation inside the prepared liposome was evaluated by measuring the hemagglutination ability of the NDV that remained outside of the liposome. Briefly, the liposomal preparation was centrifuged at 3000 rounds/minute. The amount of the virus that was not encapsulated in the liposome was measured using a Hemagglutination Test (HA). The difference between the primary HA activity of the virus, before the liposomal incorporation, and that present in the supernatant obtained from the liposomal preparation after centrifugation, reflects the amount of the incorporated virus.

Experimental bird groups and experimental design

Fifty-five specific pathogen free (SPF) breeder layer chickens were kindly donated by the Jordan Bio-Industries Center JOVAC, Amman-Jordan, at an age of 20 weeks, and housed under controlled conditions at the biological center for research (Jordan University of Science and Technology). Birds at this age are known to have a less robust immune response to antigen-specific stimulation than younger chicks.[3,6] The birds in all of the experimental groups were maintained in experimental units, under similar management conditions, with feed and water *ad libitum.* The birds were kept in the units for 2 weeks to ensure their adaptation to the new environment before the experiments began.

Treatment and maintenance were in accordance with the Animal Care Unit (Jordan University of Science and Technology) and the Use Committee (JUST-ACUC), which follow the international animal care and use guidelines.[7] Therefore, under the proposed objective, the birds were assigned to one of 4 groups as follows: a non-vaccinated group (control) (20 birds), an NDV entrapped in liposomes group (LN-NDV) (15 birds), a live attenuated NDV group (live-NDV) (10 birds), and an inactivated NDV oily adjuvant vaccine group (oil-NDV) (10 birds). The live-NDV vaccine was administered by an eye drop, while chickens in the other groups were injected subcutaneously with 0.5 mL from the different vaccine preparations. The final NDV titer in the vaccine formula that used inactivated antigen was similar and should have yielded 8 haemagglutination units for each vaccine preparation.

Sample collections and *in vivo* antigen stimulation

Blood samples from each bird were collected at 10-day intervals until day 40. After the last blood collection, all of the birds, including the control birds, were injected intravenously with 0.5 mL of crude viral antigens that had 7 HA units. This was considered an *in vivo* specific NDV antigen stimulation for the bird's immune system. Blood samples were then drawn weekly, two weeks after this *in vivo* antigen stimulation, and evaluated. The serum was harvested from the collected blood, and all serum samples were stored at –20°C for further analysis.

Detection of antibody response by hemagglutination inhibition test and ELISA

In order to detect the level of antibody titer in the collected serum sample, against the formulated ND vaccines, the HA test was done first, in order to determine the 4 HA units of the ND antigen. The HI test was performed as previously described.[3] The HI results were represented as the highest dilution of the serum that caused a complete HI for the 4 HA units of the NDV antigens. The HA was assessed by tilting the plates. The antilog 2 of the last dilution that gave a true HI was reported and considered the HI titer of the serum antibody. Specific NDV IgG was detected by performing a direct enzyme-linked immune sorbent assay (ELISA) test (JOVAC, Amman, Jordan). The commercially available ELISA kit test was carried out according to the manufacturer's instructions. The manufacturer provided NDV pre-coated plates and ready-to-use buffer and reagents. The IgG titer was predicted and calculated using the manufacturer's ELISA software.

Detection of chicken interferon-gamma in the collected chicken serum

Chicken interferon gamma (ChIFN-γ was measured using a commercial ELISA test kit (BioSource, Inc., USA). After the vaccines were given intramuscularly to the birds, the level of ChIFN-γ was detected in the serum samples that were collected at 10, 20, 30, and 40 days after vaccination, as well as twice after the *in vivo* stimulation (2 weeks). The ELISA procedure for ChIFN-γ measurement was performed according to the previously described protocol.[3] The ChIFN-γ concentration in the serum samples was calculated in correlation to the generated ChIFN-γ standard curve, as provided in the ELISA kit.

Statistical analysis

Results were compared using a two-way analysis of variance, and significant differences among means were tested using Student's t-test. For all tests and comparisons, only *P* values of less than 0.05 were considered statistically significant.

Results

Encapsulation efficacy of liposomal entrapped Newcastle disease virus

The amount of the virus that was entrapped inside the liposomes was about 97% of the quantity that was initially added to the lipid film (*i.e.*, HA=8). The leftovers of the HA activity of NDV present in the supernatant obtained after incorporation was equal to 3 (Anti-log2=8), compared to the initial HA 8 that was added to the liposome film (Anti-log=256).

Anti-Newcastle disease virus specific antibody production in response to vaccination

The HI titers, of 1:64 and above, are consid-

ered positive and specific for NDV.[3] Antibodies were detected 10 days post vaccination (DPV) and were followed for another month (*i.e.,* 10-day intervals). The antibody levels were stable for 40 days, and no significant differences were detected across time in all of the experimental groups, in either the HI test (Figure 1A), or ELISA (Figure 1B). Therefore, the average antibody titer in each group across time was reported. The antibodies that neutralized the HA activity of the NDV were negative in the control group. The antibody levels were higher in all of the experimental groups than in the control group (P<0.05). The maximum antibody titer post vaccination was detected in the group that was vaccinated with oil-NDV, followed by the group that received the live-NDV vaccine (P<0.05). The lowest antibody titer was detected in the serum obtained from the LN-NDV group (P<0.05) (Figure 1A).

The anti-NDV specific IgG, measured using ELISA (Figure 1B), mimics the antibody response profile, and the difference detected between the groups, as reported by the HI test (Figure 1A). All of the detected antibody titers in all of the experimental vaccinated groups showed a protective IgG titer (Figure 1B). The control group birds that received PBS showed no detectable anti-NDV specific IgG titer, while the LN-NDV group had the lowest positive and protective antibody titer level, which was significantly higher than the control group, but lower than all of the other experimental groups (P<0.05).

In vivo Newcastle disease virus antigen stimulation effects on anti-NDV specific antibody production

All of the groups, including the control birds, received an intravenous injection of crude NDV inactivated antigen 40 DPV. This was considered an NDV-specific *in vivo* stimulation to

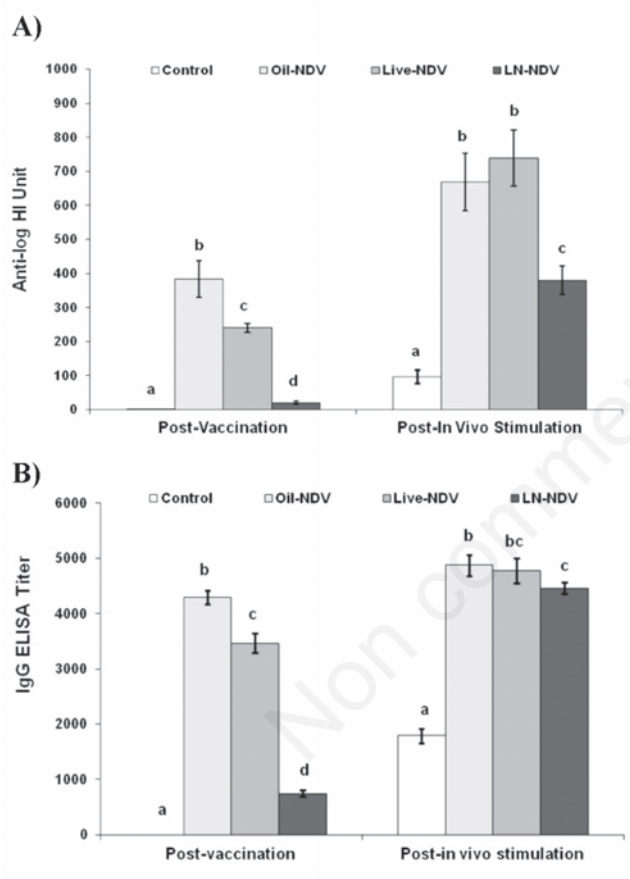

Figure 1. The antibody titer after the NDV vaccination measured by an HI test (A). The results are represented as an anti-logarithm of the HA unit. The antibody titer, as measured by ELISA (B). The figure represents the four experimental groups: the control group, the oil-NDV group that received inactivated NDV in water in an oil emulsion preparation, the live-NDV group that received live attenuated NDV, and the LN-NDV group that received inactivated ND encapsulated into liposomes. The data represent the antibody average titer over 40 days (10-day intervals) post vaccination and two measured antibody titer (2-week intervals) after the *in vivo* (intravenous) stimulation with 0.5 mL of crude viral antigens that had 7 HA units. Each value represents the mean value for each group (±SEM). Different letters indicate a significant difference between the groups within each tested time point with a P value of less than 0.05.

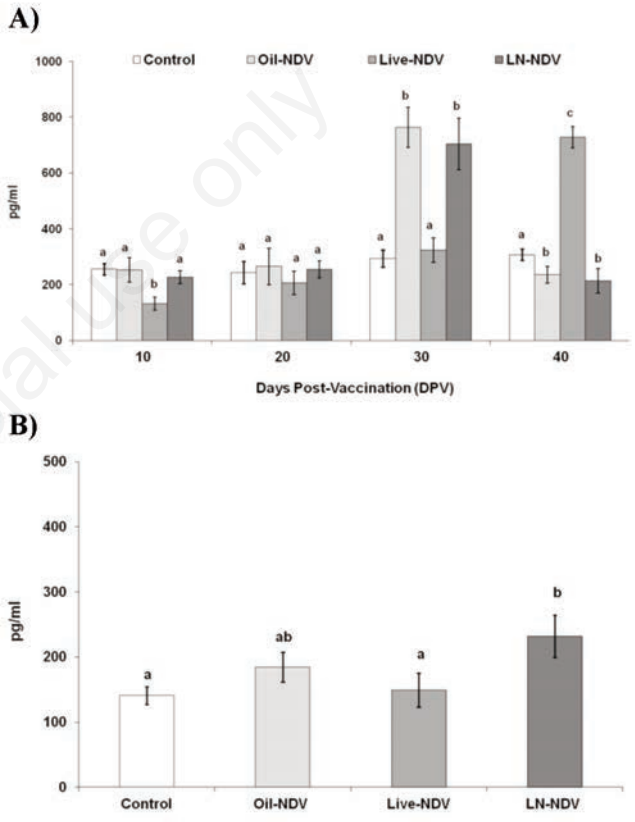

Figure 2. A) The level of interferon gamma production (pg/mL) in the bird's serum in response to the NDV vaccination. The four experimental groups were: the control group, the oil-NDV group that received inactivated NDV in water in an oil emulsion preparation, the live-NDV group that received live attenuated NDV, and the LN-NDV group that received inactivated ND encapsulated into liposome serum samples that were tested 4 times at 10-day intervals. B) The level of interferon gamma production (pg/mL) in response to the *in vivo* NDV specific stimulation. All four experimental groups were administered crude NDV whole antigen 40 days post vaccination. The data represent the average of ChIFNγ production collected twice over two weeks after the in vivo stimulation of the birds at 40 DPV with an intravenous injection of 0.5 mL of an inactivated NDV virus at an HA titer equal to 7. Each value represents the mean value for each group (±SEM). Different letters indicate a significant difference between the groups within each tested time point with a P value of less than 0.05

assess an immune response in the experimental birds. The birds that were stimulated *in vivo* with the NDV antigen were monitored for antibody production weekly for two weeks, and results were consistent with no significant differences reported over the two time points tested after the *in vivo* stimulation. Therefore, the average of antibody titer in each experimental group tested at the two time points was reported and considered as being after the *in vivo* stimulation. All of the groups that were stimulated *in vivo* with a crude NDV inactivated antigen showed a significant up-regulation in antibody titer, as measured by either an HI test (Figure 1A) or by ELISA (Figure 1B) when compared to the level of antibody recorded post vaccination ($P<0.05$).

The HI results after the *in vivo* antigen stimulation (Figure 1A) showed that the control group had the weakest positive titer, while the oil-NDV and live-NDV vaccinated groups continued to have the highest antibody production ($P<0.05$); however, both then had a similar HI antibody titer. The group that received LN-NDV had a greater boost in antibody production after the *in vivo* stimulation, but still had significantly lower antibody production than the oil-NDV and live-NDV vaccinated groups ($P<0.05$). After the *in vivo* stimulation with the NDV crude antigen, the control group started to show an HI titer, but it was significantly producing the lowest antibody response among all of the experimental groups tested by the HI test after the *in vivo* stimulation. Consistent with the HI results, the ELISA test results were similar, with several differences (Figure 1B). The up-regulation was best in the control group, where an anti-NDV specific IgG titer was not detected before the *in vivo* stimulation, but hit around an 1800 titer level after stimulation ($P<0.05$). The LN-NDV group also had an almost fivefold increase in the anti-NDV specific IgG titer levels after the *in vivo* stimulation ($P<0.05$). The anti-NDV specific IgG titer levels in the LN-NDV group after the *in vivo* stimulation became similar to the antibody production of the groups that received live-NDV or oil-NDV vaccines.

Serum levels of chicken interferon-gamma in response to Newcastle disease virus

The monitoring of ChIFN-γ post serum vaccination in both the control and experimental groups showed several significant differences across time (Figure 2A). While the control group had constant ChIFN-γ production across time, all of the other experimental groups' production peaked at different time points. ChIFN-γ release was reported in the live-NDV group; the production level was significantly lower than the experimental groups at an early time point, but then it increased over time, until reaching an obvious increment above all of the other experimental groups at 40 DPV. Both the LN-NDV and oil-NDV groups behaved similarly and showed a peak in ChIFN-γ production above the other experimental groups at only 30 days ($P<0.05$). Ten days later, these two experimental groups had significantly lower levels of ChIFN-γ production than the control group ($P<0.05$). After the *in vivo* stimulation, with an intravenous injection of inactivated NDV crude antigens, the LN-NDV group had a slight up-regulation in ChIFN-γ release in the serum compared to the later time point (40 DPV) ($P<0.05$) (Figure 2B). At the same time, the LN-NDV group had clear ChIFN-γ up-regulation above the control group. The live-NDV and oil-NDV groups had similar levels of ChIFN-γ release in the serum as the control group.

Discussion

The current work is an exploration of the effects of a liposomal NDV-based vaccine's immune response efficacy, as well as the outcome of an NDV conventional vaccination on both arms of the immune system. In addition, this work is original in that the outcome of a liposomal NDV-based vaccine is compared with the NDV conventional vaccination on both arms of the immune system over time. This study demonstrated that the liposomal delivery system for NDV is quite useful in enhancing CMI as measured by the serum IFN-γ. Additionally, both conventional vaccines, the oil-based and live attenuated NDV vaccines, showed an acceptable protective antibody titer and were able to induce an up-regulation in ChIFN-γ but at different time points post vaccination. This study is the first to present that the liposomal delivery system for a whole virus primarily results in polarizing the immune response outcome toward CMI. An interesting point revealed as well in this work was that the weak antibody response generated from the immunization with an LN-based vaccine was not a sign of immune shortage in the LN-based vaccination. Birds that received this type of vaccination seemed to be primed to later have a strong antibody response upon a second antigen exposure.

In recent years, many studies have concentrated on the role of liposomes as potential vaccine delivery vehicles that can stabilize antigens, and at the same time, act as an adjuvant.[8] It has also been established that liposome-based immunization usually gives a low-to-no antibody response and leans more toward a cell-mediated immune induction.[9,10] Provoking strong humoral immunity requires fortifying these liposomal preparations with immune stimulant compounds, such as propolis flavonoids, glycyrrhetinic acid, and epimedium polysaccharide.[10-13] The enrichment of the phospholipid content of liposomes with phosphatidylcholine (PC), and not phosphatidylserine (PS), was able to induce a stronger immune response.[14] The physicochemical properties such as a surface charge also play a role in liposome immunogenicity.[15,16] The neutral liposomes (EPC-Lip), used to deliver the NDV antigens, were able to provoke IgG antibodies against NDV more effectively than the cationic liposomes (SA-Lip) and the anionic liposomes (PS-Lip).[17]

The negatively (anionic) charged cocktail of phospholipids, as the one prepared to deliver the inactivated NDV in the current study, is expected to produce a weak anti-antibody response post vaccination.[17] Although the lowest antibody producer among all of the vaccinated groups was the LN-based vaccine preparation, it seems that this preparation was able to induce immune activation and the generation of memory cells, without provoking a substantial humoral immune response from the primary exposure, probably due to the slow release of features of the liposomal incorporated antigens. Accordingly, when the birds received the LN-NDV vaccine that was exposed to the *in vivo* NDV crude antigen stimulation, an acceptable magnitude of antibody production was reported.

The production of neutralizing antibody is important to prevent the expansion of viruses in chicken bodies, and HI titer seems to be more important to prevent the adsorption of hemagglutinin-neuraminidase (HN) proteins to the cell surface.[18] To the same extent, the CMI response is also important to eliminate the virus in chickens. Basically, ND is an acute infectious disease, and it is important to prevent the dissemination of NDV in the same population as well as to eliminate the virus in each individual. Therefore, a protective immune response to vaccination may be due to the production of antibodies (humoral immunity), the action of sensitized T lymphocytes (cellular immunity), or a combination of both.[19-21] However, the poultry industry is concerned mainly with humoral immunity measurements when evaluating vaccine efficacy, probably due to the lack of tools that can be easily applied to evaluate the CMI after vaccination. The most utilized way to evaluate CMI is measuring the lymphocyte proliferation upon recall antigen stimulation, or assessing different cytokines that represent the cell-mediated immune response activation measured by RT-PCR.[22,23] Thus, in live immunization, as in the NDV vaccination, the CMI was usually underestimated. The presence of a ChIFN-γ captured ELISA that has higher specificity and sensitivity than the available bioassay allowed the detection of ChIFN-γ in response to a viral infection *in vitro* splenocyte

cell culture and in response to NDV exposure.[3,19,24,25]

Recently, the serum levels of certain cytokines have been employed as an indicator for disease diagnosis and prognosis.[26] In humans, cytokines such as IFN- , IL-8, and IL-23 levels in serum have been labeled as immunomolecules and used for determining Th-1 and Th-17 responses against *Mycobacterium avium* complex diseases, tuberculosis, and papillomavirus infections.[26-28] The current work is the first to employ a ChIFN-γ measurement, directly in the serum, as a monitoring tool to assess the CMI response in poultry post vaccination. However, the background serum ChIFN-γ levels also need to be estimated in chickens exposed to different stressful conditions in the field and in differing bird ages and breeds. The control SPF birds had a constant level of ChIFN-γ serum production of around 300 pg/mL. Although the ChIFN-γ serum needs to be tested daily in future studies to obtain all production profiles and peaks in normal and vaccinated birds, checking the birds' serum samples for ChIFN-γ presence 4 times over 40 days might be considered a replication of sampling over time.

In the current work, we were able to show that both oil-based, and live attenuated NDV vaccines can elicit *in vivo* (serum) peaks for ChIFN-γ production post vaccination, probably from an adaptive immune activation source, at different time points of 30 days and 40 days, respectively. The production of ChIFN-γ in the serum of the groups that received the LN-based NDV vaccination resemble the production profile of the oily inactivated NDV vaccinations. An adjuvant inactivated NDV-based vaccination is usually promoted to be incompetent in cell-mediated immune response induction when compared to live-attenuated vaccination. In agreement with these results, the use of a trivalent killed vaccine, containing antigens of an infectious bronchitis virus (IBV), an infectious bursal disease virus (IBDV), and an NDV, administered intradermally to the layer flock, resulted in a strong cell mediation, represented by a delayed hypersensitivity reaction, measured at 48 hours, following a vaccination in the wattles.[21] Additionally, Rauw *et al*. have demonstrated an increment in ChIFN- production by *ex-vivo* recall stimulation for the splenocytes and peripheral blood lymphocytes after an NDV vaccination.[24,25] The differential production of ChIFN-γ among groups indicated the importance of this cytokine in the protective immunity to NDV. The late production peaks can be due to the activation of adaptive CMI. Similarly, in mice, it took CD8+ T cells 21 days post infection with a lymphocytic choriomeningitis virus to be able to accelerate antigen-triggered IFN-γ synthesis.[29]

There was no literature available for the use of an *in vivo* stimulation with inactivated NDV antigens on an immune response activation assessment. In order to assess the post-vaccination immune response, researchers typically either perform a challenge with a live velogenic NDV or rely on an *in vitro* stimulation of splenocte cell culture with inactivated or live NDV. The need for challenging experiments is still important to evaluate the potency and the efficacy of vaccines. However, this *in vivo* stimulation method might be valuable when the purpose is only to assess the immune response of birds to any vaccine component. The *in vivo* stimulation resulted in ChIFN-γ production in the LN-NDV above the cytokine levels detected in the control group (Figure 2B). The behavior of the rest of other experimental groups revealed that exposure to NDV lacks the ability to provoke a strong CMI memory response. It has been demonstrated that *Paramyxoviridae* have evolved specific proteins that inhibit interferon (IFN)-induced innate antiviral responses through the direct inhibition of cellular STAT proteins.[30] The V proteins encoded by NDV block IFN- signaling by targeting STAT1 for degradation. Although further investigation is needed, we propose that the quick reduction of ChIFN-γ levels within 2 weeks after the *in vivo* stimulation is probably due to an inhibitory mechanism that resembles the one used by NDV through V proteins on an IFN- signaling pathway.[31]

Conclusions

Finally, the analysis of different conventional NDV vaccines revealed that both oil-NDV and live-NDV vaccines were able to provoke strong humoral as well as cell-mediated immune responses. The liposomal-based vaccination might fit in programs that aim specifically to strengthen or boost the CMI against NDV without overwhelming the stimulation of primary antibody responses. Further research is needed to evaluate and improve viral liposome loading and stability and to potentially implement procedures to evaluate liposome vaccine protection efficacy and the daily precise monitoring of IFN-g production.

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