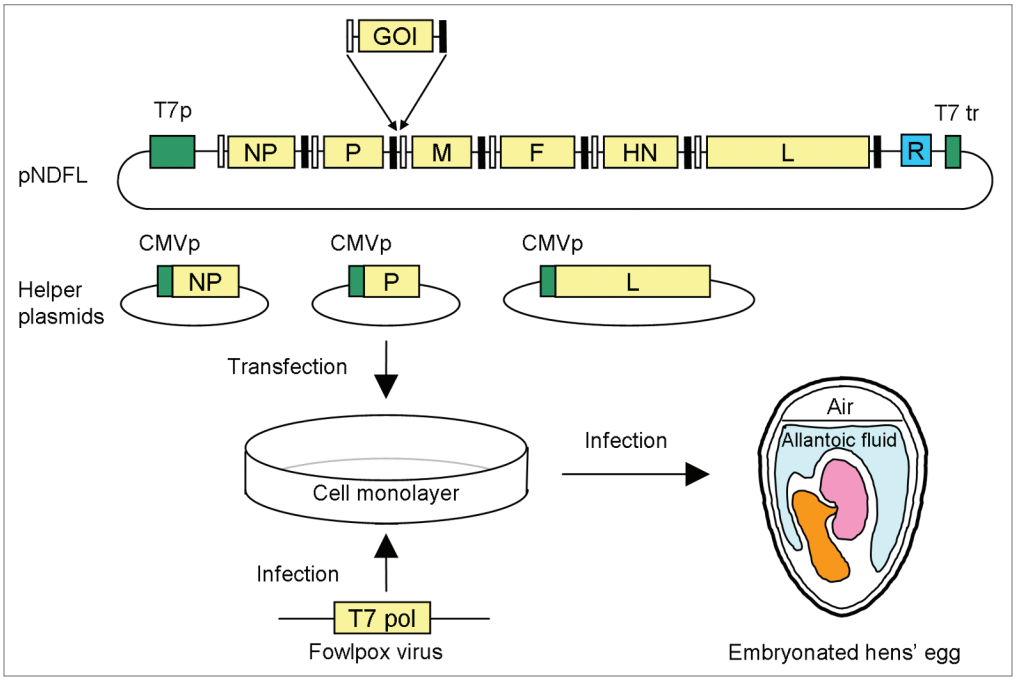

Figure 1. Construction of recombinant NDV strains. Protein coding regions (yellow bars) of foreign genes of interest (GOI) containing suitable additional gene-start and gene-end transcription regulation sequences are inserted between the P and M genes of plasmid pNDFL encoding the complete antigenomic RNA of NDV strain LaSota. The gene-start and gene-end transcription signals flanking each gene are shown as white and black boxes, respectively. The NDV genome can be transcribed from this plasmid using T7 promoter (T7 p) and terminator (T7 tr) sequences and a self-cleaving ribozyme site (R) that ensures generation of the correct 5' end. Infectious rNDV is generated by infection of cells with a fowlpox virus to supply T7 polymerase and subsequent transfection with pNDFL and three helper plasmids that encode NP, P and L proteins under control of the CMV promoter (CMVp). Infectious rNDV is further propagated on embryonated hens' eggs.

L proteins. Expression of the viral RNA is driven by T7 polymerase that is either supplied by a recombinant pox virus[4] or a stable cell line.[7] After rescue of small amounts of virus from transfected cells efficient propagation of the virus can be performed in embryonated hens' eggs. By introducing foreign genes flanked by dedicated gene-start and gene-end transcription signals in the full-length cDNA, recombinant NDV-based vectors can be produced.[9]

Effect of Administration Route on Vaccine Efficacy

NDV-vectored vaccines as well as other types of paramyxovirus-based vaccine vectors are generally administered via the respiratory route, by which they generally show good immunogenicity.[10-15] A combined intranasal/intratracheal (IN/IT) route is most often used. Importantly, DiNapoli et al.[12] recently demonstrated that delivery to the lower respiratory tract is required for effective immunization of non-human primates with NDV-based vector vaccines. From these studies, it was concluded that NDV replicates poorly in the upper respiratory tract of primates due to the relatively low temperature at this site, compared to the body temperatures of birds, the natural hosts of NDV.[12] The main interest of the authors of this work, is to develop human vaccines for emerging pathogens such as the SARS coronavirus.[11] For application of NDV-based vector vaccines in humans, delivery to the lower respiratory tract can indeed be realized by using innovative nebulizers. However, for mass applications in livestock, the use of nebulizers is not a practically feasible approach. We were therefore interested in evaluating immune responses elicited by IN administration only. Furthermore, regarding the hypothesis of DiNapoli et al.[12] we also considered the possibility that delivery of NDV by parenteral immunization (i.e., administration by any route other than the alimentary or respiratory tract) could provide the virus with the optimal temperature for replication, resulting in improved immunogenicity.

Our laboratory is evaluating NDV as a vaccine vector for the control of RVFV in livestock. We use the lentogenic LaSota strain for the production of these vaccines.[4] In a first study, calves were immunized via either the intranasal (IN) or the intramuscular (IM) route with a recombinant NDV that produces the RVFV Gn protein. Surprisingly, antibody responses against both the vector and the Gn protein were detected after IM delivery but not after delivery via the IN route.[16] In a subsequent study, we showed that immunization via the IM route with a recombinant NDV that produces both the RVFV Gn and Gc proteins protects mice against RVFV challenge infection. Importantly, a single vaccination with this vaccine was sufficient for the induction of neutralizing antibodies in sheep, the main target species of RVFV.[17] Thus, IM inoculation with our NDV-based experimental RVFV vaccines elicits high levels of antibodies in both sheep and cows and elicits protective immunity in mice.[16,17]

It was, however, unexpected to find that IN inoculation of calves did not result in

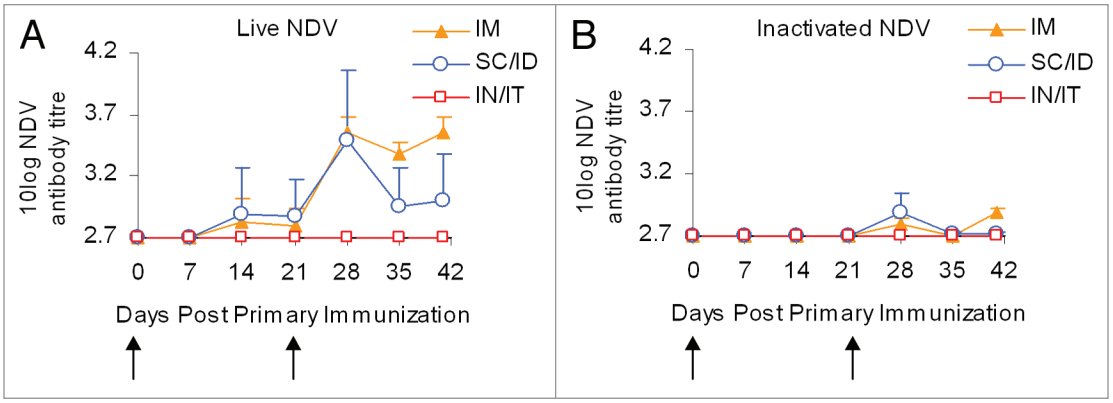

Figure 2. NDV-specific antibody responses in sheep inoculated with wildtype NDV strain LaSota via different immunization routes. Groups of 4 sheep (cross bred Texelaar x Swifter) were inoculated with live (A) or formalin-inactivated (B) NDV strain LaSota that was originally derived from the ATCC (VR-699). The virus was passaged three times in the allantoic cavity of 9- to 11-day-old embryonated hens' eggs and diluted to 10^7 $TCID_{50}$/ml in PBS prior to administration via the IN/IT, SC/ID or IM route in a volume of 2, 1 or 2 ml, respectively. When two different inoculation routes were used (IN/IT or SC/ID), the dose was equally divided between the two inoculations. Vaccinations were performed on days 0 and 21 (arrows). Serum samples were analyzed for the presence of NDV specific antibodies by ELISA.[16] Geometric mean titres and standard deviation are shown. The Y-axis intercepts at 2.7 10log titre, corresponding to the lowest serum dilution analyzed (500-fold).

any detectable antibody response.[16] In the current study, we compared the immunogenicity of wildtype (non-recombinant) NDV strain LaSota when administered to sheep via a combined IN/IT route to two parenteral immunization routes: IM and a combined subcutaneous/intradermal (SC/ID) route. In addition, we compared the immunogenicity of live and inactivated NDV when delivered via these routes. Live NDV administered via the IM route elicited significantly higher ($p < 0.001$ at 28 days post primary immunization) antibody responses as compared to the IN/IT route (**Fig. 2A**). The antibody responses elicited after SC/ID immunization were more comparable to those obtained after IM immunization (**Fig. 2A**). Thus, in line with our previous studies, parenteral immunization with live NDV was more effective when compared to delivery via the respiratory route, in this case a combined IN/IT route.

Our results seem to contrast two earlier studies in which parenteral NDV administration was compared with delivery via the respiratory tract. In the first study, immunization of mice with an NDV vector vaccine via the IN route induced more effective protective immune responses as compared to intravenous (IV) or intraperitoneal (IP) immunization.[18] In another study, immunization with NDV-based vectors that either produce the SARS coronavirus spike protein or the HN protein of human parainfluenza virus type-3 elicited higher antibody titres when delivered via a combined IN/IT route than when the same vaccines were delivered via the SC route.[12] It is important to note, however, that these studies differ from our studies with respect to the parenteral vaccination routes (IV, IP or SC versus IM or SC/ID), the inoculated species (mice or non-human primates versus cow or sheep) as well as the NDV strain used. Additional studies are clearly required to further elucidate the immune responses elicited by NDV inoculation via different routes in mammals.

The NDV-based RVFV vaccines described in our previous studies were produced in embryonated hens' eggs. The allantoic fluid of these eggs contained RVFV Gn and Gc proteins,[16,17] which could have contributed to the elicited immune responses independent of virus replication and de novo protein production. To gain insight into the role of injected proteins in the antibody response induced by our vaccines, we compared the antibody responses induced by live NDV with those elicited by formalin-inactivated NDV. The results obtained from this experiment (*cf.* **Fig. 2A and B**) underscore the notion that NDV propagation is essential for high immunogenicity in sheep and thereby show that co-injected protein is of little, if any, influence to the antibody response elicited.

Vaccine Safety

Although the immunogenicity of NDV-based vector vaccines depends on virus replication in the inoculated mammal, spread in these unnatural hosts was previously shown to be highly restricted or even absent.[10,11,19] In accordance with these findings, accidental NDV infections of mammals are rare and if these do occur they mostly remain subclinical.[19] The attenuation of NDV in mammals is primarily caused by the species-specificity of the interferon antagonist function of the V protein.[2] Consequently, spread of both lentogenic and mesogenic NDV in mammals is highly restricted and the use of both pathotypes as vaccine vectors for application in mammals is therefore considered acceptable with respect to safety for the inoculated mammal.[19] The dependence of lentogenic strains on trypsin-like proteases for infectivity makes it more difficult to grow these viruses in tissue culture. Since the introduction of foreign genes generally results in further attenuation of the viruses, it is sometimes challenging to produce recombinant lentogenic viruses with large foreign gene inserts. Therefore, vector vaccines are often based on mesogenic strains or lentogenic strains with modified F cleavage sites that confer trypsin independence. The safety for the domesticated poultry industry must, however, also be taken into consideration when applying

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