A prime-boost vaccination protocol optimizes immune responses against the nucleocapsid protein of the SARS coronavirus

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Summary

Severe acute respiratory syndrome (SARS) is a serious infectious disease caused by the SARS coronavirus. We assessed the potential of prime-boost vaccination protocols based on the nucleocapsid (NC) protein co-administered with a derivative of the mucosal adjuvant MALP-2 or expressed by modified Vaccinia virus Ankara (MVA-NC) to stimulate humoral and cellular immune responses at systemic and mucosal levels. The obtained results demonstrated that strong immune responses can be elicited both at systemic and mucosal levels following a heterologous prime-boost vaccination protocol consisting in priming with NC protein add-mixed with MALP-2 by intranasal route and boosting with MVA-NC by intramuscular route.

Keywords: SARS, modified Vaccinia virus Ankara, adjuvant, mucosal immunization, prime boost.

Introduction

The severe acute respiratory syndrome (SARS), an emerging infectious disease of humans, is caused by the SARS coronavirus (SARS-Cov), which is characterized by a high degree of transmissibility and mortality [1-3]. The modes of SARS-CoV transmission include shedding of the virus from the respiratory tract via droplets, close contact and fomites [4]. The incubation period of SARS ranges from 2 to 16 days, with a mean incubation time of 6.4 days [5]. Infected people develop influenza-like symptoms with the lung as target organ (e.g., high-grade fever, chills, myalgia, headache, and dyspnea), resulting in an up to 40% mortality rate, especially in older patients [6;7]. As evidenced by sporadic cases reported in the late 2003 and in 2004 (http://www.wpro.who.int/sars/docs/pressreleases/pr_31122003.asp and http://www.who.int/csr/don/2004_05_18a /en/index.html), SARS-CoV still remains a constant threat for epidemic outbreaks. Therefore, there is an urgent need for the development of an effective vaccine able to contain future SARS outbreaks.

So far, the SARS virus seems remarkably invariant. The genome sequences of 14 isolates from SARS patients in Singapore, Toronto, China and Hong Kong have not revealed any changes. This basically may give the chance to develop a vaccine for worldwide use. In order to generate a vaccine providing protective immunity against the SARS-CoV with pandemic potential, it would be critical to promote the elicitation of a long lasting immunity in a high percentage of vaccinated individuals.

Several vaccination approaches have been tested in experimental animal models. However, there are no vaccines available against this agent yet for human use. Currently, three main strategies have been pursued in order to develop anti-SARS vaccines. Namely, inactivated virus-based, spike protein (S)-based and nucleocapsid (NC)-based vaccines. Inactivated vaccines and immunization with S-protein-based

vaccines seem to stimulate high titers of neutralizing antibodies, as well as cellular immunity. However, they were able to provide only a limited degree of protection against infection [8-10].

Interestingly, experiences from vaccines against infectious bronchitis virus of chickens (IBV), which are the most successful and most widely used vaccines against coronaviruses, indicated that the protection against infection provided by inactivated or live attenuated vaccines via neutralizing antibodies is short lived (decline apparent after 9 weeks) and single application of the vaccines resulted in protection of less than 50% of the chicken [11]. Similarly, immunization with the IBV large S protein induced virus neutralizing antibodies, but the percentage of protected chicken was between 50-90%. On the other hand, initial immunization studies with the IBV NC protein applied as protein or DNA vaccine also induced protective immunity, obviously provided by CD8 T-cells and possibly by local antibodies [11;12]. Similar observations have been reported also from other coronavirus infections in animals [13;14]. Even if the basis for this protection against coronavirus infection is not well understood, it could be a basis for an alternative vaccine development against the SARS-CoV by using the NC protein.

To promote protection against both infection and disease of the lung tissue infecting SARS-CoV, it would be essential to stimulate also the elicitation of robust mucosal immune responses at the entry site. Therefore, in the present work we evaluated different NC-based vaccination protocols for their ability to stimulate humoral and cellular immune responses at systemic and mucosal levels. To this end, mice were immunized with homologous or heterologous prime-boost vaccination regimens based on recombinant NC protein co-administered with the mucosal adjuvant MALP-2 or expressed by the highly attenuated modified Vaccinia Ankara (MVA) virus. The obtained results demonstrated that strong immune responses can be elicited both at systemic and mucosal levels following a heterologous prime-boost vaccination protocol consisting

in priming with NC protein add-mixed with a MALP-2 derivative by intranasal route and boosting with MVA-NC by intramuscular route. These data suggest that this prime-boost approach might be useful to confer protection against SARS.

Materials and methods

Production of MVA-NC and His-tagged-NC fusion protein

To generate the MVA-NC construct the sequence of the SARS-NC gene has been cloned into the *Sma*I site of MVA transfer plasmid pIII-E3L-mPH5 [15]. After transfection/infection of BHK cells with pIII-E3L-mPH5-SARS-NC and MVA∆E3L, clonally pure MVA-SARS-NC has been selected by consecutive rounds of plaque purification in CEF. Subsequently high titer vaccine stocks have been prepared from infected CEF cultures by purification through sucrose cushions [16]. His-tagged SARS-CoV NC protein was expressed in *Escherichia coli* BL21 (Invitrogen) after induction with 0.02% L-arabinose. The bacterial cells were lysed (6 M GdnHCl, 20 mM Na_3PO_4, 500 mM NaCl, pH 7.8) and lysates were loaded on a Ni-nitrilotriacetic acid affinity column (Invitrogen). After several washing steps, His-tagged proteins were recovered with elution buffer containing 8 M urea, 20 mM Na_3PO_4, 500 mM imidazole (pH 6.3). The eluted protein was refolded by dialysis against sodium acetate (pH 5.2) and quantified by Bradford assay (Coomassie protein assay reagent; Pierce; Bonn, Germany). The LPS content of the protein preparations was below 500 pg/µg, as determined by the HEK-Blue™ LPS Detection kit (InvivoGen; San Diego, USA).

Immunization and sample collection

BALB/c mice (*n* = 5; Harlan Winkelmann GmbH, Borchen, Germany) were immunized by intranasal or intramuscular route on day 0 and 14 (see Table 1). Animals received 10^8 PFU of MVA-NC or MVA alone and 10 µg of the NC protein, respectively. When immunizing with the NC protein by the mucosal route, vaccine formulations contained 0.5 µg of a pegylated synthetic derivative of the TLR2/6 agonist MALP-2 (macrophage-activating lipopeptide of 2 kDa from *Mycoplasma fermentans*) as mucosal adjuvant [17;18]. This molecule is composed by a double fatty acid with a substituted cystein which is bounded via a carboxyl group to a monomethoxy polyethylene glycol rest. The resulting conjugate is soluble in water, bio-compatible and non immunogenic. Parenteral vaccination with the NC protein was performed using alum as adjuvant. Serum samples were collected on days -1, 13 and 25, whereas broncho-alveolar lavages and spleen cells were sampled on day 25.

Evaluation of NC-specific antibody responses

Sera were tested for NC-specific IgG by ELISA. In brief, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc Roskilde, Denmark) were coated with 50 µl/well of NC (2 µg/ml) in coating buffer (bicarbonate, pH 9.6). After overnight incubation at 4°C, plates were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.4) for 1 h at 37°C. Serial 2-fold dilutions of pooled sera in PBS/1% BSA were added (100 µl/well), and plates were incubated for 2 h at 37°C. After four washes, secondary biotinylated antibodies were added followed by 1 h incubation at 37°C. After six washes, 50 µl/well (1:1000) of peroxidase conjugated streptavidin (Pharmingen) was added, and plates were further incubated for 45 min at room temperature. After final six washes, the substrate ABTS [2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer containing 0.1% H_2O_2 was added, and plates were

incubated for 30 min at room temperature. The end-point titers were expressed as the last dilution giving an A_{405} of 0.1 U above the A_{405} of negative controls. The IgG isotypes present in serum samples were determined by ELISA as previously described [19], using as secondary antibodies biotin-conjugated rat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, UK).

Determination of total and NC-specific IgA

The amounts of total and NC-specific IgA present in broncho-alveolar and vaginal lavages of individual mice were determined by ELISA, as previously described [20]. To establish the IgA standard curve, plates coated with goat anti-mouse IgA (Sigma Chemie) as capture antibody were incubated with serial dilutions of purified mouse IgA (Dianova, Hamburg, Germany). As secondary antibody, biotinylated goat anti-mouse IgA (Sigma Chemie) was used, plates were developed as described above. To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized and expressed as percentage of NC-specific IgA with respect to the total amount of IgA present in the sample.

Proliferation assays

To investigate T-cell proliferation, spleen cells were isolated at day 25 after immunization, pooled within each group and adjusted to 5×10^6 cells/ml in complete medium (RPMI supplemented with 10% fetal calf serum). Cells were then seeded at 100 µl/well in flat-bottomed 96-well micro titer plates (Nunc), which were incubated for 4 days in the presence of 10, 20 and 40 µg/ml of NC protein, respectively. During the final 18 h of culture, 1 µCi of [^{3}H]-thymidine (Amersham International, Freiburg, Germany) was added to each well. Afterwards, cells were harvested on paper filters (Filtermat A;

Wallac, Freiburg, Germany) by using a cell harvester (Inotech, Wohlen, Switzerland) and the amount of [^{3}H]-thymidine incorporated into the DNA of proliferating cells was determined by a scintillation counter (Wallac 1450, Micro-Trilux).

ELISPOT

To determine the amount of IFNγ, IL-2 and IL-4 secreting cells, the murine IFNγ, IL-2 and IL-4 ELISpot kits (BD Pharmingen) were used according to the manufacturer's instructions. In brief, flat bottomed 96-well plates with a 0.45 µm hydrophobic High Protein Binding Immobilon-P-Membrane were coated with the corresponding capture antibody (anti-IFNγ, anti- IL-2 or anti-IL-4) and stored over night at 4°C. To remove unbound capture antibodies plates were washed once and unspecific binding sites were saturated by incubating with blocking solution for 2 h at room temperature (RT). Afterwards, splenocytes (1x10^6/well) were incubated at 37°C in an atmosphere containing 5% CO_2 for 24 h (IFN-γ) or 48 h (IL-2 and IL-4) in the absence or presence of the NC protein (20 µg/ml). As negative control, cells were cultured in RPMI complete medium without stimulants. For the stimulation of 0.5 x 10^6 cells/well, ConA (positive control) was diluted in RPMI (final concentration 5 µg/ml). After incubation, plates were washed twice with deionised water including a soaking for 5 min followed by three washes using wash buffer (0.05% Tween 20/PBS). To detect the captured cytokines, the corresponding biotinylated detection antibody was added and incubated for 2 h at RT. After additional three washing steps, horseradish peroxidase (HRP) was added and plates were incubated for 1 h at RT. After final four washing steps spots were developed for 5 to 60 min using substrate solution (333.3 µl of AEC stock solution +10 ml 0.1 mM acetate solution + 5 µl H_2O_2). The reaction was stopped by washing the plates with deionised water. After drying the plates for 2 h at RT in the dark, spot forming units

(SFU) were counted using the automated ELISPOT ImmunoSpot S4 Analyzer (CTL-Europe GmbH, Aalen, Germany) and analyzed using the C.T.L. ImmunoSpot image analyzer software v3.2. Results are expressed as SFU for 1 x 10^6 spleen cells.. The spots produced by the non re-stimulated cells served as background and were subtracted from the spots produced by the re-stimulated cells. Only ratios stimulated versus non-stimulated spots above >2 were further analyzed.

Statistical analysis

The significance of the differences observed in the immunogenicity studies was analyzed using the Student´s unpaired *t* test or the non-parametric Mann-Whitney rank sum test (SigmaStat for Windows V3.10, Systat Software, Inc., San Jose, USA), $P \leq 0.05$ was considered as significant.

Results

Generation and characterization of the vaccine strain MVA-SARS-NC and the SARS-NC protein

MVA-SARS-NC was generated by insertion of the SARS-NC expression cassette into the transfer plasmid pIII-E3L-mPH5 and direct homologous recombination to deletion site III within the genome of MVAΔE3L. (Fig.1A). PCR analysis of viral genomes confirmed genetic purity and stability of the newly generated vaccine strain MVA-SARS-NC (Fig. 1B). As expected, the E3L locus resulted in a PCR product of 1,400 bp (lane 2) in comparison to the 1,800 bp of the MVA wild type strain (lane 1). PCR of the insertion site (deletion III) revealed a fragment of 2,700 bp, which corresponds to the insertion of

SARS-NC and E3L (lane 4), whereas MVA wild type yields a product of 700 bp, as expected for the empty insertion site (lane 5). Furthermore, correct expression of the SARS-NC protein was assessed after infection of chicken embryo fibroblast cells with MVA-SARS-NC by Western blot analysis (Fig.1C). Only in lysates of cells infected with the recombinant MVA a specific signal of 47 kDa (lane 3) corresponding to NC was detected, whereas MVA or mock infection did not lead to any signal.

Recombinant NC was cloned and expressed in *E. coli* as an N-terminally poly-histidine tagged protein (see material and methods) [21]. Purification using IMAC affinity columns and refolding yielded high amounts of soluble and very pure protein (>95%) with no detectable degradation products as judged by SDS-PAGE and Coomassie-blue staining (data not shown). Proper reactivity as antigen in immunoblot was assessed using various polyclonal anti-NC antisera, including also sera from human SARS patients (data not shown). Immunogenicity of recombinant NC was tested by immunization of rabbits which resulted in antisera detecting NC very specifically [22].

Immunization with the NC protein stimulates strong NC-specific antibody responses

The stimulation of protective antibody responses is essential in order to efficiently prevent viral infection. Thus, we analyzed the immunogenic potential of different vaccine formulations in different immunization protocols. All vaccine formulations (Table 1) were well tolerated by the animals, which do not show alterations in the weight, food intake or general behavior. Furthermore, we have not observed any obvious pathologic modifications of organs, such as lung, liver or spleen of the vaccinated animals (data not shown). High titers of NC-specific antibodies were stimulated after intramuscular immunization with the NC protein co-administered with alum on day 0 and 14. Similar

IgG responses were observed following a protocol in which mice were primed with NC plus alum on day 0, followed by an heterologous boost with MVA-NC by intramuscular route on day 14 (Fig.2A). In contrast, animals vaccinated by the intranasal route showed poor IgG responses and a significant increment in NC-specific antibody titers was only observed in the group in which mice were primed with NC co-administered with MALP-2 by intranasal route, followed by an heterologous intramuscular boost of MVA-NC (Fig.2A).

Interestingly, while intramuscular immunization of mice with NC co-administered with alum elicited a Th2 immune response, as indicated by the dominant IgG1 isotype, a mixed Th1-Th2 response was stimulated after boosting with MVA-NC (Fig.2B). Furthermore, when immunizing animals by the intranasal route with NC co-administered with MALP-2 followed by an intramuscular injection of MVA-NC, a Th1-dominant response was stimulated, as indicated by the increase in the IgG2a isotype (Fig.2B).

We further evaluated the elicitation of mucosal responses in vaccinated animals. A significant ($P \leq 0.04$) increase in the levels of NC-specific secretory IgA (sIgA) was only detected in broncho-alveolar lavages of mice vaccinated twice with NC co-administered with MALP-2 or primed with NC+MALP-2 and boosted with MVA-NC (Fig.2C). No sIgA have been detected in mice immunized by the parenteral route (data not shown).

Immunization with the NC protein stimulates strong NC-specific cellular responses

Following immunization by the parenteral route, the strongest cellular responses were obtained when priming with NC admixed with alum was followed by a booster injection of MVA-NC (Fig. 3, $P \leq 0.001$). On the other hand, when comparing groups of mice immunized by the intranasal route, the strongest proliferative responses were observed

in animals receiving two times NC protein co-administered with MALP-2 (Fig. 3). However, intranasal vaccination with only a single dose of NC protein co-administered with MALP-2 also stimulated strong cellular responses, when boosted with MVA-NC by intramuscular route (Fig. 3). The observed results were statistically significant in respect to values of mice receiving NC protein alone ($P \leq 0.001$). No significant differences were observed comparing the group receiving NC alone and the control groups, receiving PBS and MVA (Fig. 3).

Then, we investigated the cytokines produced by splenocytes from vaccinated animals. The number of IFNγ-producing cells was only significantly increased in animals receiving the prime-boost protocols (Fig. 4A). The highest numbers were detected in mice primed with recombinant NC protein with alum and boosted with MVA-NC by intramuscular route, followed by those primed with NC protein and MALP-2 by intranasal route and boosted with MVA-NC. Interestingly, no differences were observed between animals receiving empty MVA and those receiving two doses of MVA-NC by intramuscular route. On the other hand, intranasal immunization with NC co-administered with MALP-2 followed by either a homologous or heterologous (i.e., MVA-NC) boost resulted in a significant increment in the number of IL-2 secreting cells (i.e., homologous boost: $P = 0.002$, heterologous boost: $P \leq 0.05$; Fig. 4B). To a lesser extent, IL-2 secreting cells were also increased in mice receiving the systemic prime-boost protocol. Finally, homologous parenteral immunization with NC admixed with alum was the most efficient regime to enhance IL-4 production ($P = 0.006$) when compared with control groups, followed by the heterologous and homologous prime-boost mucosal vaccination protocols (Fig. 4C).

Discussion

The majority of the experimental vaccines recently developed against the SARS-CoV aimed at initiating a sterilizing immunity via virus neutralizing antibodies. This included the use of inactivated SARS virus and the S protein, as well as different delivery systems thereof, such as DNA and viral vectors (Adenovirus, Rabies, Parainfluenzae, MVA-Vaccinia). These animal trials demonstrated the potential of the S protein for promoting the induction of neutralizing antibodies and, in some cases, also protection against viral challenge [23;24]. Several immunization studies have also been performed with the NC protein of SARS CoV applied as protein, DNA or in viral vectors (e.g., Adenovirus, MVA). The good immunogenicity of the NC antigen could be shown by the induction of antibody and/or T-cell responses [25-32]. In this work, we performed a side-by-side comparison of different strategies to optimize the immune responses against the NC-protein of the SARS-CoV. The live attenuated MVA vector was used for protein delivery to achieve good and long lasting systemic and local T-cell responses [33]. For the induction of humoral immune responses, recombinant NC protein was co-administered with a systemic or a mucosal adjuvant by different routes (intramuscular and intranasal). Special attempts were also carried out to fine-tune and broaden the elicited responses by combining the different candidates in prime-boost vaccination protocols. The inclusion of MVA-NC in the heterologous prime-boost protocols was based in the well-known capacity of recombinant MVA to boost cellular responses in a previously primed host. Prime-boost regimens using different viral vectors expressing the same recombinant antigen proved very efficient in enhancing the target antigen specific immune responses in comparison to homologous vector immunizations, most likely because these approaches circumvent the problem of anti-vector immunity [34-36]. In this context, priming with NC protein co-administered with a mucosal adjuvant,

followed by a boost with MVA-NC appealed as the more promising approach to promote both humoral and cell mediated responses against NC. This would be expected to lead to a better control of viral infection at both mucosal and systemic level.

Intramuscular immunization of mice with the NC protein admixed with alum stimulated strong humoral immune responses with no significant differences between the conventional (twice NC protein + alum) and the prime-boost (NC protein + alum prime, MVA-NC boost) protocols (Table 1). However, in mice receiving the prime-boost protocol, the T helper response pattern was switched from a dominant Th2 to a mixed Th1-Th2 respect to animals vaccinated with NC + alum alone. In contrast to that observed at humoral level, significantly stronger proliferative responses in comparison to the control mice receiving MVA alone ($P \leq 0.001$) were only observed in animals receiving the prime-boost protocol. The same was true in terms of the observed increment in the number of IFN-γ producing cells. Hence, the MVA-based boost was critical to modulate the elicited immune response towards a mixed Th1/Th2 response pattern, as well as to optimize the cellular responses. Nevertheless, vaccination by the parenteral route was not able to induce relevant amounts of antibodies in the respiratory tract.

On the other hand, when immunizing by the intranasal route, although the overall responses were slightly weaker, serum IgG responses were observed in animals receiving either the homologous (NC protein + MALP-2 based) or the heterologous (NC protein + MALP-2 intranasal priming and MVA-NC intramuscular based) vaccination protocols. Both protocols were also proven effective in terms of stimulating local production of NC-specific sIgA in the broncho-alveolar lavages of vaccinated mice ($P \leq 0.04$). Interestingly, these vaccination protocols were even more effective than the parenteral prime-boost protocols in terms of stimulating proliferative responses ($P \leq 0.001$), with the strongest response in mice vaccinated twice with NC + MALP-2.

However, when analyzing the cytokine production of splenocytes from immunized mice, a significant increment in the number of IFN-γ producing cells was only observed in mice receiving the heterologous prime-boost protocols respect to the control groups ($P \leq 0.001$). Furthermore, the highest increment in the number of IL-2-producing cells in response to antigen stimulation was detected in mice receiving the mucosal formulations, followed by those receiving the parenteral heterologous prime-boost protocol ($P \leq 0.002$). As expected, IL-4 production was maximal in mice immunized with NC protein and alum by intramuscular route ($P \leq 0.01$). Thus, our data demonstrated that the heterologous prime boost protocol based on intranasal priming with NC+MALP-2 and intramuscular boosting with MVA-NC is able to induce good humoral and cellular immune responses at systemic and mucosal level. The strength of the observed immune responses was similar or even better than that reported in former studies (e.g., using DNA- or Adenovirus-based approaches alone) [27;30]. Furthermore, these experiments indicate that one MVA-NC boost is enough to stimulate a single NC protein prime, and is clearly superior to a homologous MVA-NC prime/boost [32].

There is a general consensus that vaccination by the mucosal route offers several advantages over parenteral immunization since mucosal vaccination promotes immune responses at both systemic and mucosal levels, whereas parenteral immunization only stimulates systemic responses. Recently, it has been shown that in fatal SARS cases predominantly alveolar pneumocytes are infected, as demonstrated by the detection of the SARS-CoV nucleoprotein [37]. Thus, the stimulation of an efficient local immune response at the portal of entry is highly desirable for a pathogen like the SARS-CoV. In fact, this strategy can lead not only to protection against disease but also against infection (i.e., colonization), thereby contributing to reduce the risk of horizontal transmission. Although the stimulation of S protein-specific antibody responses seems to be essential in order to protect against SARS infection [24;38;39], there is the

possibility of antibody-dependent enhancement and exacerbation of the disease, which in turn underlines the need for alternative vaccines [40]. The use of NC protein as an alternative vaccine approach may also become attractive by the fact that nucleoproteins from different animal coronaviruses share antigenic cross-reactivity, which may be of interest for a vaccine against a “human” virus with an animal origin [41].

The present study shows that an approach characterize by mucosal priming with protein and systemic boosting with MVA-NC is superior to either the homologous mucosal or the parenteral regimens, in that it was the only one inducing serum antibodies, lung sIgA and cellular responses. Usually, the production of IFN-γ, a Th1 cytokine associated with cell-mediated immunity and resistance to intracellular pathogens, is dramatically increased during SARS-Cov infection, whereas IL-4, the dominant Th2 cytokine, which promotes humoral immunity that protects against extra cellular microbial infections, is decreased after onset of SARS-Cov infection. There is also an up-regulation of pro-inflammatory cytokines, such as tumor necrosis factor alpha, IL-1ß and IL-6, which might enhance disease outcome [42;43]. Thus, the stimulation of a Th1 dominant response with increased number of IL-2 and IFN-γ producing cells by the mucosal/systemic protein/MVA-based prime-boost protocol appeals as an additional advantage of this promising vaccination approach.

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FIGURE LEGENDS

Figure 1 Construction of MVA-SARS-NC. (A) Schematic representation of the MVA-ΔE3L genome and transfer plasmid for insertion into deletion III. (B) PCR-analysis of MVA-SARS-NC at the E3L locus and insertion site (Del III). Viral DNA has been extracted and subjected to MVA specific PCR using primer pairs for the E3L locus and site of deletion III, respectively. Arrowhead indicates SARS-NC specific signal. (C) Expression of SARS-NC in chicken embryo fibroblasts after infection with recombinant MVA. Sub-confluent monolayer were infected with 10 IU MVA or MVA-SARS-NC and harvested after 24 h in Laemmli-buffer. Lysates of infected cells were subjected to 10% polyacrylamide sodium dodecyl sulfate gel electrophoresis and proteins transferred to nitrocellulose membranes. Detection was carried out using rabbit anti-SN antibodies at a 1:2000 dilution. *E. coli* expressed SARS-NC protein (NC) served as positive control, MVA infected or mock infected chicken embryo fibroblasts (wt, C) served as negative controls. Arrowhead indicates SARS-NC specific signal. kD: molecular mass standards.

Figure 2 Humoral immune responses in mice vaccinated with the NC protein of SARS. A) Analysis of NC-specific IgG titers in sera of vaccinated mice. The end-point titres were expressed as the mean of the reciprocal $\log_{10}$ of the last dilution (end point dilution) of sera giving an A405 of 0.1 U above the values of negative controls within each immunization group. B) Detection of the NC-specific IgG isotype present in the sera from vaccinated mice 25 days after the first immunization. C) Antigen-specific IgA antibodies in broncho-alveolar lavages of immunized mice. Results are expressed as the percentage of antigen-specific IgA antibodies with respect to total IgA. SEM is indicated by vertical lines. The obtained results are statistically significant (Student´s *t*-test) when compared with the values for the control groups (NC and MVA alone) at $P \leq 0.03$ (*) and $P \leq 0.04$ (**), respectively.

Figure 3 Cellular immune responses stimulated in vaccinated animals. Results are expressed as the difference between values (average of triplicates) from stimulated and non-stimulated samples (cpm). SEM is indicated by vertical lines. The obtained results are statistically significant (Student´s *t*-test) when compared with the values for the control groups (i.e. PBS and empty MVA) at $P \leq 0.001$ (*).

Figure 4 Characterization of cytokine producing cells stimulated by the NC protein. Spleen-derived lymphocytes were cultured for 16 h with 20 ug/ml of the protein. (A) INF-γ (B) IL-2 and (C) IL-4 production was determined by ELISPOT. The SEM is indicated by vertical lines. Results are expressed as spots forming units per 10^6 cells above background. The observed differences were statistically significant in comparison with the control groups PBS and MVA alone (*) at $P \leq 0.001$ (INF-γ), $P \leq 0.002$ (IL-2) and $P \leq 0.02$ (IL-4).

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