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# A new subunit vaccine based on nucleoprotein nanoparticles confers partial clinical and virological protection in calves against bovine respiratory syncytial virus

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ABSTRACT

Human and bovine respiratory syncytial viruses (HRSV and BRSV) are two closely related, worldwide prevalent viruses that are the leading cause of severe airway disease in children and calves, respectively. Efficacy of commercial bovine vaccines needs improvement and no human vaccine is licensed yet. We reported that nasal vaccination with the HRSV nucleoprotein produced as recombinant ring-shaped nanoparticles ($N^{SRS}$) protects mice against a viral challenge with HRSV. The aim of this work was to evaluate this new vaccine that uses a conserved viral antigen, in calves, natural hosts for BRSV. Calves, free of colostral or natural anti-BRSV antibodies, were vaccinated with $N^{SRS}$ either intramuscularly, or both intramuscularly and intranasally using Montanide™ ISA71 and IMS4132 as adjuvants and challenged with BRSV. All vaccinated calves developed anti-N antibodies in blood and nasal secretions and N-specific cellular immunity in local lymph nodes. Clinical monitoring post-challenge demonstrated moderate respiratory pathology with local lung tissue consolidations for the non-vaccinated calves that were significantly reduced in the vaccinated calves. Vaccinated calves had lower viral loads than the non-vaccinated control calves. Thus $N^{SRS}$ vaccination in calves provided cross-protective immunity against BRSV infection without adverse inflammatory reaction.



## 1. Introduction

Human and bovine respiratory syncytial viruses (HRSV and BRSV) are two closely related, highly infectious, worldwide prevalent viruses that are the leading cause of acute lower respiratory tract disease in children and calves, respectively [1]. RSV is a negative strand RNA virus that belongs to the *Pneumovirus* genus within the *Paramyxoviridae* family. The frequency and seriousness of BRSV diseases are regarded as the principal health problem in calf rearing worldwide and are responsible for large economic losses in dairy and beef farming. The frequency of BRSV infections is very high in cattle less than 1-year-old and the virus may be responsible for more than 60% of the epizootic respiratory diseases observed in dairy herds and up to 70% in beef herds [2]. Mortality caused by BRSV infections ranges generally between 0.5% and 3% but can reach up to 20% in some outbreaks [3]. The control of BRSV infections is a high priority for animal health and farming organizations, not only for economic reasons, but also due to the impact on animal welfare. Several commercial BRSV vaccines, including modified-live virus and inactivated single fraction are available for use in cattle. Their efficacy needs improvement in terms of duration of protection, clinical and virological protection. Even though the commercial bovine vaccines probably have reduced the prevalence of infection, BRSV continues to circulate in cattle populations.

No commercial vaccine is available against HRSV, a pathogen of major importance in infants. HRSV induced-bronchiolitis is the most common cause of infant hospitalization in industrialized countries and is a suspected risk factor of recurrent wheeze and asthma in later life [4]. The main reason for the lack of human

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vaccine is the dramatic failure in the late sixties of a formalin inactivated HRSV vaccine that not only failed to protect against subsequent infection but also induced exacerbated disease in children [5]. Vaccine augmented disease has also been described in calves and some commercial BRSV vaccines were withdrawn from the market for that reason [6,7]. Other obstacles to vaccination exist, such as the need to immunize immunologically immature young infants and the presence of maternal antibodies that can have a strong suppressive effect on the outcome of vaccination, both in animals and humans.

Experimental models in rodents have been developed to find out the immune correlates of protection versus disease exacerbation and help the conception of safe RSV vaccines. Altogether these studies highlight the delicate tuning between cytotoxic anti-viral CD8 T cells and RSV-specific antibodies, which, although generally protective against RSV infection, may both have deleterious effect [8]. For instance, poorly neutralizing antibodies with low avidity for the protective RSV epitopes can lead to enhanced respiratory syncytial virus disease [9]. The RSV-F and G glycoproteins, which are situated at the surface of the virions, are the targets of neutralizing antibodies. Research on HRSV subunit vaccines has essentially focused on these two proteins, by using chimeric FG glycoprotein, full-length F proteins or a recombinant protein containing the central antigenic domain of the HRSV G protein fused to the C-terminal end of the albumin-binding domain of the streptococcal G protein [10]. However, recombinant G and F or chimeric FG were often found to cause enhancement of lung pathology upon RSV challenge, in association with the priming of Th2 cells [1,11].

In addition to the risk of disease exacerbation by vaccination, another critical issue for human or bovine RSV vaccination is the variability of the viral isolates circulating worldwide. An effective bovine or human vaccine should protect against all of them. The nucleoprotein (N) that covers the viral RNA genome, forming the viral nucleocapsid, is the most conserved of RSV proteins and is a major target of the cellular immune response against RSV [12–14]. Thus, contrary to F or G antigens, N based vaccines offer the possibility of T-cell-mediated cross-protective immunity against circulating RSV. Strategies aimed at using N in a vaccine to stimulate T cell immunity have focused on live-attenuated virus vector and on DNA vaccine. Vaccination of mice with recombinant vaccinia virus encoding the HRSV N protein induced partial protection [15,16]. Similarly, immunization of young calves with a recombinant vaccinia virus expressing the BRSV N protein induced non-neutralizing antibodies and primed BRSV-specific proliferative T response and IFN-γ production that resulted in reduction of viral replication in the upper and lower respiratory tract [17]. DNA immunization by two administrations of plasmids encoding BRSV-F and N proteins primed a strong cell-mediated immunity in calves, which drastically reduced viral replication, clinical signs and pulmonary lesions after a highly virulent challenge [18]. More recently a nucleocapsid-based DNA prime–protein boost vaccination was shown to confer protection against BRSV replication and lung pathology [19].

Compared to DNA, or live-attenuated vector vaccines, subunit vaccines are safer because they do not present the risk of replication/integration of genetically modified material. However subunit vaccines using the nucleoprotein (N) have been poorly investigated, in part because a recombinant N was difficult to produce as a soluble protein. We have set up an original technology to engineer circular nanoparticles composed of 10–11 recombinant N [20], the 3D structures of which have just been solved [21]. These nanoparticles are named $N^{SRS}$ for sub-nucleocapsid ring structures [20]. Intranasal vaccination of mice with HRSV $N^{SRS}$ nanoparticles primes N-specific CD4 and CD8 T cells and significantly reduces titers of RSV in the lungs of mice following HRSV challenge without signs of disease exacerbation [22]. One major limit of the mouse model is the absence of respiratory disease in response to RSV infection. Therefore the objectives of the present study were to evaluate the potency of $N^{SRS}$ as a vaccine in calves that are the natural host for BRSV and that display clinical respiratory symptoms and lung lesions upon infection. Because the N amino acid sequence is highly conserved between bovine and human RSV strains (≈94% amino acid identity), we took it as an opportunity to test whether $N^{SRS}$ from HRSV strain Long would provide cross-protective immunity against viral challenge with a BRSV strain. The data presented in this study showed that vaccination with the nano-rings $N^{SRS}$ partially protected against both respiratory disease and virus replication upon BRSV challenge without signs of vaccine-mediated disease exacerbation.

## 2. Materials and methods

### 2.1. Plasmid constructions

The pGEX-PCT (coding for residues 161–241 of the C-terminal fragment of the phosphoprotein, named PCT, fused to glutathione-S-transferase) and pET-N plasmids which contain sequences from the HRSV Long strain have been described previously [20]. Random-primed cDNA synthesis was done using SuperscriptII (GIBCO, Invitrogen Life Science, France) and 1 μg of total cytoplasmic RNA isolated from bovine Turbinate cells infected with the A2Gelfi strain of BRSV [23,24]. The cDNAs were amplified by PCR with high fidelity PfuTurbo Polymerase (5U, Stratagene, Agilent Technologies, France) and 100 ng of the following primers:

N-A2G+: 5′-GAGGAGCCATGGCTCTTAGCAAGGTCAAACTAAATG-3′;
N-A2G−: 5′-GAGGAGCTCGAGTCACAATTCCACATCATTATCTTTGG-3′;
P-A2G+: 5′-GAGGGATCCATGGCTGCTCGTGATGGTATAAGAGATG-CCATG-3′;
P-A2G−: 5′-GAGGAGCTCGAGTCAGAAATCTTCAAGTGATAGATCA-TTGTC-3′.

The amplified full-length cDNA coding for BRSV N protein was digested subsequently by NcoI and XhoI and cloned into pET-28a(+) vector (Novagen, Merck Chemicals products, Germany). The PCT coding for amino acid residues 161–241 of BRSV P protein was digested subsequently by BamHI and XhoI and inserted into the pGEX-4T3 expression vector (Pharmacia, France). Constructs were verified by sequencing.

### 2.2. Expression and purification of recombinant HRSV and BRSV proteins from E. coli

*E. coli* BL21(DE3) (Novagen, Merck Chemicals products, Germany) cells were co-transformed with the pGEX-PCT and pET-N plasmids coding for either BRSV or HRSV proteins. Recombinant protein expression was induced by IPTG and proteins were purified by glutathione–Sepharose affinity (Pharmacia, France). HRSV N + PCT complexes were separated from glutathione–Sepharose beads by biotinylated-thrombin cleavage in Tris 10 mM pH 8.5, NaCl 140 mM and thrombin was removed by the Thrombin Cleavage Capture kit according to manufacturer's instructions (Novagen, Merck Chemicals products, Germany). This protocol allows the purification of recombinant HRSV N proteins via their capacity to interact with the C-terminal fragment of P fused to GST (named GST-PCT) as previously described [20]. According to this procedure, 10 to 11 N proteins assemble into ring-shaped structures containing RNA subsequently named $N^{SRS}$ for sub-nucleocapsid ring structures [20]. Two hundred and fifty milligrams of HRSV $N^{SRS}$ were produced.

### 2.3. Adjuvant and vaccine formulation

For i.m. injection, $N^{SRS}$ was formulated in Montanide™ ISA71 VG (SEPPIC, Air Liquide, France) at a final concentration of 1 mg/ml. Montanide™ ISA 71 VG is a blend of oil and an ester from mannitol sugar and oleic fatty acid (anhydromannitol octadecenoate ether) with specific emulsifying properties due to its sugar polar head, its non-ionicity and the specificity of fatty acid chains of the surfactant system. The oleic acid and the sugar polar part used are from a vegetable origin. Experimental small scale vaccine emulsion was performed using silverson L4RT with tubular system mixer. The ratio of Montanide™ 71 VG/aqueous phase was 7 g of adjuvant/3 g antigenic phase. Montanide™ 71 VG was added first in a beaker and the head of the silverson was placed in the oil with agitation at 1000 rpm. The appropriate amount of antigenic phase (at the same temperature than the Montanide™ 71 VG) was then added progressively, and the rotation speed was increased to 5000 rpm for 3 min, with gentle moving of the beaker. To control that $N^{SRS}$ were not denatured in emulsion with Montanide™ ISA71 VG, 0.1 ml of butanol was added to 1 ml of emulsion, resulting in separation of aqueous and organic phases. Proteins present in the aqueous phase were dosed and imaged by negative-stain microscopy with a Philips CM12 microscope operated at an accelerating voltage of 120 kV.

For i.n. administration, $N^{SRS}$ was formulated in 25% (V/V) Montanide™ IMS 4132 VG (SEPPIC, Air Liquide, France) at a final concentration of 5 mg/ml. Montanide™ IMS 4132 VG is a ready to dilute water-soluble vaccine adjuvant. Montanide™ IMS are an association of apolar amphiphile nanoparticles combined with a soluble immunostimulant. All raw materials used in this formulation have monographs in different pharmacopeia and/or are already used in injectables for human. This adjuvant has been specifically selected for the intranasal trial due to its high spreadability. Experimental vaccine formulation was done by a simple dilution of the antigenic media in the adjuvant under gentle magnetic steering.

### 2.4. Virus and inoculum preparation

BRSV isolate 3761 (BRSV-3761) was isolated from a nasal swab of a calf with distress respiratory syndrome in 2003 [1]. The virus was then replicated for five passages in Bovine Turbinate cells (American Type Culture Collection, CRL 1390) and was amplified by 3 passages in newborn calves to give the BRSV-3761 inoculum. Passages in newborn calves were performed as follows: a 2-days-old calf, deprived of colostrum and maternal antibodies, was inoculated by intranasal and intratracheal routes with $10^6$ PFU of BRSV-3761. Calf was euthanized under anesthesia 5 days later and bronchoalveolar lavage (BAL) was performed in the lung with 500 ml of MEM medium supplemented with enrofloxacin (0.02 μg/ml, Baytril 5%, Bayer, France) and fungizone (2.5 μg/ml, Invitrogen Life Science, France). This BAL was snap frozen at −180 °C. The same method was used to obtain and store BAL at the second and third passages. The challenge inoculum of the present study consisted of the BAL at the third passage and was free of the following bovine respiratory pathogens: *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis*, Bovine Viral diarrhea virus (BVDV), bovine parainfluenza type 3, bovine Adenovirus 3, bovine coronavirus, and bovine herpesvirus 1. The titre of the challenge inoculum was $5 \times 10^3$ PFU/ml. Infectivity of the inoculum was controlled after challenge ($3.8 \times 10^3$ PFU/ml, when tested 6 h after experimental infection).

### 2.5. Experimental design (Table 1)

Twenty-four Normandy × Holstein breed male calves were selected at birth, reared in isolation unit (A2 level of bio safety, INRA Experimental Platform of Infectiology, Nouzilly, France) from birth to euthanasia and allocated to specific units, according to experimental groups. Animals were housed in biocontainment facilities as prescribed by the guidelines of the European Community Council on Animal Care (86/609/CEE) and under the authority of licence issued by the Direction des Services Vétérinaires (accreditation number 31–234). Calves were colostrum deprived until 3 days after birth and then received a substitute of colostrum (CER Marloie, Belgium) by oral route for 4 days to protect them against enteritic pathogens. They were fed with commercial milk for first age (Sanders Ouest SAS, Champagne, France). Antibiotics (1 mg/kg cefquinome, Cobactan, Scherring-Plough Intervet, France) were administrated from birth to 7 days. Absence of maternal antibodies against BRSV was confirmed by IgG detection (indirect BRSV ELISA, LSI, Lissieu, France) in blood of calves at 7 days after birth. BRSV ELISA was also performed each week before inoculation, to rule out natural BRSV infection during rearing. Absence of BVDV in calves was assessed at birth and one week before challenge by negative detection of the BVDV p80-125 antigen (Serelisa BVDV-BD, Synbiotics, Lyon, France) and by negative RT-PCR [25]. All calves remained healthy during the 3-month period before challenge. At the end of the experiment all calves were found seronegatives for bovine parainfluenza type 3.

Calves were randomly allocated in three groups. They were 1-month-old ±10 days at day of vaccination (considered as day 0). The first group (8 calves) was vaccinated twice at 3 weeks interval with 2 mg of $N^{SRS}$ protein with Montanide™ ISA71 VG adjuvant by the intramuscular route (2 ml, left flank). The second group (8 calves) received twice at 3 weeks interval 2 mg of $N^{SRS}$ protein with Montanide™ ISA71 VG adjuvant by the intramuscular route (2 ml, left flank) and 10 mg of $N^{SRS}$ protein with Montanide™ IMS 4132 VG adjuvant by the intranasal route (1 ml per nostril, using a nebulizator device for medical use, MADgic700, Wolfe Tory Medical, Utah, USA). The doses of antigen were defined according to one preliminary experiment done in calf to test the safety and immunogenicity of the $N^{SRS}$/adjuvant formulations (not shown). The last group (8 calves) was untreated and served as negative control for the two vaccination regimen. Three weeks after the final vaccination, all calves were challenged with $10^5$ PFU of the BRSV-3761 inoculum by intranasal nebulization (10 ml, tracherine IBR vaccine nebulizator) and intratracheal route (10 ml, Intraflon 2 catheter, Vycon, France). Two calves per group were euthanized under general anesthesia overdose (5 mg/kg ketamine followed by 15 mg/kg pentobarbital sodium) 6 days post-challenge (day 48), the remaining being euthanized 20 days post-challenge (day 62).

### 2.6. Clinical examination

Calves were observed for clinical signs of respiratory tract disease from 3 days prior infection to 20 days post-infection. Clinical assessments were made at the same time twice a day by the same veterinarian. Calves were examined for body temperature, nasal discharge, coughing, decrease appetite, general state, abnormal breathing, respiratory rate and abnormal lung sounds. Clinical scores were done for each calf as already described [26] with slight modifications. Rectal temperatures and respiratory frequencies were evaluated separately. Scores for respiratory rates (RR/min) were 0 (RR < 35), 1 (35 < RR < 45), 2 (45 < RR < 60) and 4 (RR > 60). A score between 0 (normal), 1 (mild) or 2 (severe) was attributed for nasal discharge, coughing, decrease appetite, general state, dyspnoea, and abnormal lung sound parameters, respectively. A fold coefficient of 3, 1, 3, 2, 2, 3 and 3 was subsequently attributed for respiratory rate, nasal discharge, coughing, decrease appetite, general state, dyspnoea, and abnormal lung sound parameters, respectively.

**Table 1**
Study design.

| Group label (no.) | Vaccination (s) | | Challenge | No. of euthanized calves | |
|---|---|---|---|---|---|
| | Day 0 | Day 21 | Day 42 | Day 48 | Day 62 |
| No vaccine ($n=8$) | – | – | BRSV | 2 | 6 |
| $N^{SRS}$ i.m. ($n=8$) | $N^{SRS}$/ISA71 i.m. | $N^{SRS}$/ISA71 i.m. | BRSV | 2 | 6 |
| $N^{SRS}$ i.m. + i.n. ($n=8$) | $N^{SRS}$/ISA71 i.m. $N^{SRS}$/IMS4132 i.n. | $N^{SRS}$/ISA71 i.m. $N^{SRS}$/IMS4132 i.n. | BRSV | 2 | 6 |

### 2.7. Fluid and tissue samples collected

To follow antigen-specific antibody responses, nasal swabs in PBS 0.1% Tween and anti-proteases (Complete Mini, Roche Applied Science, Indianapolis, USA) and blood samples were collected at days 0, 20, 41 and 62. To monitor virus detection after challenge, nasal swabs were collected daily from days 39 to 62 from each animal in 1 ml of RLT-buffer (Qiagen S.A., France) for real time RT-PCR or in 1 ml PBS buffer for commercial EIA assay (Speed® ReSpiVB BVT, La Seyne-sur-Mer, France).

Complete necropsies of calves were performed immediately after euthanasia at days 48 and 62. Lymphatic nodes (prescapular, tracheo-bronchial and mediastinal) were dissected out and processed for subsequent T cell assays. Lung macroscopic lesions were recorded on a standard lung diagram and expressed as % pneumonic consolidation of the cranial lobes (photographs were taken). BAL was performed with 500 ml D-MEM supplemented with antibiotics. After cell numeration, $2 \times 10^5$ BAL cells were cyto-centrifuged (Cytospin 4, Shandon, Thermo Scientific, France) on Superfrost plus slides (SFPLUS-42, Milan, France) for May-Grünwald-Giemsa staining, and $2 \times 10^6$ cells were fixed in Cyto-Chex (Streck, NE, USA) for flow cytometry analysis. The left-over BAL cells were lysed in RLT-buffer (RNeasy Mini, Qiagen S.A., France) for RNA extraction. Microscopic analysis was performed on tissue samples from the right cranial lobes of lungs, fixed in formaldehyde, embedded in paraffin, 4-μm sectioned, deparaffinized and counterstained with hematoxylin/eosin/saffran, analyzed and photographed. Examination for bacterial infection was performed on the same tissue samples after Gram staining. Samples of cranial lobe of the lungs were also collected in RNAlater (Qiagen S.A., France) for subsequent BRSV quantification by real time RT-PCR.

### 2.8. Real time RT-PCR

Virus shedding in nasal swabs was quantitatively determined by a real time RT-PCR assay according to Boxus et al. [27] except that quantitative analysis of BRSV RNA was performed relative to the bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressed housekeeping gene [28]. The same RT-PCR was used for quantification of BRSV in BAL and lung tissues.

### 2.9. Immunostaining of BRSV antigens in lung tissue sections

Paraffin-embedded lung tissue sections were deparaffinized, rehydrated in Tris 0.05 M pH 7.4 with 0.2% $CaCl_2$ and then permeabilized with 0.02% Saponin (Sigma–Aldrich, France). BRSV antigens were detected using MoAb IgG2b anti RSV-F (clone B016, AbD Serotec, Germany) diluted 1:100 in Tris 0.05 M pH 7.4, 0.2% $CaCl_2$ and 0.02% Saponin. An irrelevant isotype-matched mouse Ab was used as a control for non-specific staining. Binding of primary Ab was revealed by adding HRP-conjugated anti-mouse IgG followed by the insoluble peroxydase substrate 3,3′-Diaminobenzidine (SigmaFast™, Sigma–Aldrich, France). The tissue sections were then counterstained with hematoxylin.

### 2.10. Flow cytometry analysis of BAL lymphocytes

One million BAL cells were incubated for 20 min in RPMI containing 10% horse serum (RPMI-HS) on ice. BAL cells were then stained for 30 min on ice with mouse MoAbs anti-bovine CD4 (IgG2a, clone ILA11, VMRD, WA, USA), CD8 (IgM, clone BAQ111A, VMRD, WA, USA) and CD45RO (IgG3, clone ILA116, VMRD, WA, USA), or matching isotype control mouse antibodies, all diluted 1:500 in RPMI-HS. BAL cells were washed and then incubated for another 30 min with anti-isotype antibodies conjugated to fluorochromes (FITC anti-IgG3, PE anti-IgG2a, Cy5 anti-IgM, Invitrogen Life Science, France). Cells were then fixed in 10% CellFIX (BD Biosciences, France). All samples were analyzed on a FACScalibur (BD Biosciences, France) collecting data on at least 20,000 lymphocytes gated according to their forward and side scatter features.

### 2.11. Preparation of lymph node cells

Lymph nodes were mechanically disrupted for cell dissociation in sterile RPMI-1640 medium plus 10% fetal calf serum (FCS) at 4 °C. The recovered cells were filtered through a sterile 100 μm cell strainer (BD Biosciences, France) and washed twice in RPMI-1640 medium plus 10% FCS. PBMC and lymph node cells were finally suspended in X-vivo 15 medium (BioWhittaker, Lonza, Switzerland) supplemented with 1% FCS, 2 mM L-glutamine, 100 U/ml Penicillin and 0.1 mg/ml Streptomycin and cultivated *in vitro* for T cell proliferation or IFN-γ detection assays.

### 2.12. Antigen-specific lymphoproliferation assays

Proliferation assays were carried out in 96-well flat-bottomed plates. Isolated lymph node cells were seeded in triplicate at $3 \times 10^5$ cells per well with or without $N^{SRS}$ (10 μg/ml final concentration). Plates were incubated at 37 °C in 5% $CO_2$ for 96 h, then pulsed overnight with 1 μCi [$^3$H]-thymidine per well. Cells were then collected on filter mats using a cell harvester (Filtermate, PerkinElmer, France) and radioactivity was measured in a liquid scintillation luminescence counter (MicroBeta TriLux, Wallac Inc., Gaithersburg, MD, USA). Results were expressed as stimulation indexes (cpm of stimulated cells over cpm of unstimulated control cells).

### 2.13. IFN-γ production

Lymph node cells were plated in 96-well plates (Falcon 3072) in triplicates at $3 \times 10^5$ cells per well and incubated at 37 °C, in 5% $CO_2$, with or without $N^{SRS}$ (10 μg/ml final concentration). Supernatants were harvested at 72 h and the IFN-γ content was tested using a specific ELISA test (Bovigam, Biocor, Melbourne, Australia), according to manufacturer's instructions. Results were expressed as stimulation indexes ($OD_{450\,nm}$ of $N^{SRS}$-stimulated cells over OD of unstimulated control cells).

### 2.14. Detection of $N^{SRS}$-specific bovine antibody by ELISA

Individual sera and nasal secretions were assayed for N-specific antibodies (total Ig, IgG1 and IgA) by ELISA. Microtiter plates (Immulon 2HB, Thermo Labsystems, France) were coated

overnight at 4 °C with N antigen (200 ng per well in 100 μl carbonate–bicarbonate buffer 0.1 M, pH 9.5). Plates were washed five times with PBS 0.05% Tween 20 between each step of the assay. After coating, the remaining protein binding sites were saturated with 5% horse serum in PBS 0.05% Tween 20 (PBS-T-HS) for 1 h at 37 °C. Samples were serially diluted threefold in PBS-T-HS starting at 1:30 for sera and 1:3 for nasal secretions and incubated for 2 h at 37 °C. Antigen-bound Abs were detected using HRP-conjugated anti-bovine Ig(H + L), HRP-conjugated sheep anti-bovine IgG1 or rabbit anti-bovine IgA (AbD Serotec, Germany) diluted 1:1000, incubated for 1 h at 37 °C, followed when required by an incubation with goat anti-rabbit Ig HRP-conjugated. The TMB substrate (Kirkegaard & Perry Laboratories Inc., MD, USA) was added and the reaction was stopped after 10 min by 1 M phosphoric acid. The absorbance was measured at 450 nm with an ELISA plate reader (MRX Revelation, Dynex Technologies, Germany). The results were expressed as endpoint antibody titers calculated by regression analysis plotting dilution versus $A_{450}$ (regression curve $y = (b + cx)/(1 + ax)$ using Origin software). Endpoint titers were calculated as the highest dilution giving twice the absorbance of negative control sample.

Alternatively, plates were coated with a lysate from BRSV-infected or mock-infected Turbinate cells in PBS. Lysate was obtained by treating the BRSV-infected or mock-infected Turbinate cells with 1% *n*-Octyl glucoside (Sigma–Aldrich, France) and 5 mM EDTA in 10 mM, pH 7.6 Tris saline buffer. Serum samples were diluted 1:270 and incubated on alternate rows with BRSV-infected and mock-infected Turbinate lysates, and the ELISA was performed as described above. To measure anti-BRSV-specific binding, background antibody binding to control lysate was substracted from binding to BRSV-infected cell lysate.

### 2.15. Statistics

All data were expressed as arithmetic mean ± standard error of the mean (SEM). Statistical analysis on immune parameters was performed using non-parametric Mann–Whitney *U*-test (http://elegans.swmed.edu/~leon/stats/utest.html). Levels of significance are indicated on the graphs with stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Peak clinical signs and viral load values were compared by one-way analysis of variance. Logarithmic transformation was applied to fulfill the conditions of variances in homogeneity and normality when necessary. A three-factor split-plot ANOVA test was used to calculate the effect of the factors 'day' and 'vaccination' between groups. For significant results, a Bonferroni's test among contrast was then used to compare the two conditions at each day post-challenge.

## 3. Results

### 3.1. Nano-rings were obtained with N from HRSV Long strain, formulated with Montanide™ adjuvants and tested for their immunogenicity

We have previously shown that soluble RNA-nucleoprotein complexes forming nano-rings ($N^{SRS}$) can be purified from bacteria expressing recombinant N and the C-terminal region (residues 161–241) of P protein (PCT) from the HRSV Long strain [20]. The same protocol was used in order to purify BRSV N proteins. The BRSV N protein was co-expressed with PCT from either HRSV or BRSV origin. As shown Fig. 1a, the BRSV N protein (strain A2Gelfi) was only recovered in the unsoluble fraction of bacterial lysates, and attempts to purify it by co-expression with GST-PCT were unsuccessful, either using PCT from BRSV or HRSV origin. On the other hand, the HRSV N protein was soluble and efficiently purified by BRSV PCT fused to GST (Fig. 1a).

The N proteins of human strain Long and bovine strain 3761 are highly conserved since they share 93.6% of sequence amino acid identity and 99.2% of amino acid sequence similarity (Fig. 1b). Thus we used the $N^{SRS}$ nano-rings derived from the HRSV Long strain as a vaccine candidate against BRSV infection. To control that $N^{SRS}$ were not degraded after emulsification with Montanide™ ISA71 VG or Montanide™ IMS4132 VG, the proteins present in the two formulations were analyzed by SDS-PAGE native gel electrophoresis and electron microscopy as described previously [20]. In both cases, the N protein was recovered as SRS (data not shown).

Intramuscular injection is the classical way to vaccinate bovine. Our previous data in mice showed that nasal vaccination with the nano-rings $N^{SRS}$ was the most efficient immunization route for preventing HRSV replication in lung [22]. Thus we decided to administer the $N^{SRS}$ vaccine via both intramuscular and intranasal route at the same time (group $N^{SRS}$ i.m. + i.n.) and test the benefit of nasal vaccination by comparison with a group of calves receiving the vaccine by intramuscular injection only (group $N^{SRS}$ i.m.). All calves were vaccinated twice at 3 weeks interval. A third group of calves was left untreated (group "no vaccine"). No adverse clinical reactions were observed after the first or second immunizations.

To monitor the immunogenicity of the vaccine regimen, N-specific antibodies were investigated in serum samples and nasal secretions (Fig. 2). Calves vaccinated with $N^{SRS}$ either i.m. or i.m. + i.n. displayed anti-N Ab in serum and in nasal secretions, detectable after the first immunization and increasing after the booster immunization (Fig. 2a and b). Anti-N Ab titers were not different between the two vaccinated groups. The nasal anti-N Ab response was of IgG1 isotype (Fig. 2c), with lower anti-N IgA titers arising mostly after the booster immunization (Fig. 2d). Nasal anti-N Ab titers were higher in the group $N^{SRS}$ i.m. + i.n. compared to $N^{SRS}$ i.m. ($p < 0.05$ for Ig titers and $p < 0.01$ for IgA titers, at day 41). Non-vaccinated control calves had significant anti-N Ab titers in blood and nasal secretion only after viral challenge (Fig. 2a and b, $p < 0.01$ between days 0–41 and day 62, Ig titers in the no vaccine group). The antibodies elicited upon $N^{SRS}$ vaccination were also able to recognize the native viral N as shown by their specific binding to BRSV-infected Turbinate cells as antigen ($OD_{\times 100}$ at day 41 were $22 \pm 10$, $287 \pm 64$ and $188 \pm 41$, for groups no vaccine, $N^{SRS}$ i.m. and $N^{SRS}$ i.m. + i.n., respectively, $p < 0.01$ between vaccinated and non-vaccinated calves).

### 3.2. $N^{SRS}$ vaccination reduced clinical symptoms and extension of lung lesions upon BRSV challenge

After challenge, all calves showed mild clinical signs of upper respiratory tract infection that were essentially characterized by slight mucous nasal discharge, except for one calf of group $N^{SRS}$ i.m. which remained healthy. Cough was observed in two non-vaccinated calves for 2–3 days. Moderate hyperthermia was observed in all calves with no statistical differences between the 3 groups (data not shown). Respiratory signs consisted of moderate to high increased respiratory rates and mild dyspnoea with increasing lung sounds. Dyspnoea was also associated with wheezes and crackles in some calves. Among the 6 calves of the non-vaccinated control group, one calf was slightly affected, 4 calves were moderately ill and one calf developed a marked dyspnoea, with abnormal breathing, discordance, surrounding lung sounds of the cranial lobe, wheezes and crackles. For the six calves of group $N^{SRS}$ i.m., one calf showed no clinical signs, 2 calves developed very mild respiratory symptoms and 3 calves developed a moderate dyspnoea. Finally, 3 calves of the group $N^{SRS}$ i.m. + i.n. developed very mild respiratory signs and the other 3 showed a moderate dyspnoea. The mean clinical scores are shown in Fig. 3. Statistical analyses (three-factor

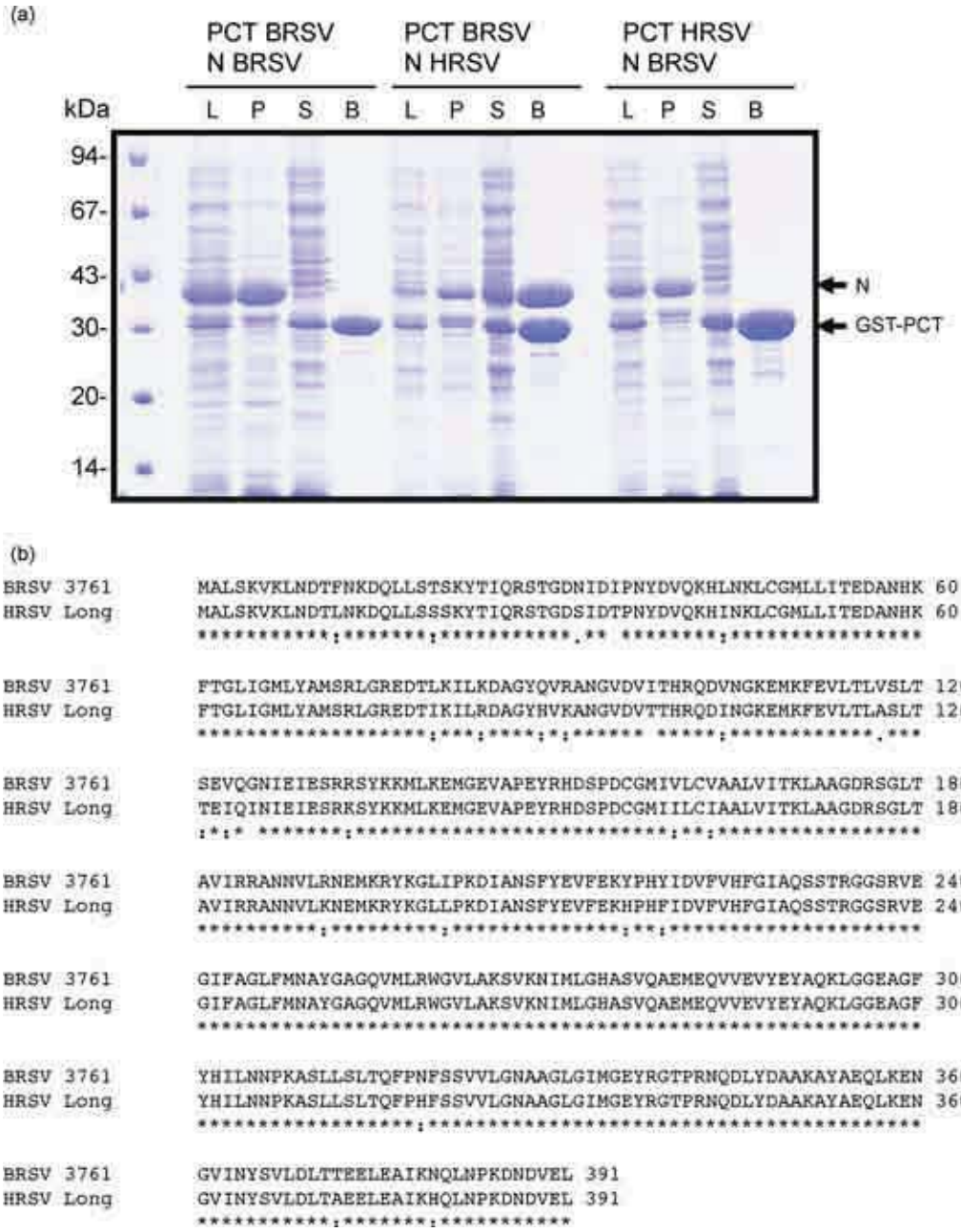


**Fig. 1.** Production of soluble N from HRSV or BRSV origin. (a) Coomassie blue-stained SDS-PAGE analysis of GST-PCT and N proteins from HRSV (strain Long) and BRSV (strain A2Gelfi) expressed in *E. coli*. Cell lysates (L) were centrifuged and the soluble (S) or unsoluble (P) fractions were run on a 12% polyacrylamide gel. Proteins were purified by glutathione–Sepharose affinity from the cell lysates and the proteins pulled-down with the sepharose beads (B) were analyzed on the same gel. GST-PCT from HRSV or BRSV together with the HRSV N protein were soluble, while BRSV N was only found in the unsoluble fraction. The HRSV N protein was efficiently purified by the BRSV PCT fragment.
(b) N protein sequence comparison between HRSV Long strain and BRSV 3761 strain with the ClustalW2 sequence alignment program. Stars and points indicate amino acid identities and similarities (two dots indicate strong similarity, one dot weak similarity), respectively.

**Table 2**
Clinical signs and lung lesions post-BRSV challenge.

| | Clinical signs (days) | | | Lung lesions at day 6 |
|---|---|---|---|---|
| | Onset ($n=6$) | Peak ($n=6$) | Duration ($n=6$) | Extent (%) of consolidation in cranial left/right lobes per calf |
| No vaccine | [a]1.4 ± 0.3 | 6.2 ± 0.7 | 18.2 ± 0.6 | 10/10; 20/15 |
| $N^{SRS}$ i.m. | [b]2.5 ± 0.3* | 7.5 ± 0.6 | 13.5 ± 2.5 | 5/5; 5/5 |
| $N^{SRS}$ i.m. + i.n. | 2.5 ± 0.3* | 8.7 ± 0.4* | 14.0 ± 1.5* | 5/0; 5/5 |

[a] Data (onset, peak, duration) are given as mean ± SEM.
[b] Stars indicate significant differences using the non-parametric Mann–Whitney *U*-test ($p$ one-tailed) between the vaccinated groups ($N^{SRS}$ i.m. or $N^{SRS}$ i.m. + i.n.) and the non-vaccinated group. Onset, peak and duration were not significantly different between the $N^{SRS}$ i.m. and $N^{SRS}$ i.m. + i.n. vaccinated groups.

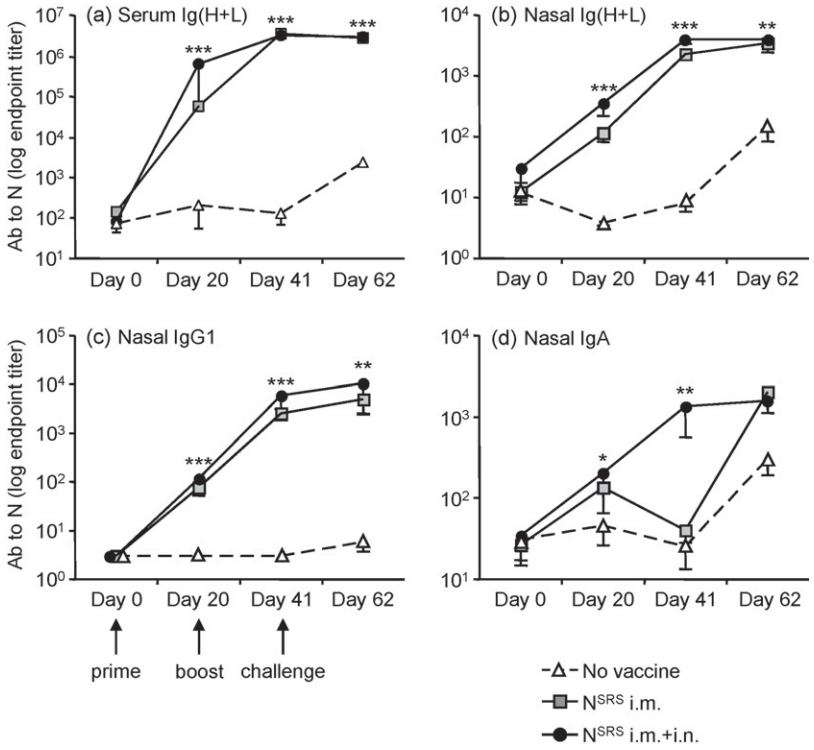


**Fig. 2.** N-specific antibody responses elicited in serum and nasal secretion upon $N^{SRS}$ vaccination and BRSV challenge. Calves were vaccinated twice with $N^{SRS}$ i.m. or i.m. + i.n. (day 0 and 21) followed by challenge with BRSV (day 42). (a) Serum Ig(H + L) and (b) nasal Ig(H + L) titers to N were measured by an ELISA endpoint assay. N-specific IgG1 and IgA Ab were quantified in nasal secretions (c and d). Data are expressed as mean ± SEM and plotted with a logarithmic scale. Stars indicate significant differences between the two vaccinated group ($N^{SRS}$ i.m. and i.m. + i.n.) and the non-vaccinated one.

split-plot ANOVA test) indicated group and time effects between the non-vaccinated group and the groups $N^{SRS}$ i.m. or $N^{SRS}$ i.m. + i.n. No differences were found between the two vaccinated groups ($N^{SRS}$ i.m. and $N^{SRS}$ i.m. + i.n.). Significant reduction of mean clinical scores (Bonferroni's test among contrast, $p < 0.05$) was found for the group $N^{SRS}$ i.m. at days 5 and 6, for the group $N^{SRS}$ i.m. + i.n. at days 5–7 post-infection when compared to the non-vaccinated group (Fig. 3). Onset, peak and duration of clinical scores were calculated for each group (Table 2), showing delayed onset, peak and shorter duration of clinical symptoms in the two vaccinated groups. Thus the calves vaccinated with $N^{SRS}$, either i.m. only or i.m. + i.n., were partly protected against the respiratory disease caused by virus challenge.

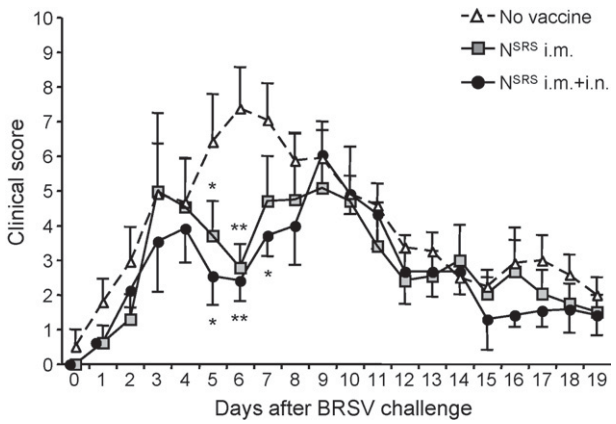


**Fig. 3.** Clinical scores following BRSV challenge. Respiratory rhythm, anorexia, presence of nasal discharge, lung sounds, cough and demeanour were recorded daily after challenge and clinical scores were calculated. Data represent means ± SEM ($n = 6$) in each group from day 0 (challenge) to day 19 after challenge. Stars indicate significant differences between the two vaccinated group ($N^{SRS}$ i.m. and i.m. + i.n.) and the non-vaccinated one.

Two out of 8 calves of each group were euthanized on day 6 after challenge. The lungs were examined and the extent of macroscopic lesions was recorded. For all animals gross lesions were restricted to the cranial lobes except for one non-vaccinated calf showing lesions also in the middle and accessory lobes. Some patchy areas were atelectasic, collapsed, deep red and rubbery in texture. Extension of the lung lesions of calves is detailed in Table 2 for the right and the left cranial lobes respectively. To summarize, the extension of consolidation lesions varied between 10% (Fig. 4a, right cranial lobe) and 20% for unvaccinated calves while it was estimated to be 5% for the vaccinated calves (Fig. 4b, right cranial lobe). No macroscopic lesions were found for calves euthanized on day 20 post-challenge.

Histological examination of lung tissue sections (sample from right cranial lobe, taken at the site of macroscopic lesions) revealed typical bronchointerstitial pneumonia (Fig. 4c and e) characterized by necrotizing bronchiolitis, formation of bronchiolar epithelial syncitia and proliferative alveolitis in 2 calves from the non-vaccinated group. In contrast, lung tissue sections from 3 out of 4 vaccinated calves (2/2 $N^{SRS}$ i.m. + i.n. and 1/2 $N^{SRS}$ i.m.) showed limited cellular infiltration in the peribronchiolar and bronchiolar areas, with minimal densification of the alveolar areas (shown of one $N^{SRS}$ i.m. + i.n. calve, Fig. 4d). Besides, most of the bronchiolar lumina were clear of cellular debris (Fig. 4f). The presence of BRSV-infected cells was revealed by immunostaining on the same lung tissue sections. BRSV-specific staining was found in the epithelial cells of the bronchioles from either vaccinated or non-vaccinated calves (Fig. 4g and h, brown staining).

### 3.3. $N^{SRS}$ vaccination reduced BRSV loads in nasal secretions

The replication of BRSV in the respiratory tract of infected calves was further investigated by real time RT-PCR on BAL cells and

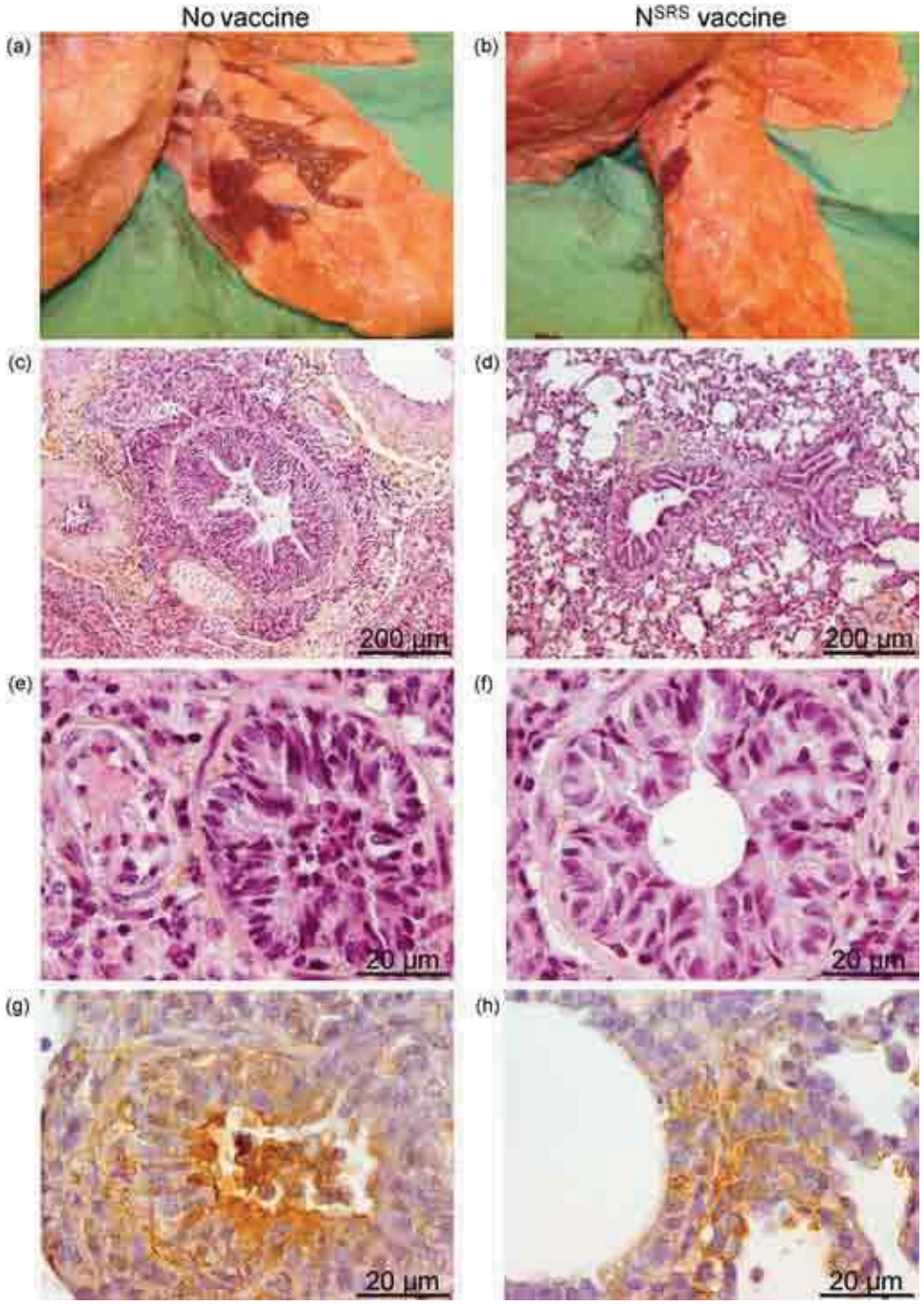


**Fig. 4.** Macroscopic and microscopic lung lesions following BRSV challenge. On day 6 post-BRSV challenge (peak of clinical scores), two calves per group were euthanized and their lungs dissected out for macroscopic analysis of lesions (a and b). Lung pieces were sampled in the right cranial lobe at the border between red atelectatic collapsed pulmonary areas and healthy tissue, fixed in formalin and embedded in paraffin. Histological examination of sections counterstained with hematoxylin/eosin/saffran showed areas of bronchointerstitial pneumonia with proliferative alveolitis in non-vaccinated calves. This marked infiltration of inflammatory cells was observed in the alveolar, peribronchiolar and bronchiolar areas (c) and was associated to a necrotizing bronchiolitis (e). Bronchiolar lumen contained sloughed necrotic epithelial cells and sometimes multinucleate syncytial cells closely associated with the bronchiolar epithelium, and few inflammatory cells infiltrating the bronchiolar epithelium. Similar sections in vaccinated calves showed alveolar functional areas with minimal thickening of alveolar septa (d) and bronchiolar lumen clear of cellular debris (f). The same lung tissue sections were stained for BRSV antigens with an anti-F monoclonal antibody (brown staining) and counterstained with hematoxylin (pale blue staining). The control immunohistochemical reaction with an isotype-matched irrelevant mouse IgG was negative (data not shown). Immunohistochemical staining of BRSV-F revealed virus-infected bronchiolar epithelial cells (g and h) with viral antigens among the necrotic cells sloughed the bronchiole lumen (g). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lung samples (right cranial lobe). At day 6 post-infection, viral RNA was detected in BAL cells and lung tissue of the two euthanized calves of each group with no significant differences between groups (Fig. 5a). These data are in agreement with the finding of BRSV-infected cells in lung tissue sections by immunostaining. No virus could be detected at day 20 post-infection in BAL and lung of any calves.

The kinetic and amount of virus shedding was monitored in nasal secretions on a daily basis post-challenge. BRSV RNA was detected in nasal secretions of all infected calves (Fig. 5b and c) with

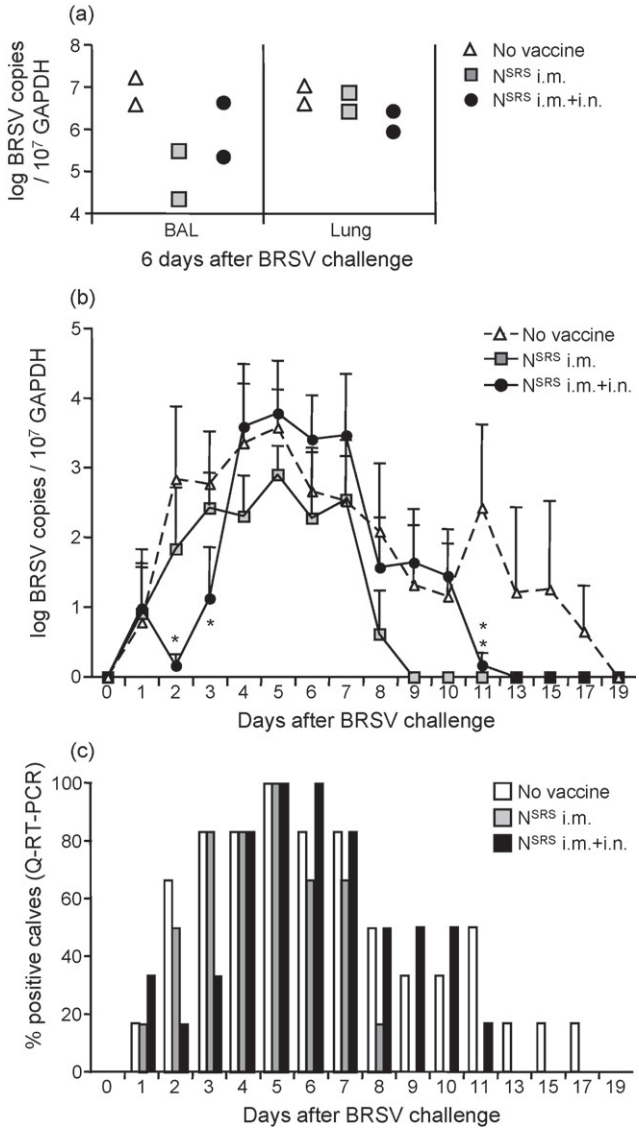


**Fig. 5.** BRSV RNA detection and viral load following challenge. The viral loads were examined by performing quantitative real time RT-PCR on total RNA extracted from BAL cells and lung pieces collected on two calves per group euthanized on day 6 after challenge (a) and from the nasal swabs sampled daily from the day of challenge up to 19 days after (b and c). Viral load is expressed as the log of BRSV copies per $10^7$ GAPDH cDNA (mean ± SEM, $n = 6$). Stars indicate significant differences between the two vaccinated group ($N^{SRS}$ i.m. and i.m. + i.n.) and the non-vaccinated one. The daily percentage of positive calves per group is shown (c).

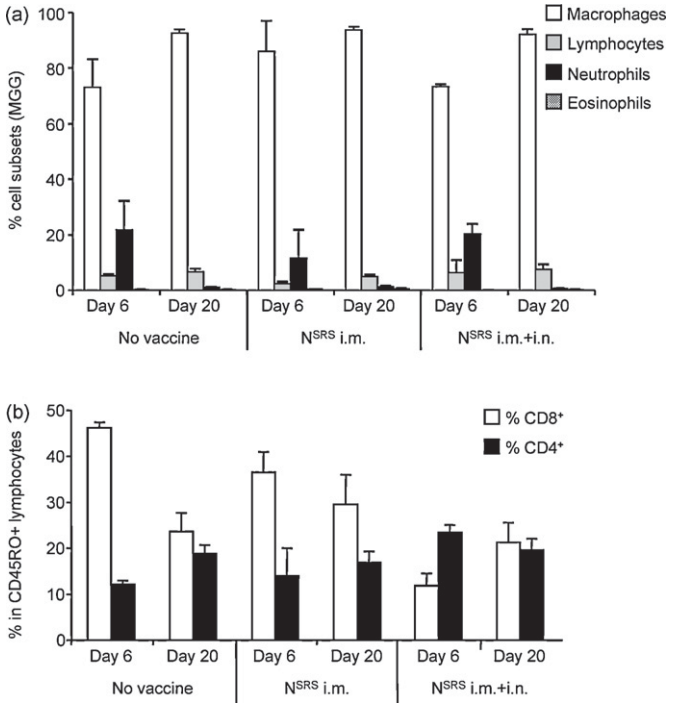


**Fig. 6.** Cell subsets recruited to the BAL following BRSV infection. Two calves per group were euthanized 6 days after BRSV challenge and the other calves were euthanized 20 days after challenge. Lungs were dissected out of the thoracic cage and lavaged with 500 ml of medium. The cells present in BAL were collected by cyto-centrifugation. The cellular composition of the BAL was established after May-Grünwald-Giemsa coloration and numeration of macrophages, lymphocytes, neutrophils and eosinophils (a). BAL cells were labeled with anti-CD45RO, CD4 and CD8 antibodies and analyzed by flow cytometry to determine which T lymphocyte subsets were recruited to the lung upon infection (b). 200,000 events were acquired, gated on lymphocytes according to FSC/SSC and $CD45RO^+$ criteria (at least 5000 events were gated). Data are mean ± SEM, $n = 2$ at day 6 and $n = 6$ at day 20.

a peak of virus shedding at day 5 post-challenge ($10^{3.6\pm1}$, $10^{2.9\pm0.7}$ and $10^{3.8\pm1.2}$ copies/$10^7$ copies of GAPDH for groups no vaccine, $N^{SRS}$ i.m., and $N^{SRS}$ i.m. + i.n., respectively). No significant differences were found between the two vaccinated groups. However, statistical analyses (three-factor split-plot ANOVA test) indicated group and time differences between the two vaccinated groups and the non-vaccinated group. Lower amounts of viral RNA were found in group $N^{SRS}$ i.m. at days 4 ($p = 0.06$) and 11 ($p < 0.05$) and in group $N^{SRS}$ i.m. + i.n. at days 2, 3 and 11 ($p < 0.05$, Fig. 5b). Importantly the duration of viral excretion in nasal swabs was reduced in the vaccinated groups, BRSV being detected in swabs from days 1 to 17 post-infection in non-vaccinated calves versus days 1–8 in group $N^{SRS}$ i.m., and days 1–11 in group $N^{SRS}$ i.m. + i.n. (Fig. 5c). In addition, the detection of BRSV proteins in nasal secretions by EIA assay on five calves of each group at days −1, 0, 2 to 7, 9, 11, 13 and 15 post-infection showed that less calves were found positives on a daily basis in the two vaccinated groups compared to the non-vaccinated group (data not shown).

### 3.4. $N^{SRS}$ vaccination and BRSV challenge was associated with few granulocytes and mixed T cell subsets in BAL

Respiratory infection is usually accompanied by an influx of lymphocytes and granulocytes into the lungs. BAL cells were collected from calves euthanized on days 6 ($n = 2$) and 20 ($n = 6$) after challenge and the percentages of macrophages, lymphocytes, neutrophils and eosinophils were determined after May-Grünwald-Giemsa staining. Six days after infection, we observed neutrophils in all BAL whether or not the calves had been vaccinated (18 ± 4%, $n = 6$, Fig. 6a, black bars). Twenty days post-challenge, the percentages of neutrophils in BAL ranged between 0.1 and 2.5%, without any significant differences between the three groups (no vaccine, $N^{SRS}$ i.m. and $N^{SRS}$ i.m. + i.n.). No eosinophils were found in BAL at 6 and 20 days post-challenge (Fig. 6a).

To get an insight into the pattern of T cell responses in the lung upon challenge, CD4 and CD8 T lymphocytes in BAL were monitored by flow cytometry analysis (Fig. 6b). Lymphocytes were gated according to their low FSC/SSC features and memory/activated lymphocytes were subsequently gated on the basis of CD45RO expression. The proportion of $CD4^+$ and $CD8^+$ cells within gated $CD45RO^+$ lymphocytes was determined. For the non-vaccinated calves experiencing a primary BRSV infection, $CD8^+$ effector lymphocytes were found rapidly and more abundantly than their $CD4^+$ counterpart into the airways (Fig. 6b, day 6). The same pattern of T cell subsets on day 6 post-challenge (% $CD8^+ > CD4^+$ mem-

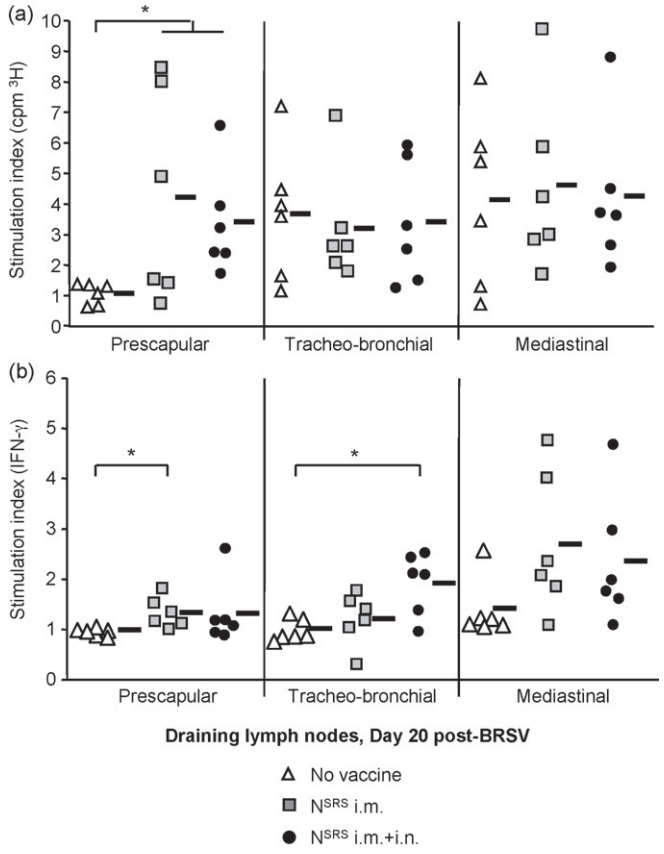


**Fig. 7.** N-specific memory T cell responses following vaccination and challenge. The lymph nodes draining the site of i.m. vaccination (prescapular) and the upper and lower respiratory tract (tracheo-bronchial and mediastinal, respectively) were dissected out on day 20 after BRSV challenge and processed to isolate lymph node cells. (a) *In vitro* lymphocyte proliferation was evaluated by measuring [$^3$H]thymidine incorporation after $N^{SRS}$ or mock antigenic restimulation for 96 h and values were expressed as stimulation index (SI). Individual SI is plotted for each group (square, circle, triangle) and the mean is shown next (black line). (b) IFN-γ was measured in the supernatant of lymph node cells cultivated for 72 h with $N^{SRS}$ or with medium only (mock). Results are expressed as SI and data displayed individually as in (a).

ory/effector cells) was observed for the $N^{SRS}$ i.m. vaccinated calves whereas calves from the group vaccinated with $N^{SRS}$ i.m.+i.n. tended to have more memory/effector $CD4^+$ than $CD8^+$ T cells into their airways. By day 20 post-challenge, $CD45RO^+$ $CD4^+$ and $CDR45RO^+$ $CD8^+$ T cells were found in same proportion among BAL cells without significant differences between groups.

### 3.5. $N^{SRS}$ vaccination and BRSV challenge primed N-specific T cells in calves

In primary BRSV infection of calves, N is a known target of cell-mediated immunity [29]. Thus we tested whether $N^{SRS}$ vaccination had primed memory T cells responses that could be revealed post-challenge. Leukocytes were isolated from lymph nodes on day 20 post-BRSV challenge and then restimulated *in vitro* with $N^{SRS}$. N-specific cellular responses were measured by proliferation index based on tritiated thymidine incorporation (Fig. 7a). To get insight into the function of T lymphocytes elicited upon $N^{SRS}$ immunization and BRSV challenge, their capacity to make IFN-γ was assessed by ELISA in lymph node cell culture supernatant (Fig. 7b).

N-specific T cell responses were primed in the prescapular lymph node draining the site of i.m. vaccination with $N^{SRS}$. Only $N^{SRS}$ vaccinated calves displayed a N-specific proliferative response (stimulation index of $4.2 \pm 1.4$ and of $3.4 \pm 0.7$, $N^{SRS}$ i.m. and $N^{SRS}$ i.m.+i.n. respectively, versus $1.1 \pm 0.1$ for non-vaccinated calves, $p < 0.05$). Accordingly the capacity of T cells isolated from the prescapular LN to make IFN-γ was significantly higher in the vaccinated calves than in the non-vaccinated one ($p < 0.05$% between no vaccine and $N^{SRS}$ i.m.).

Cells isolated from the lymph nodes draining the upper airways (tracheo-bronchial LN) and the lung (mediastinal LN) proliferated in response to $N^{SRS}$ and the mean proliferation index of the two vaccinated groups was not different from the non-vaccinated group, suggesting a T cell priming mainly due to BRSV infection. However when T cell memory responses were monitored by IFN-γ production in tracheo-bronchial LN, the vaccinated calves had higher responses than the non-vaccinated one ($p < 0.05$% between no vaccine and $N^{SRS}$ i.m.+i.n.). The strongest IFN-γ production was recorded in the mediastinal LN and tended to be higher for T cells isolated from vaccinated calves (SI of $1.4 \pm 0.2$, $2.7 \pm 0.6$ and $2.4 \pm 0.5$ for no vaccine, $N^{SRS}$ i.m. and $N^{SRS}$ i.m.+i.n. respectively, $p = 0.07$ between no vaccine and $N^{SRS}$ i.m. and $p = 0.06$ between no vaccine and $N^{SRS}$ i.m.+i.n.). Thus the IFN-γ response in the lymph nodes draining the airways was suggestive of memory T cells primed by $N^{SRS}$ vaccination and boosted upon BRSV challenge.

## 4. Discussion

No RSV vaccine is yet licensed for human use and the inactivated or attenuated vaccines commercialized for bovine have a limited efficacy and a short duration of protective immunity. Difficulties of RSV vaccine development include the lack of a relevant animal model for human, the need to immunize immunologically immature young infants or calves with maternal RSV antibodies, the impact of RSV variability on vaccination and the risk of vaccine-associated disease enhancement. We were the first to publish an efficient and safe vaccination strategy against RSV using the nucleocapsid protein alone as a vaccine antigen, under the form of soluble nanoparticles referred to as $N^{SRS}$ [22]. In this previous study done in mice we have demonstrated that $N^{SRS}$ is highly immunogenic when delivered via the nasal route and that the immune response primed upon vaccination is protective against an HRSV challenge [22]. In the present study, we investigated the potency of $N^{SRS}$ as a vaccine in calves that are the natural hosts for BRSV and display clinical respiratory symptoms and lung lesions upon infection.

Our findings indicate that calves vaccinated with $N^{SRS}$ were partially protected against the respiratory disease caused by a virus challenge. Significantly lower clinical scores were observed for two to three days in the vaccinated calves compared to the non-vaccinated calves and the duration of clinical signs was reduced in the vaccinated calves. At the precise site of virus-induced lesions, in the cranial lobe of the lungs, the vaccination with $N^{SRS}$ reduced the extent of local inflammatory consolidation. The vaccination with $N^{SRS}$ reduced the duration of viral shedding and the frequency of virus-secreting calves on a daily basis but it did not prevent viral shedding in nasal secretion, nor viral replication in lung. There was no difference between the two vaccination regimen (i.m. only or i.m.+i.n.) for their capacity to reduce clinical scores and viral load.

Importantly the degree of protection conferred by vaccination with $N^{SRS}$ was not associated with markers of disease exacerbation (like eosinophilia) as is reported when vaccinating calves with FI-BRSV or live-BRSV [6]. Indeed no eosinophils and very few neutrophils were found in broncho-alveolar lavages of calves autopsied 3 weeks after challenge.

Several points of discussion could explain the partial protection of calves by vaccination with $N^{SRS}$, whatever the protocol used.

Clear respiratory symptoms and lung lesions were induced upon BRSV challenge but they were not severe. As frequently published with BRSV challenge models [30–32], it is difficult to reproduce the severe clinical signs or lesions observed upon natural infections.

In calves normally bred in farms and not in isolation units like in the present study, bacterial or virus co-infections complicate the classical BRSV disease. The bovine parainfluenza virus 3 (BPIV-3), which is widespread in 2–8-month-old cattle, reduces pulmonary defences [33,34] and thus may enhance the severity of the BRSV pathogenicity. Both viruses, BRSV and BPIV-3, are important predisposing factors in the development of bacterial bronchopneumonia in cattle. In addition intrinsic host parameters seem to control the severity of the disease since severe pathology is associated with dysfunctions of the host's response [8]. In this study, we used a BRSV 3761 inoculum which was previously shown successful in reproducing severe respiratory signs after intranasal and intratracheal injections of 1 and 3-month-old Prim'Holstein calves (G. Meyer, unpublished results; [18]). This inoculum contained BRSV with few passages in cell cultures and with 3 cycles of amplification in newborn calves, a condition also shown by others to induce severe respiratory disease in calves [35,36]. By comparison with previous successful experiments, failure to reproduce respiratory distress syndrome in this study could be related to host intrinsic parameters. Indeed, another study, using the same inoculum and crossed Prim'Holstein/Normandy calves of same origin, also failed to reproduce respiratory distress [19].

The nucleoprotein subunits ($N^{SRS}$) used in this study were from HRSV origin. The gene encoding the nucleoprotein is shown to be one of the most conserved between BRSV and HRSV with an average of ≈94% amino acid identity. The nucleoprotein from BRSV is recognized by bovine CD8+ T cells but the precise CTL epitopes have not been defined yet [29]. In human, HLA-B07, HLA-B08 and HLA-A02 restricted epitopes were mapped in the nucleoprotein [12,14] and interestingly their amino acid sequence is fully conserved among various HRSV field isolates and with BRSV strains [14]. The mechanisms of CTL cross-reactivity have been recently investigated with well-characterized CTL epitopes from HIV showing that biochemically similar amino acid substitutions do not drastically affect recognition by TCR [37]. Some level of cross-protection between HRSV and BRSV has been demonstrated in the cotton rat model in which BRSV was tested as a possible Jennerian vaccine against HRSV [38]. Among the viral antigens that may be cross protective between BRSV and HRSV, the BRSV-F (81% amino acid identity with HRSV-F), delivered as DNA vaccine, was shown to protect mice against an HRSV challenge [39]. The present study brings new data to support the hypothesis of common B or T epitopes between BRSV and HRSV nucleoproteins. Indeed, in our study, primary BRSV infection resulted in antibody and cellular immunity that could be revealed with an $N^{SRS}$ coated ELISA assay or following an *in vitro* boost with $N^{SRS}$, respectively. Conversely serum Ab from $N^{SRS}$ vaccinated calves reacted against a BRSV-infected cell lysate. This indicates that recombinant N from HRSV origin assembling into nano-rings and the nucleocapsid protein N from BRSV strain 3761 displayed at least some common epitopes and that vaccination with $N^{SRS}$ from HRSV protein sequences provide significant cross-protection against BRSV challenge in calves.

Finally, several parameters such as the dose of vaccine, the type of adjuvant and the route of administration may have influenced the degree of protection. The oil based adjuvants in our $N^{SRS}$ vaccine are from the Montanide™ range that are present in several veterinary vaccines used in the field to eradicate viral diseases (e.g. foot and mouth disease) in many countries and for decades. In the present study, we aimed at inducing a strong cellular response therefore we selected a dedicated mineral oil based adjuvant for intramuscular vaccination (ISA 71 VG). For intranasal vaccination we selected one adjuvant from the Montanide™ IMS technology (IMS 4132 VG) that has physical properties (flow-ability, viscosity) permitting an easy delivery in animal nostril.

The intramuscular route is a classical way of injection for inactivated vaccines. As it is true for live virus, intranasal vaccination with live-BRSV or modified-live-BRSV vaccine has been shown to be more efficacious in reducing viral shedding than intramuscular administration in young calves [40,41]. Moreover, a single intranasal vaccination has previously been shown to prime calves in the face of maternal antibodies [41]. Commercially available modified-live-BRSV vaccines that were formulated and licensed for parenteral use were shown to induce partial protection when administered intranasally [42]. Recently a single intranasal dose of a bivalent modified-live vaccine was shown to reduce nasal shedding of BRSV after challenge at 10 or 21 days post-vaccination, despite low BRSV neutralizing antibody titers detected after vaccination [32].

Thus we have chosen the two vaccination regimen used in the present study based on the hypothesis that administration of $N^{SRS}$ via the nasal route would strengthen any level of protection conferred by the intramuscular vaccination. However no differences in clinical and viral protection were observed between calves vaccinated intramuscularly only, versus intramuscularly plus intranasally. This could be explained by a weak response to the intranasal vaccination or a masking of the immune response induced after intranasal vaccination by those obtained after intramuscular vaccination. Unfortunately, due to a restricted number of BRSV seronegative calves, it was not possible to have a supplementary group of calves vaccinated only by the intranasal route.

By itself, the intramuscular administration of $N^{SRS}$ powerfully stimulated mucosal and systemic Ab responses and cellular immunity. The intranasal administration of $N^{SRS}$ given with the i.m. immunization increased some immune responses at the level of the upper respiratory tract: memory T cells producing IFN-γ in tracheo-bronchial lymph node and antigen-specific IgA in nasal secretion.

The expression of CD45RO is considered a reliable marker to monitor activated CD4 and CD8 T cells in bovine [43,44]. In the context of primary infection with BRSV, CD8 T cells are the predominant subset recruited to the airways [45] and prior vaccination with inactivated or live-attenuated virus can modify the pattern of T cell responses [46]. We had reported previously a preferential priming of CD4 responses following challenge with HRSV-A2 of mice vaccinated intranasally with $N^{SRS}$ [22]. Thus it is possible that the intranasal delivery of $N^{SRS}$ was responsible for the early presence of $CD45RO^+$ $CD4^+$ T cell in bovine airways post-challenge of the i.m. + i.n. vaccinated calves.

However, the nasal vaccination might not have been efficient enough to prime protective local anti-viral immunity. This could be related to the type of adjuvant used, the delivery device/route, the antigen itself. Additional studies will be necessary to improve intranasal vaccination with $N^{SRS}$ by testing several doses of antigen in association with adjuvants used for intranasal delivery and to compare results with those obtained after intramuscular vaccination.

What are the immune correlates of the viral and clinical protection afforded by the $N^{SRS}$ vaccination?

We have shown in our previous study in mice that the antibodies raised against N were not neutralizing and thus unlikely to be involved in the anti-viral protection. Cellular immunity mediated by virus-specific CD8 T cells is required to clear BRSV from the lungs of infected calves in a primary infection [47,48] and N is one of the main targets of $CD8^+$ T cell responses to BRSV [29]. In the present study we have shown that $N^{SRS}$ vaccination primed antigen-specific T cell memory responses, characterized by their capacity to proliferate and secrete IFN-γ. In other studies implying $N^{SRS}$ vaccination in mouse or lamb animal models we have shown that N-specific memory CD8 and CD4 T cell are primed ([22] and S. Riffault unpublished results). Recently a vaccination regimen using the nucleoprotein in a DNA prime–protein boost protocol was shown to be superior to DNA or protein vaccination alone to prime

antigen-specific CD8 memory T cells, to protect against BRSV replication and to reduce lung pathology [19]. However, because DNA vaccination is not authorized in human, the use of N protein alone for vaccination of newborn children could be an interesting alternative approach. Moreover $N^{SRS}$ can be produced in large amount in bacteria and their nanoring structure is very stable either at 4 °C or 20 °C (our unpublished observations), making their production and storage cheap and easy.

Setting up the right conditions for cross-protective cellular immunity against conserved antigens is a growing challenge in the vaccinology field nowadays (e.g. universal Influenza vaccine). The nucleoprotein subunit approach described in the present study is efficient for inducing cross-protective immunity against RSV. Interestingly the $N^{SRS}$ structures are very potent at stimulating antibody responses both at the systemic and mucosal levels. We have recently obtained the X-ray 3D structure of the $N^{SRS}$ [21] and have been able to map exposed sites on the nano-rings to which other antigenic motifs can be grafted. Our next goal will be to improve the degree of protection by using RSV nucleocapsid nanoparticles grafted with peptidic epitopes from the BRSV fusion- (F) and glyco- (G) proteins in order to trigger neutralizing antibody responses in addition to anti-N cellular responses.

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*Conflict of interest*: None.

## References

[1] Meyer G, Deplanche M, Schelcher F. Human and bovine respiratory syncytial virus vaccine research and development. Comp Immunol Microbiol Infect Dis 2008;31(2–3):191–225.
[2] Valarcher JF, Taylor G. Bovine respiratory syncytial virus infection. Vet Res 2007;38(2):153–80.
[3] Elvander M. Severe respiratory disease in dairy cows caused by infection with bovine respiratory syncytial virus. Vet Rec 1996;138(5):101–5.
[4] Openshaw PJ, Yamaguchi Y, Tregoning JS. Childhood infections, the developing immune system, and the origins of asthma. J Allergy Clin Immunol 2004;114(6):1275–7.
[5] Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969;89(4):405–21.
[6] Antonis AF, Schrijver RS, Daus F, Steverink PJ, Stockhofe N, Hensen EJ, et al. Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: exploring the parameters of pathogenesis. J Virol 2003;77(22):12067–73.
[7] Kimman TG, Sol J, Westenbrink F, Straver PJ. A severe outbreak of respiratory tract disease associated with bovine respiratory syncytial virus probably enhanced by vaccination with modified live vaccine. Vet Q 1989;11(4):250–3.
[8] Openshaw PJ, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. Clin Microbiol Rev 2005;18(3):541–55.
[9] Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15(1):34–41.
[10] Goetsch L, Plotnicky-Gilquin H, Aubry JP, De-Lys P, Haeuw JF, Bonnefoy JY, et al. BBG2Na an RSV subunit vaccine candidate intramuscularly injected to human confers protection against viral challenge after nasal immunization in mice. Vaccine 2001;19(28–29):4036–42.
[11] van Drunen Littel-van den Hurk S, Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol 2007;17(1):5–34.
[12] Goulder PJ, Lechner F, Klenerman P, McIntosh K, Walker BD. Characterization of a novel respiratory syncytial virus-specific human cytotoxic T-lymphocyte epitope. J Virol 2000;74(16):7694–7.
[13] Cherrie AH, Anderson K, Wertz GW, Openshaw PJ. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J Virol 1992;66(4):2102–10.
[14] Venter M, Rock M, Puren AJ, Tiemessen CT, Crowe Jr JE. Respiratory syncytial virus nucleoprotein-specific cytotoxic T-cell epitopes in a South African population of diverse HLA types are conserved in circulating field strains. J Virol 2003;77(13):7319–29.
[15] King AM, Stott EJ, Langer SJ, Young KK, Ball LA, Wertz GW. Recombinant vaccinia viruses carrying the N gene of human respiratory syncytial virus: studies of gene expression in cell culture and immune response in mice. J Virol 1987;61(9):2885–90.
[16] Connors M, Collins PL, Firestone CY, Murphy BR. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J Virol 1991;65(3):1634–7.
[17] Taylor G, Thomas LH, Furze JM, Cook RS, Wyld SG, Lerch R, et al. Recombinant vaccinia viruses expressing the F, G or N, but not the M2, protein of bovine respiratory syncytial virus (BRSV) induce resistance to BRSV challenge in the calf and protect against the development of pneumonic lesions. J Gen Virol 1997;78(Pt 12):3195–206.
[18] Boxus M, Tignon M, Roels S, Toussaint JF, Walravens K, Benoit MA, et al. DNA immunization with plasmids encoding fusion and nucleocapsid proteins of bovine respiratory syncytial virus induces a strong cell-mediated immunity and protects calves against challenge. J Virol 2007;81(13):6879–89.
[19] Letellier C, Boxus M, Rosar L, Toussaint JF, Walravens K, Roels S, et al. Vaccination of calves using the BRSV nucleocapsid protein in a DNA prime-protein boost strategy stimulates cell-mediated immunity and protects the lungs against BRSV replication and pathology. Vaccine 2008;26(37):4840–8.
[20] Tran TL, Castagne N, Bhella D, Varela PF, Bernard J, Chilmonczyk S, et al. The nine C-terminal amino acids of the respiratory syncytial virus protein P are necessary and sufficient for binding to ribonucleoprotein complexes in which six ribonucleotides are contacted per N protein protomer. J Gen Virol 2007;88(Pt 1):196–206.
[21] Tawar RG, Duquerroy S, Vonrhein C, Varela PF, Damier-Piolle L, Castagne N, et al. Crystal structure of a nucleocapsid-like nucleoprotein-RNA complex of respiratory syncytial virus. Science 2009;326(5957):1279–83.
[22] Roux X, Dubuquoy C, Durand G, Tran-Tolla TL, Castagne N, Bernard J, et al. Sub-nucleocapsid nanoparticles: a nasal vaccine against respiratory syncytial virus. PLoS One 2008;3(3):e1766.
[23] Valarcher JF, Bourhy H, Gelfi J, Schelcher F. Evaluation of a nested reverse transcription-PCR assay based on the nucleoprotein gene for diagnosis of spontaneous and experimental bovine respiratory syncytial virus infections. J Clin Microbiol 1999;37(6):1858–62.
[24] Valarcher JF, Bourhy H, Lavenu A, Bourges-Abella N, Roth M, Andreoletti O, et al. Persistent infection of B lymphocytes by bovine respiratory syncytial virus. Virology 2001;291(1):55–67.
[25] Hamel AL, Wasylyshen MD, Nayar GP. Rapid detection of bovine viral diarrhea virus by using RNA extracted directly from assorted specimens and a one-tube reverse transcription PCR assay. J Clin Microbiol 1995;33(2):287–91.
[26] Espinasse J, Peel JE, Voirol MJ, Schelcher F, Valarcher JF. Absence of circulating TNF alpha in experimental bovine pneumonic pasteurellosis. Vet Rec 1993;132(12):303–4.
[27] Boxus M, Letellier C, Kerkhofs P. Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. J Virol Methods 2005;125(2):125–30.
[28] Leutenegger CM, Alluwaimi AM, Smith WL, Perani L, Cullor JS. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan polymerase chain reaction. Vet Immunol Immunopathol 2000;77(3–4):275–87.
[29] Gaddum RM, Cook RS, Furze JM, Ellis SA, Taylor G. Recognition of bovine respiratory syncytial virus proteins by bovine CD8+ T lymphocytes. Immunology 2003;108(2):220–9.
[30] Belknap EB, Baker JC, Patterson JS, Walker RD, Haines DM, Clark EG. The role of passive immunity in bovine respiratory syncytial virus-infected calves. J Infect Dis 1991;163(3):470–6.
[31] Salt JS, Thevasagayam SJ, Wiseman A, Peters AR. Efficacy of a quadrivalent vaccine against respiratory diseases caused by BHV-1, PI3V, BVDV and BRSV in experimentally infected calves. Vet J 2007;174(3):616–26.
[32] Vangeel I, Antonis AF, Fluess M, Riegler L, Peters AR, Harmeyer SS. Efficacy of a modified live intranasal bovine respiratory syncytial virus vaccine in 3-week-old calves experimentally challenged with BRSV. Vet J 2007;174(3):627–35.
[33] Bryson DG, Adair BM, McNulty MS, McAliskey M, Bradford HE, Allan GM, et al. Studies on the efficacy of intranasal vaccination for the prevention of experimentally induced parainfluenza type 3 virus pneumonia in calves. Vet Rec 1999;145(2):33–9.
[34] Adair BM, Bradford HE, McNulty MS, Foster JC. Cytotoxic interactions between bovine parainfluenza type 3 virus and bovine alveolar macrophages. Vet Immunol Immunopathol 1999;67(3):285–94.
[35] West K, Petrie L, Konoby C, Haines DM, Cortese V, Ellis JA. The efficacy of modified-live bovine respiratory syncytial virus vaccines in experimentally infected calves. Vaccine 1999;18(9–10):907–19.
[36] Woolums AR, Anderson ML, Gunther RA, Schelegle ES, LaRochelle DR, Singer RS, et al. Evaluation of severe disease induced by aerosol inoculation of calves with bovine respiratory syncytial virus. Am J Vet Res 1999;60(4):473–80.

[37] Frankild S, de Boer RJ, Lund O, Nielsen M, Kesmir C. Amino acid similarity accounts for T cell cross-reactivity and for "holes" in the T cell repertoire. PLoS One 2008;3(3):e1831.
[38] Piazza FM, Johnson SA, Darnell ME, Porter DD, Hemming VG, Prince GA. Bovine respiratory syncytial virus protects cotton rats against human respiratory syncytial virus infection. J Virol 1993;67(3):1503–10.
[39] Taylor G, Bruce C, Barbet AF, Wyld SG, Thomas LH. DNA vaccination against respiratory syncytial virus in young calves. Vaccine 2005;23(10):1242–50.
[40] Kimman TG, Westenbrink F, Straver PJ. Priming for local and systemic antibody memory responses to bovine respiratory syncytial virus: effect of amount of virus, virus replication, route of administration and maternal antibodies. Vet Immunol Immunopathol 1989;22(2):145–60.
[41] Woolums AR, Brown CC, Brown Jr JC, Cole DJ, Scott MA, Williams SM, et al. Effects of a single intranasal dose of modified-live bovine respiratory syncytial virus vaccine on resistance to subsequent viral challenge in calves. Am J Vet Res 2004;65(3):363–72.
[42] Ellis J, Gow S, West K, Waldner C, Rhodes C, Mutwiri G, et al. Response of calves to challenge exposure with virulent bovine respiratory syncytial virus following intranasal administration of vaccines formulated for parenteral administration. J Am Vet Med Assoc 2007;230(2):233–43.
[43] Bembridge GP, MacHugh ND, McKeever D, Awino E, Sopp P, Collins RA, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology 1995;86(4):537–44.
[44] Hogg AE, Worth A, Beverley P, Howard CJ, Villarreal-Ramos B. The antigen-specific memory CD8+ T-cell response induced by BCG in cattle resides in the CD8+ gamma/deltaTCR-CD45RO+ T-cell population. Vaccine 2009;27(2):270–9.
[45] McInnes E, Sopp P, Howard CJ, Taylor G. Phenotypic analysis of local cellular responses in calves infected with bovine respiratory syncytial virus. Immunology 1999;96(3):396–403.
[46] Antonis AF, Claassen EA, Hensen EJ, de Groot RJ, de Groot-Mijnes JD, Schrijver RS, et al. Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary bovine respiratory syncytial virus infection. Vaccine 2006;24(10):1551–61.
[47] Taylor G, Thomas LH, Wyld SG, Furze J, Sopp P, Howard CJ. Role of T-lymphocyte subsets in recovery from respiratory syncytial virus infection in calves. J Virol 1995;69(11):6658–64.
[48] Gaddum RM, Cook RS, Thomas LH, Taylor G. Primary cytotoxic T-cell responses to bovine respiratory syncytial virus in calves. Immunology 1996;88(3):421–7.