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Intranasal vaccination with recombinant CD protein and adamantylamide dipeptide as mucosal adjuvant enhances pulmonary clearance of *Moraxella catarrhalis* in a murine experimental model

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Running title: Intranasal Vaccination with rCD Protein and AdDP

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Abstract

Moraxella catarrhalis causes acute otitis media in children and lower respiratory tract infections in adults and elderly. The presence of antibodies against the highly conserved outer membrane protein CD correlates in children with protection against infection, suggesting its usefulness as vaccine antigen. However, native CD is difficult to purify and it is still unclear if recombinant CD (rCD) constitutes indeed a valid alternative. We performed a side-by-side comparison of the immunogenicity and efficacy of vaccine formulations containing native and rCD with adamantylamide dipeptide as mucosal adjuvant. Intranasal vaccination of mice stimulated the production of high CD-specific antibody titers in sera and sIgA in mucosal lavages, which cross-recognized both antigens. While vaccination with native CD increased the number of IL-2 and IFN-γ producing cells, rCD mainly stimulated IL-4 secreting cells. Nevertheless, efficient bacterial clearance was observed in the lungs of challenged mice receiving either native or rCD (96% and 99%, respectively). Thus, rCD is a promising candidate for incorporation in vaccine formulations against *M. catarrhalis*.

Introduction

In the last two decades, *Moraxella catarrhalis* has emerged as an important mucosal pathogen (35). In children, it is one of the etiological agents of sinusitis, bronchitis, pneumonia and acute otitis media (AOM) (18, 23). In our hospital, between 1994 and 2001 the main etiological bacterial agents isolated from children middle ear fluids were *Haemophilus influenzae* (45%) and *Streptococcus pneumoniae* (39%), which remained almost constant during this period, followed by *M. catarrhalis*, which raised from 4 to 11%. In adults, it is one of the etiological agents of recurrent infections, particularly in patients with chronic obstructive pulmonary disease (COPD), being responsible for approximately 30% of the new cases (37). The clinical management of patients infected with *M. catarrhalis* also represents a problem, since high costs are associated with established therapies and there is a global emergence of antibiotic resistant strains (35). Therefore, a vaccine able to block bacterial infection at mucosal level would represent an invaluable tool.

Between the eight major outer membrane proteins (OMPs) of *M. catarrhalis*, the proteins C and D represent two different stable forms of the same protein (11, 35). A single gene codes for a protein with a predicted molecular weight of 46 kDa, which migrates as a doublet in SDS-PAGE in the 60 kDa range (2, 28). CD is an integral porin-like protein that plays a role in bacterial attachment to host cell associated mucins, promoting bacterial ascension through the Eustachian tube (15, 20, 21). The CD protein is considered a promising vaccine candidate because it is surface exposed and highly conserved (17, 30). The presence of exposed epitopes on its surface suggests that antibodies could bind CD on the intact

bacteria (24, 30). The importance of antibody responses directed against CD is further supported by clinical evidence. In fact, patients recovered from a *M. catarrhalis* infection with high levels of antibodies against CD are less susceptible to re-infections than those with low levels or no antibodies against CD (22, 26).

Thus, the potential of CD as candidate vaccine antigen has been explored in the past. Purified CD protein induced antibodies in guinea pigs and mice that not only bound to intact *M. catarrhalis*, but also exhibited *in vitro* bactericidal activity against the pathogen (41). However, the fastidious growth properties of *M. catarrhalis* and the relatively poor expression of this protein render particularly difficult the large-scale production of native CD (nCD). Therefore, the production of a recombinant CD protein (rCD) constitutes the only valid alternative for mass vaccine production. In this context, a previous report suggested that rCD might be a potentially useful candidate antigen (20, 27). However, side-by-side comparisons between the recombinant and the native antigen were not performed, making extremely difficult to assess whether rCD represents indeed a valid alternative. In addition, this study was performed injecting the rCD emulsified with incomplete Freund's adjuvant intra Peyer's patches (27), thereby rendering more difficult to predict responses under standard vaccination schedules in humans.

It was demonstrated that intranasally administered antigens trigger better immune responses in the respiratory tract and in the middle ear than those administered orally or parenterally (16). Thus, it seems particularly attractive to assess the potential of a CD-based formulation administered by this route, using a mucosal challenge model with bacterial clearance as a read-out. Unfortunately, the use of this route generally induces relatively poor immune responses, with the

exception of naturally acquired infections. However, this can be overcome by the use of mucosal adjuvants.

We have previously demonstrated that the mucosal adjuvant adamantylamide dipeptide (AdDP) (3, 5) enhance the immune responses against the OMP of *H. influenzae* P6 when co-administered by intranasal route. This led to the elicitation of a protective response against pulmonary or middle ear challenge with virulent bacteria (5). Thus, in the present work we performed a side-by-side comparison of the immunogenicity and efficacy of vaccine formulations containing nCD and rCD with AdDP as mucosal adjuvant. The obtained results demonstrated that a vaccine candidate based on rCD and AdDP stimulates an immune response able to promote efficient bacterial clearance after a pulmonary challenge of mice with a virulent *M. catarrhalis* strain.

Materials and Methods

Animals

BALB/c mice (aged 8-12 weeks) were purchased from Gador Laboratories (Buenos Aires, Argentina) and Harlan-Winkelmann GmbH (Borchen, Germany) and maintained under standard conditions. All experiments were approved by the local authorities.

110

111 ***Cell cultures***

112 Spleen cells were grown in RPMI 1640 supplemented with 10% fetal bovine
113 serum (FBS), 100 U/ml of penicillin, 50 µg/ml of streptomycin, $5x10^{-5}$ M 2-
114 mercaptoethanol and 1 mM L-glutamine (Gibco BRL, Karlsruhe, Germany).

115

116 ***Bacterial strains and growth conditions***

117 Pathogenic strains of *M. catarrhalis* were isolated from the middle ears of
118 children with long-term otitis media with effusion at the Ricardo Gutiérrez
119 Children's Hospital. The strains were maintained in pure skim milk as well as in
120 brain-heart infusion (BHI) broth plus glycerol 50% (vol/vol) at -80°C until used. One
121 strain (ARG2003-1) was selected for CD purification, cloning and challenge studies
122 based on its clearance rates in lungs of BALB/c mice. *M. catarrhalis* was streaked
123 onto BHI agar or were grown in BHI broth and then were incubated overnight (ON)
124 at 37°C in 5% CO_2. *Escherichia coli* strains XL1-blue and BL21 (DE3) were used
125 for cloning and expression of recombinant protein. Luria-Bertani broth or agar (20)
126 was used for the cultivation of *E. coli* strains.

127

128 ***Adjuvant***

129 AdDP was synthesized by Bachem (Bubendorf, Switzerland), according to
130 good manufacturing practice (GMP) guidelines, as described elsewhere (3).

131

132 ***SDS-PAGE and Western blot analysis***

133 SDS-PAGE was performed according to the methods of Laemmli (19).
134 Proteins present on the gels were detected by using the Coomassie blue stain.
135 Immunoblot analysis was performed with mouse antiserum raised against nCD or
136 rCD, as previously described (7).

137

138 ***Purification of native CD***

139 Native CD was purified according to a method described elsewhere (41)
140 with minor modifications. In brief, *M. catarrhalis* was grown ON in BHI broth.
141 Bacteria were harvested by centrifugation at 10,000 *g* for 20 min at 4°C. The pellet
142 was resuspended in 50 mM Tris-HCl, pH 8.0, and the cells were disrupted by
143 sonication and centrifuged at 26,000 *g* for 20 min at 4°C. The supernatant
144 containing soluble bacterial proteins was discarded. The pellet was washed twice
145 with buffer A (50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 10 mM
146 EDTA) and twice with 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS. The SDS-
147 insoluble pellet was then resuspended in buffer A. Urea was added to a final
148 concentration of 6 M and the mixture was heated at 60°C for 30 min. Under these
149 conditions nCD was solubilized and remained soluble after ON dialysis at 4°C
150 against 50 mM Tris-HCl, pH 8.0. The nCD protein was found to be >95% pure as
151 judge by SDS-PAGE and scanning densitometry, according to the analysis
152 performed using the multi-Analyst software (Bio-Rad laboratories, Inc). The protein

153 concentration was determined by use of a protein assay kit (BioRad Laboratories),
154 according to the manufacturer's instructions.

155

156 ***Construction and purification of rCD protein***

157 Restriction and modification enzymes were purchased from New England
158 Biolabs. DNA manipulations were performed as described by Sambrook et al. (29),
159 and PCR amplifications were performed with a PCR reagent kit (Perkin-Elmer),
160 according to the manufacturer's instructions. A DNA fragment encoding the mature
161 CD protein was amplified from the chromosomal DNA of the *M. catarrhalis* isolate
162 strain by use of PCR. The primer complementary to the 5´ end of the CD gene, 5´-
163 GCGGGATCCGGTGTGACAGTCAGCCCACTACTA-3´, was designed to contain a
164 *Bam*HI site (underlined). The reverse primer, 5´-
165 CGCGTCGACTTGAACAATCATATCTTTGGTTTG-3´, was designed to contain the
166 3´ end of the coding sequence and a *Sal*I site (underlined). The resulting PCR
167 products was ligated into the vector pCR2.1-TOPO (TOPO TA Cloning; Invitrogen),
168 and then transformed into the *E. coli* strain XL-1 blue. The fragment encompassing
169 the CD gene was digested with *Bam*HI and *Sal*I, subsequently cloned into the
170 expression vector pET23a(+) (Novagen) to generate the plasmid pET23-CD, which
171 was then transformed into *E. coli* BL21 (DE3) strain. The sequence of the insert
172 was verified by DNA sequence analysis. The amino acid sequence has 99%
173 identity with the two previously reported CD proteins (15, 25). At nucleotide level
174 the sequence of our strain (GenBank accession number EF093799) differed in 9

175 and 7 nucleotides with respect to the published sequences (GenBank accession
176 numbers AY493741 and L10755, respectively). CD expression was verified by use
177 of SDS-PAGE analysis and Coomassie blue stain, before and after the induction of
178 the promoter T7 with isopropyl-β-D-thio-galactopyranoside (IPTG). Overexpression
179 and purification of the His-tagged CD protein was performed under denaturing
180 conditions, according to Qiagen protocols. The identity of the purified rCD was
181 verified by Western blot analysis using mice anti-sera raised against nCD. The
182 purity and protein concentration were determined as described above for the nCD
183 protein.

184

185 ***Immunization schedules and sample collection***

186 At days 0, 7 and 21, groups of 5-10 mice were vaccinated by intranasal inoculation
187 (10 µl/nostril) with either nCD or rCD (40 µg/dose) and AdDP (200 µg/dose) as a
188 mucosal adjuvant diluted in sterile PBS. Control groups received either AdDP
189 alone or PBS. No endotoxin activity was detected (<1 ng/ml) using the HEK-Blue™
190 LPS detection kit (InvivoGen). On day 31, serum samples were collected from
191 blood of the tail vein, and stored at –20°C until used. Then, mice were sacrificed to
192 obtain bronchoalveolar lavage (BAL), nasal lavage (NAL), and middle-ear lavage
193 (MEL) samples. NAL samples were obtained by gently flushing the nasal cavities
194 from the posterior opening of the nose with 100 µl of PBS with 40 µM of PMSF
195 each cavity after removing the mandible. BAL samples were obtained by irrigation
196 with 400 µl of PBS with 40 µM of PMSF, using a blunted needle inserted into the

197 trachea after tracheotomy. MEL samples were obtained by irrigation of each
198 tympanic cavity twice with 50 µl of PBS with 40 µM of PMSF. The recovered
199 washes were centrifuged at 10,000 x *g* for 5 min to remove debris and
200 supernatants were collected and stored at -20°C until used. Spleens were removed
201 and pooled for analysis.

202

203 ***Detection of CD-specific antibodies by ELISA***

204 CD-specific antibody titers in NAL, BAL, MEL and serum samples were
205 determined by ELISA. In brief, 96-well Nunc-Immuno MaxiSorp assay plates
206 (Nunc) were coated with 0.5 µg/well nCD or rCD in coating buffer (carbonate [pH
207 9.4]) for 2 h at 37°C and then washed four times with PBS-0.05% Tween 20 (PBS-
208 T). Plates were blocked with PBS-T plus 1% bovine serum albumin (BSA) for 2 h at
209 37°C. Serial 2-fold dilutions of samples in PBS-T were added (50 µl/well) and
210 plates were incubated ON at 4°C. After four washes, antibody binding was
211 revealed by use of either horseradish peroxidase-conjugated goat anti–mouse IgG
212 or phosphatase alkaline–conjugated α-chain–specific rabbit anti–mouse IgA
213 antibodies (ICN), as secondary antibodies. Plates were incubated for 2 h at 37°C,
214 and, after 4 washes, the reactions were developed by use of 2,2´-azino-bis(3-
215 ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer (pH 4.35)
216 containing 0.01% H_2O_2 or *p*-nitrophenyl phosphate in 10 mM diethanolamine (pH
217 9.5) containing 0.5 mM $MgCl_2$. The absorbance was read at a wavelength of 405

218 nm. End-point titers were expressed as the reciprocal log_2 of the last dilution, which
219 provided an OD >0.1 units above the background (pre-immune sera).

220

221 ***Bactericidal assay***

222 The bactericidal capacity of the antibodies present in serum and BAL fluids from
223 immunized mice was assessed as previously described (27). In brief, pooled sera
224 and BAL samples were heat-inactivated by incubation at 56°C for 30 min. Bacteria
225 were grown in BHI broth at 37°C with shaking until they reach an OD_{600} of 0.2.
226 Then, the culture was diluted 1:4000 in sterile PCGM (3.26 mM NaH_2PO4, 12.8
227 mM $NaHPO_4$, 125 mM NaCl, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1% gelatin) and
228 20 µl were mixed with 10 µl of diluted sera and 10 µl of PBS, and incubated at RT
229 for 30 min. Then, 10 µl of complement, prepared by adsorbing normal human
230 serum with protein G to remove IgG, were added and the reaction was incubated
231 at 37°C for further 30 min. Finally, 200 µl of PCGM were added and 50 µl reactions
232 were plated onto agar plates in duplicates. The plates were incubated ON and
233 colonies were counted the next day. The bactericidal titer was defined as the
234 highest dilution wherein at least 50% killing was observed. Viable bacterial counts
235 at the time complement added were determined for each experiment. Appropriate
236 controls were included in the assay, namely: bacteria and complement in the
237 absence of serum or BAL fluid to ensure that the complement source was not
238 killing bacteria; bacteria plus either serum or BAL in the absence of complement to

ensure that the samples were not toxic to the bacteria; bacteria and buffer alone to ensure viability of bacteria.

Determination of IL-2, IL-4 and IFN-γ secreting cells

The number of IL-2, IL-4 and IFN-γ secreting cells was determined by ELISPOT (Becton-Dickinson). Spleen cells were added at a final concentration of $5x10^5$ and $1x10^6$ cells/well, and incubated in quadruplicates in the absence or presence of either nCD or rCD (10 µg/ml). To determine the number of $CD8^+$ IFN-γ secreting cells, $CD4^+$ cells were depleted using Dynabeads M-450 epoxy (Dynal Biotech) coated with anti-CD4 antibodies (clone L3T4, BD Pharmingen), according to the manufacturers' instructions (4). Spots were scanned with the ImmunoSpot Series 3A Analyzer and counted using the ImmunoSpot image analyzer software v3.2 (C.T.L.).

Measurement of cellular proliferation.

Proliferation assays were performed in triplicates, as previously described (6). Briefly, spleen cells ($5x10^5$ cells/well) were incubated for 4 days in the presence of rCD. Eighteen hours before harvest 1 µCi of ^{3}H-Thymidine (Amersham International, Freiburg, Germany) was added to each well. Cells were harvested on paper filters (Filtermat A; Wallac, Freiburg, Germany) by using a cell-harvester (Inotech, Wohlen, Switzerland), and the amount of incorporated ^{3}H-Thymidine was determined by a γ–scintillation counter (Wallac 1450, Micro-Trilux).

261

262 ***Bacterial challenge studies***

263 Bacteria were grown ON onto BHI plates in 5% CO_2, harvested, and washed
264 twice and resuspended in saline. After 8 days of the last boost, mice were
265 anesthetized by ip injection with 100 µl of PBS containing 2.5 mg of ketamine, and
266 a bolus inoculum of $5x10^8$ CFU of live bacteria in 50 µl of saline was introduced
267 into the lungs via an intratracheal cannula. The concentration of the inoculum was
268 estimated by the OD_{600} and confirmed by determining the number of CFU, by
269 plating serial dilutions on BHI plates. Mice were sacrificed 4 h after lung inoculation
270 and the intact lungs were excised, placed in 200 µl of sterile saline, and
271 homogenized (27). The efficiency of the bacterial clearance was established by
272 determining the number of viable bacteria present in lung homogenates plating
273 serial dilutions of the samples on BHI plates and incubated at 37°C with 5% CO_2
274 (5, 27).

275

276 ***Statistical analysis***

277 In the immunogenicity and protection studies the significance of the
278 differences observed between two groups was determined by Student's unpaired
279 two-tailed *t*-test on transformed data (log_{10} or log_2), and between three or more
280 groups by one-way analysis of variance (ANOVA) with Tukey-Kramer as multiple
281 comparisons test. Viable bacterial counts were compared by nonparametric Mann-
282 Whitney U test between two groups and Krustal-Wallis test with Dunn's multiple

comparisons test for three or more groups. For parametric or log transformed data, results were expressed as mean ± standard error of the mean (SEM), whereas for nonparametric data results were expressed as median and ranges. Differences were considered significant at $P<0.05$.

Results

Characterization of the rCD protein

The rCD protein was evaluated by SDS-PAGE and densitometry, as well as by Western blot analysis using serum samples obtained from mice vaccinated with nCD or rCD (Fig. 1A and B). These approaches allowed us to determine the purity and identity of the rCD preparation used in the vaccination studies. The obtained results demonstrated that rCD could be efficiently purified by Ni^{+2}-agarose affinity chromatography (>95% of purity), and that this recombinant protein is specifically recognized by antibodies raised against both nCD and rCD. In addition, total proteins of *M. catarrhalis* were analyzed by Western blot using an antiserum raised against rCD, thereby confirming the cross reactivity of both proteins (Fig. 1C). This was further supported by competitive binding inhibition assays, which showed that nCD specifically binds to antibodies raised against rCD, blocking their capacity to bind to immobilized rCD protein (data not shown).

Intranasal immunization with nCD or rCD co-administrated with AdDP induces the elicitation of humoral responses both in serum and mucosal secretions.

High titers of CD-specific antibodies were stimulated after intranasal immunization with either nCD or rCD co-administered with AdDP (Fig. 2). There were not statistically significant differences between the end point titers observed in both groups, thereby confirming that under our experimental conditions rCD is as immunogenic as nCD. On the other hand, no significant differences were observed in the IgG titers when sera from rCD or nCD vaccinated animals were tested using plates coated with either nCD or rCD (data not shown). This further confirms the ability of rCD-specific antibodies to recognized nCD. The levels of CD-specific serum IgA were slightly higher in animals vaccinated with rCD than in those receiving nCD (Fig. 2), however, the differences were not statistically significant ($P>0.05$). No CD-reacting antibodies were observed in control animals.

To evaluate the capacity of the different formulations to promote the elicitation of a mucosal immune response in vaccinated mice, the presence of CD-specific secretory IgA (sIgA) was analyzed in mucosal lavages (Fig. 3). Significant increments in the levels of CD-specific sIgA were observed in NAL, BAL and MEL of mice vaccinated with rCD. In contrast, mice vaccinated with nCD showed a weaker CD-specific sIgA response in NAL and BAL than mice vaccinated with rCD, and anti-CD sIgA was not detected in their MEL.

The quality of the antibodies stimulated after vaccination was assessed by evaluating the bactericidal activity of sera and BAL. Interestingly, the sera and BAL from mice immunized with rCD showed bactericidal activity up to a dilution of 1:64, and 1:4, respectively, whereas no activity was detected when samples from nCD vaccinated mice were tested (Table 1). The pre-immune sera from vaccinated and control mice showed no bactericidal activity.

Cellular responses stimulated after intranasal vaccination with nCD and rCD.

The cellular immune responses induced by vaccination were first evaluated by assessing the proliferative capacity of spleen cells after *in vitro* re-stimulation with either nCD or rCD. Similar proliferative responses were observed in animals immunized with nCD or rCD alone (Fig. 4A). The co-administration of nCD or rCD protein with AdDP resulted in the induction of a stronger proliferative response (Fig. 4A). No statistic significant differences were observed in mice vaccinated with nCD or rCD. We also analyzed the number of IL-2-secreting cells present in spleens of immunized mice by ELISPOT. The cells were increased in mice receiving either nCD or rCD, with stronger responses in those vaccinated with AdDP as adjuvant (Fig. 4B). Interestingly, the responses were significantly stronger (P<0.001) in animals vaccinated with nCD alone or co-administered with AdDP than in the immunization groups receiving rCD or rCD plus AdDP (Fig. 4B).

We then analyzed the number of IL-4-secreting cells present in spleens from vaccinated mice after *in vitro* re-stimulation with either nCD or rCD. The number of IL-4-secreting cells was not increased in animals immunized with nCD, whereas a

slight increment was observed when cells from rCD-vaccinated mice were tested (Fig. 4C). On the other hand, the presence of AdDP in the formulation resulted in an increased number of IL-4-secreting cells, which was significantly stronger in mice receiving rCD ($P<0.05$) (Fig. 4C). These results are in agreement with those obtained for IL-2-secreting cells (Fig. 4B).

Finally, we determined the number of IFN-γ-producing cells in vaccinated animals. As shown in Fig. 4D, the frequency of $CD8^+$ IFN-γ-secreting cells was higher in mice vaccinated with nCD protein plus AdDP than in the PBS control group ($P<0.01$) and in mice receiving rCD ($P<0.05$) or nCD alone. Interestingly, the co-administration of AdDP with rCD resulted in a reduction in the number of IFN-γ-secreting cells respect to animals receiving rCD alone (Fig. 4D).

Intranasal immunization with nCD or rCD enhances the pulmonary clearance of M. catarrhalis.

The comparative efficacy of nCD- and rCD-based vaccine formulations was evaluated by studying the pulmonary clearance of a clinical isolate of *M. catarrhalis* after challenge. Mice immunized with either nCD- or rCD-protein based vaccine showed more efficient bacterial clearance in the lungs ($P<0.03$), in comparison to control mice (8.8×10^4 and 1.8×10^4 CFU/lungs, respectively, versus 2.6×10^6 CFU/lungs) (Fig. 5). In fact, a reduction of 96% and 99% in the number of viable bacteria was observed in nCD- and rCD-vaccinated mice, respectively. These results indicate that both nCD and rCD are able to stimulate a similar degree of bacterial clearance ($P>0.05$).

Discussion

There is a clear need for a vaccine able to prevent diseases caused by *M. catarrhalis*. Like most human pathogens, *M. catarrhalis* gains entrance to the body via the mucosal surface. Thus, it would be desirable to stimulate a local immune response at the portal of entry, since sIgA plays a major role in the defense against mucosal pathogens (38). In this context, mucosal vaccination can lead to the stimulation of humoral and cellular immune responses at both mucosal and systemic levels. However, despite the concept of a common mucosal immune system, whereby immune cells activated at one site disseminate to remote mucosal tissues, there is a significant degree of compartmentalization. This constraints the choice of vaccination route for inducing effective immune responses at the specific effector sites (14, 16). Intranasal vaccination seems particularly attractive for stimulating responses in the respiratory tract. However, administration of soluble antigens by this route generally does not induce effective immune responses. This problem can be overcome by using mucosal adjuvants (10, 31, 39, 40). However, there are safety concerns associated with the use of some of these molecules in humans (*e.g.*, toxicity, retrograde homing to neural tissues) (34).

The implementation of vaccines against *M. catarrhalis* based on a native antigens, such as the proposed OMP CD is, unfortunately, not suitable for large scale production under GMP conditions. However, a previous study showed that intra Peyer's patches injection of rCD emulsified in Freund's adjuvant enhances pulmonary clearance of *M. catarrhalis* in a mouse model (27). Purification of rCD

396 can be implemented in a simple and cost effective manner. Nevertheless, the
397 selection of a recombinant immunogen as a vaccine component is not only based
398 on the capacity to purify large amounts of antigen to homogeneity, but also on the
399 demonstration that the recombinant protein retains intact the immunological
400 properties of the native protein. In this context, no comparative studies have been
401 performed testing nCD and rCD.

402 In the present study, we have performed a side-by-side evaluation of the
403 immunogenicity and efficacy of nasal vaccine formulations based on nCD and rCD.
404 Our results show that intranasal immunization with CD using a mucosal adjuvant
405 with an adequate safety profile, such as AdDP, induces an efficient CD-specific
406 sIgA response in the middle ear and lungs, as well as strong humoral and cellular
407 systemic responses. Immunoblot assays also showed that both rCD and nCD are
408 cross-recognized by antibodies raised after vaccination with either nCD or rCD.
409 Vaccination with the rCD and AdDP resulted in a stronger stimulation of IL-4
410 secreting cells than in animals immunized with nCD plus AdDP. On the other hand,
411 vaccination with nCD and AdDP stimulated a significant increment in the number of
412 IL-2 and IFN-γ producing cells. Thus, the use of rCD seems to result in a stronger
413 Th_2 polarization, which is in agreement with the improved sIgA responses
414 observed in mucosal territories.

415 Although *M. catarrhalis* is a human restricted pathogen, the pulmonary
416 clearance murine model is widely accepted as cost-efficient screening tool for new
417 vaccine candidates (9, 12, 13, 27, 32, 36). Thus, we exploited it to evaluate the
418 efficacy of the CD-specific responses stimulated after intranasal immunization with

rCD or nCD plus AdDP. Our results demonstrated that there is an enhanced bacterial clearance in the lung in both nCD- and rCD-vaccinated mice challenged with *M. catarrhalis*. No statistic significant differences were observed in bacterial clearance between animals immunized with nCD or rCD. Thus, it seems that enhanced bacterial clearance correlates with the presence of CD-specific IgG in serum from vaccinated mice. However, no bactericidal activity was observed when sera from mice immunized with nCD were tested. This suggests that effector mechanisms other than bactericidal antibodies are responsible for the clearance observed in nCD-vaccinated mice. In this context, it is important to highlight that efficient production of IFN-γ was only observed in nCD vaccinated animals. These findings are not unusual in vaccinology, since different effector mechanisms seems to be stimulated by different licensed vaccines against whooping cough (1, 8, 33).

In conclusion, our comparative study strongly supports the use of rCD as antigen for mucosal vaccine formulations against *M. catarrhalis*, particularly in combination with AdDP as adjuvant. Interestingly, we have recently shown that intranasal immunization with recombinant P6 protein and AdDP confers protection against otitis media and lung infection by non-typeable *H. influenzae* (5). These results suggest that it might be possible to use AdDP for a combined nasal vaccine aimed at protecting against respiratory infections caused by *H. influenzae* and *M. catarrhalis.*

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587 **Figure Legends**

588

589 **Fig. 1.** Characterization of nCD and rCD proteins. (A) Total proteins from *E. coli*
590 BL21 (pET23-CD) were analyzed for rCD expression on a 10% SDS-PAGE by
591 Coomasie blue stain. *E. coli* BL21 (pET23-CD) before (lane 1) and after (lane 2)
592 induction. (B) rCD from cell extract was purified by Ni^{+2}-agarose chromatography,
593 separated onto a 10% SDS-PAGE, and visualized by Coomassie blue stain (lane
594 1) and Western blot using mouse antiserum raised against rCD (lane 2) and nCD
595 (lane 3). (C) Total proteins from *M. catarrhalis* were analyzed by Western blot
596 using a mouse antiserum raised against rCD. The position of the standards for
597 molecular weight (kDa) is indicated on the left. IPTG, isopropyl-β-D-
598 thiogalactopyranoside.

599

600 **Fig. 2.** CD-specific serum antibodies after intranasal immunization with nCD or rCD
601 using AdDP as mucosal adjuvant. Data are the reciprocal log2 of the mean end-
602 point titer, the standard error of the mean is indicated by vertical lines. Differences
603 were statistically significant at $P<0.0001$ (*) and $P<0.0005$ (**) respect to the
604 control groups.

605

606 **Fig. 3.** CD-specific secretory IgA antibodies in bronchoalveolar (BAL), nasal (NAL)
607 and middle-ear (MEL) lavages obtained from mice after intranasal immunization
608 with nCD or rCD using AdDP as adjuvant. Data are the reciprocal log2 of the mean
609 end-point titer; the standard error of the mean is indicated by vertical lines.

610 Differences were statistically significant at $P<0.005$ (*) and $P<0.01$ (**) respect to
611 the control group.
612
613 **Fig. 4.** Cellular immune responses stimulated in mice after vaccination with either
614 nCD or rCD using AdDP as adjuvant. (A) Proliferation was assessed after 4 days
615 of in vitro re-stimulation of spleen cells in the presence of the CD protein, by
616 measuring ^{3}H-Thymidine incorporation (c.p.m.). Results are expressed as the
617 mean cpm from triplicates subtracted of background values from non-stimulated
618 cells. (B, C) Total splenocytes were incubated for 40 h in the presence of either
619 nCD or rCD and the numbers of (B) IL-2- and (C) IL-4-producing cells was
620 determined by ELISPOT. (D) $CD4^{+}$-depleted splenocytes were incubated for 16 h
621 in the presence of either nCD or rCD and the number of IFN-γ-producing cells was
622 determined by ELISPOT. Results are expressed (B, C and D) as spot forming units
623 (SFU)/10^{6} cells in stimulated samples subtracted of background from non-
624 stimulated cells. The standard error of the mean is indicated by vertical lines.
625 Differences between the experimental groups were statistically significant at
626 $P<0.05$ (*), $P<0.01$(**) and $P<0.001$ (***).
627
628 **Fig. 5.** *M. catarrhalis* recovery from lung homogenates obtained from mice
629 intranasally vaccinated with either nCD or rCD co-administered with AdDP.
630 Immunized mice were challenged by intratracheal route with $5x10^{8}$ CFU of *M.*
631 *catarrhalis*. Bacterial clearance was assessed after 4 h by plating serial dilutions of
632 the lung homogenates onto BHI agar plates. Data are expressed as mean

633 CFU/lung; the standard error of the mean is indicated by vertical lines. (*)
634 Differences were statistically significant at *P*<0.03 respect to the AdDP control
635 group.
636

TABLE 1: Bactericidal activity of sera and BAL of mice immunized with nCD or rCD using AdDP as mucosal adjuvant

Vaccination group	Tested sample	Bactericidal titer[a]	
		Pre-immune	Post-immune
PBS	Sera	<2	<2
	BAL	<2	<2
nCD + AdDP	Sera	<2	<2
	BAL	ND[b]	<2
rCD + AdDP	Sera	<2	64
	BAL	ND	4

[a]Bactericidal titers are expressed as the reciprocal of the highest dilution of either serum or BAL capable of killing at least 50% of bacteria compared with controls. The results were obtained from duplicate bacterial plating and no significant variations were found between the two plates. [b]ND: not determined.