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Development and characterization of attenuated metabolic mutants of *Bordetella bronchiseptica* for applications in vaccinology

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Summary

Bordetella bronchiseptica is an important pathogen causing a number of veterinary respiratory syndromes in agriculturally important and food-producing confinement-reared animals, resulting in great economic losses annually amounting to billions of € worldwide. Currently available live vaccines are incompletely satisfactory in terms of efficacy and safety. An efficient vaccine for livestock animals would allow reducing the application of antibiotics, thereby preventing the massive release of pharmaceuticals into the environment. Here, we describe two new potential vaccine strains based on the BB7865 strain. Two independent attenuating mutations were incorporated by homologous recombination in order to make negligible the risk of recombination and subsequent reversion to the virulent phenotype. The mutations are critical for bacterial metabolism, resistance to oxidative stress, intracellular survival and *in vivo* persistence. The resulting double mutants BB7865 *risA aroA* and BB7865 *risA dapE* were characterized as promising vaccine candidates, which are able to confer protection against colonization of the lower respiratory tract after sub-lethal challenge with the wild type strain.

Keywords: *B. bronchiseptica*, metabolic mutant, vaccination, vaccine.

Introduction

Bordetella bronchiseptica infects a broad range of mammalian hosts, causing acute or chronic respiratory infections, often resulting in oligosymptomatic or asymptomatic clinical forms (Goodnow, 1980). *B. bronchiseptica* constitutes both, a primary etiological agent and a predisposing factor for secondary infections, thereby exacerbating disease symptoms provoked by other pathogens (e.g. bacteria and viruses), thus resulting in a broad range of diseases, such as pneumonia and atrophic rhinitis in swine, infectious tracheobronchitis in dogs (kennel cough), and bronchopneumonia in other domestic animals like cats (Mattoo and Cherry, 2005). *B. bronchiseptica* efficiently colonizes the ciliated respiratory epithelium and persists in the lower respiratory tract for several weeks, thereby establishing chronic infections (Mattoo and Cherry, 2005). The persistence of *B. bronchiseptica* is facilitated by the expression of virulence determinants tightly controlled by a two-component regulatory system encoded in the *bvgAS* locus.

Until now, most *B. bronchiseptica* strains are sensitive to aminoglycosides such as amikacin and gentamycin, as well as to extended-spectrum third-generation penicillins, and tetracyclines (Woolfrey and Moody, 1991; Mattoo and Cherry, 2005). However, antimicrobial therapy seems to be of limited value in animals (Bemis et al., 1977; Speakman et al., 2000; Zhao et al., 2011). In addition, antibiotic treatment of food animals could also have dramatic consequences for human health (Aarestrup et al., 2008). Therefore, prophylaxis of diseases caused by *B. bronchiseptica* through vaccination rather than by treatment of infected animals seems to be the only

reasonable choice. Current vaccines against *B. bronchiseptica* are given by the systemic route stimulating protective antibody responses against severe forms of disease (Gopinathan et al., 2007). However, these vaccines do not prevent *B. bronchiseptica* infections. Thus, despite vaccination animals continue to be carriers, which in turn predispose them to other infectious agents (Sukumar et al., 2009). Furthermore, recent studies indicated that immunity elicited after parenteral vaccination is less protective than infection-induced immunity. Hence, the induction of mucosal immunity seems to be crucial in order to prevent subsequent infections (Wolfe et al., 2007). To stimulate protective immune responses it might be also essential to maintain the expression of the virulence factors necessary during the infection process. A strategy which enables attenuation without compromising the antigenicity of a vaccine strain is based on the disruption of metabolic pathways, such as inactivation of the genes encoding for the 5-enolpyruvyl-shikimate-3-phosphate synthase (*aroA*) and the N-succinyl-DAP desuccinylase (*dapE*), which are crucial for bacterial *in vivo* growth and survival (Gilvarg, 1960; Dugal et al., 1992; Jungnitz et al., 1998; McArthur et al., 2003).

B. bronchiseptica not only invades both nonphagocytic and phagocytic cells (Guzman et al., 1994a; Forde et al., 1998), but also efficiently persists within these cells (Guzman et al., 1994b; Schipper et al., 1994; Banemann and Gross, 1997; Zimna et al., 2001). This in turn facilitates bacterial escape from host clearance mechanisms and might subsequently promote chronic infection (Schipper et al., 1994; Harvill et al., 1999). The underlying mechanisms allowing *B. bronchiseptica* to survive intracellularly seem to be *bvg*-independent, as *bvg* mutants survived to a similar or even greater extent than their wild type counterparts (Guzman et al., 1994b; Schipper et al., 1994; Banemann and

Gross, 1997). Mutants in the two-component regulatory system RisAS, which is required for bacterial resistance to oxidative stress, exhibited impaired intracellular survival and *in vivo* persistence (Jungnitz et al., 1998). In vaccine development, the impairment of the capacity to survive intracellularly of a given mutant is an important correlate for an attenuated phenotype, but it might also lead to a reduced *in vivo* survival (i.e. decreased antigen load), which might result in impaired immune responses.

Thus, our strategy was to target critical metabolic pathways of *B. bronchiseptica* in combination with a mutation in the *ris* locus. The double mutants showed an adequate safety profile and conferred protective immunity, representing promising attenuated vaccines against this pathogen, as well as scaffold for multicomponent live veterinary vaccines.

Results

Double attenuated mutants of *B. bronchiseptica* showed impaired intracellular survival

Single and double *B. bronchiseptica* mutants were generated as described in Experimental procedures. The ability of single and double mutants in the selected target genes to invade and persist within eukaryotic cells was evaluated using the human epithelial cell line (HeLa), as well as the murine macrophage- and DC-like cell lines J774A.1 and CB1, respectively (Fig. 1). The single metabolic mutant BB7865 *aroA* exhibited a considerable impaired capacity to persist within HeLa cells (23% of the wild

type after 24 h). In contrast, dysfunction of the N-succinyl-DAP succinylase (*dapE*) seems to affect the intracellular survival of BB7865 *dapE* to a lesser extent (50%). As expected, this capacity has been further impaired in the double mutants (BB7865 *risA aroA*, 17% and BB7865 *risA dapE*, 39%; Fig. 1). A similar survival pattern has been obtained evaluating the survival capacity within the murine derived professional antigen presenting DC-like CB1 cells (Fig. 1). Again, the strains harboring the *dapE* mutation seem to be slightly more efficient in intracellular survival, as compared to the strains harboring the *aroA* mutation (18% and 17% vs 14% and 14%, respectively). However, in contrast to what has been observed in HeLa cells, this capability was not further impaired in the double mutants (Fig. 1). Interestingly, the BB7865 *aroA* mutant exhibit a considerably improved *in vitro* survival within J774A.1 cells, even after 24 h of infection (146% of the wild type strain, respectively; Fig. 1). This higher recovery could be related to lower levels of toxicity, associated with lower expression of the autotransporter protein pertactin (PRN) by this mutant, as *B. bronchiseptica* has been reported to mediate the cytotoxicity to macrophages via *bvg*-regulated factors, including PRN (Forde et al., 1999). Furthermore, the BB7865 *dapE* mutant was also able to persist within J774A.1 cells more efficiently, as compared to the other cell types (80% of the parental strain). In contrast, only a few CFU of the BB7865 *risA* strain were present 24 h after infection (10%), which is in accordance with previous observations (Jungnitz et al., 1998). However, similar to what was observed for HeLa cells, the introduction of a second mutation further reduced the capacity of intracellular survival of strains BB7865 *risA aroA* and BB7865 *risA dapE* (45% and 11% of the values obtained for BB7865). Interestingly, while the *dapE* mutants seemed to be more efficient in surviving within

both HeLa and CB1 cells, as compared to the *aroA* mutants, the latter persists more efficiently within J774A.1 cells (Fig. 1).

Taken together, destruction of metabolic pathways by mutation resulted in an impaired capacity of the mutant strains to survive within eukaryotic cells. In addition, introduction of a second mutation (*risA*) further reduced this ability.

Vaccination of mice with live attenuated derivatives of *B. bronchiseptica* by i.n. route induces strong humoral and cellular immune responses

Previous studies demonstrated the importance of both humoral and cellular immunity in prevention of *B. bronchiseptica* infections (Pishko et al., 2004; Kirimanjeswara et al., 2005; Pilione and Harvill, 2006). Clearance of this pathogen from the respiratory tract is mainly triggered by opsonizing antibodies (IgG2a subclass), Th1 immune responses characterized by high IFN- γ production, and TLR4-mediated recruitment and activation of immune cells, followed by neutrophil-mediated CR3- and Fc γ R-dependent phagocytic killing mechanisms (Pishko et al., 2004; Kirimanjeswara et al., 2005; Pilione and Harvill, 2006). Thus, we evaluated the immune responses triggered following vaccination with the attenuated derivatives of *B. bronchiseptica*.

Consistent with previous studies (Gueirard et al., 1996), IgG production was detected in all of the groups between 11-18 days following priming (Fig. 2). Although after the challenge the highest titers were observed in mice immunized with the single mutants BB7865 *aroA*, BB7865 *dapE* and BB7865 *risA*, similar titers were stimulated by the double mutants BB7865 *risA aroA* and BB7865 *risA dapE*. Interestingly, while similar or even increased levels of IgG1 compared to the values of IgG2a were stimulated

following immunization with the mutant strains, IgG2a was the predominant subclass present 10 days after challenge of vaccinated mice with the wild type strain BB7865 (Fig. 3). These findings affirm the results of Gopinathan et al. showing that vaccination and infection with *B. bronchiseptica* induced different type of antibody responses (Gopinathan et al., 2007). However, only low levels of *B. bronchiseptica*-specific secretory IgA (sIgA) could be observed in mucosal secretions of vaccinated animals (Fig. 4). Furthermore, no significant levels of antigen-specific sIgA were detected in lung homogenates of immunized mice, as compared to the control group. Conversely, the highest sIgA titers were observed in bronchoalveolar lavage fluids (BALF) of immunized mice, whereupon BB7865 *aroA* stimulated the strongest sIgA response, followed by BB7865 *risA dapE*, BB7865 *risA* and BB7865 *dapE* , whereas no sIgA have been detected in BALF of mice immunized with BB7865 *risA aroA* (Fig. 4). On the other hand, immunization with the double mutants seemed to stimulate higher sIgA titers in nasal washes (NW), as compared to those of mice vaccinated with the single mutants (Fig. 4).

Besides humoral immune responses, an efficient stimulation of cellular immunity seems to be critical to confer optimal protection against *B. bronchiseptica*. In this context, the clearance of this pathogen is known to be facilitated by a Th1-dominated cellular response, but there is increasing evidence that *B. bronchiseptica* is able to redirect the polarization of the adaptive immune response from a dominant Th1 towards a Th17 response pattern (Siciliano et al., 2006). Interestingly, BB7865 *aroA* was the only strain stimulating the same cytokine profile as the wild type strain, showing high values of IL-17 producing cells followed by IFN- γ producing cells (Fig. 5). In contrast, all other mutant strains stimulated a Th1-dominated cytokine pattern, as indicated by the high numbers

of IFN- γ producing cells. In line with the observations of previous studies, only very low levels of IL-4 secretion were observed in response to stimulation with inactivated bacteria in all immunization groups, thereby confirming their limited capacity to promote Th2 helper responses. The same was true in terms of induction of IL-2 secreting cells. Only modest and no significant differences were detected between experimental groups following vaccination (Fig. 5).

Vaccination of mice using the novel attenuated *B. bronchiseptica* strains inhibits colonization of the lower and upper respiratory tract after challenge with wild type *B. bronchiseptica*

To investigate the capacity of the generated mutants to trigger protective immune responses following vaccination by intranasal (i.n.) route, bacterial loads in NW and lung homogenates of immunized mice challenged with the wild type strain BB7865 were investigated. We also assessed the capacity of the mutants to persist following immunization. To distinguish between BB7865, BB7865 *risA* and their attenuated derivatives bacterial suspensions were plated on appropriate selective media and the number of CFU were determined. Remarkably, while the single mutants BB7865 *aroA* and BB7865 *dapE* were able to persist over the whole course of the experiment (Fig. 6A, C, E, G), introduction of a second mutation in the vaccine strains further reduced their ability to persist within the host since the double mutants BB7865 *risA aroA* and BB7865 *risA dapE* were cleared within 14 and 21 days from both, the lower and upper respiratory tract, respectively (Fig. 6B, D, F, H). However, vaccination with all tested mutants resulted in an efficient clearance of the wild type strain from the upper and

lower respiratory tract (Fig. 6). Immunization with the single mutants BB7865 *aroA* and BB7865 *dapE* resulted in an about 1,000-fold reduction in the number of CFU from lungs, as compared to the values observed in the control group receiving PBS alone (Fig. 6E, G). Clearance of the wild type strain from the upper respiratory tract of immunized animals was also statistically significant compared to the control group (Fig. 6A, C). Although vaccination with the double mutant BB7865 *risA aroA* was slightly less efficient at stimulating clearance in the lower respiratory tract (about 200-fold reduction of bacterial load), immunization with the BB7865 *risA dapE* strain resulted in a complete clearance of the wild type strain from the lungs of infected mice (Fig. 6F, H). On the other hand, vaccination of mice with BB7865 *risA aroA* seems to be slightly more efficient at protecting mice against colonization of the upper respiratory tract by the wild type strain (factor 40 compared to unvaccinated control group) with respect to immunization with BB7865 *risA dapE* (factor 7 compared to unvaccinated control group) (Fig. 6B, D).

Immunization of mice using the novel attenuated *B. bronchiseptica* strains reduces neutrophil influx in the lungs following challenge with wild type bacteria

To evaluate the safety profile of the new attenuated mutants, BALF of vaccinated animals were collected at different time intervals to perform a kinetic analysis of the local influx of different cellular subpopulations (Fig. 7). In all experimental groups except the control group (non-vaccinated), an early self-limited influx of lymphocytes and neutrophils was observed on day 3 after immunization (Fig. 7A). This initial recruitment of neutrophils to the lungs during the course of *B. bronchiseptica* infections seems to be

triggered by TLR4 (Mann et al., 2004). While the influx of neutrophils completely subsided already after 7 days in animals vaccinated with the BB7865 *aroA* mutant, it persisted in animals receiving BB7865 *dapE*, BB7865 *risA*, BB7865 *risA aroA* and BB7865 *risA dapE* for 7 to 13 days, and in the case of BB7865 *dapE* between 14 and 21 days (Fig. 7B, C). In addition, BALF of vaccinated mice also showed an influx of monocytic cells, whereas the counts for eosinophils were negligible at all-time points. Interestingly, on day 21 (Fig. 7D) a second cellular influx was observed in all groups, which was accompanied by an increment in the number of epithelial cells. This could be explained by a progressive damage of the epithelial integrity as a result of a secondary release of bacterial products and/or desquamation of infected cells, which in turn could act as chemo-attractants for lymphocytes and neutrophils. However, only marginal levels of epithelial cells have been observed in BALF of mice immunized with BB7865 *aroA*, suggesting only moderate tissue damage (Fig. 7D). Following challenge of vaccinated animals with the wild type *B. bronchiseptica* strain, a strong influx of neutrophils was observed. Interestingly, the influx seemed to be less massive in BALF of mice immunized with the double mutants (Fig. 7E). Furthermore, vaccinated animals showed a significant reduction in the number of neutrophils compared to naïve mice already on day 3 post challenge and values returned to baseline levels already on day 10 post challenge (Fig. 7E-G). In contrast, the strongest influx of neutrophils and lymphocytes was observed in BALF of control animals (PBS + challenge) returning to baseline levels on day 21 after the challenge (Fig. 7H). Subsequent to the challenge, a strong increment was also evident in the counts for monocytes/macrophages with the highest values reached on day 3 post challenge (Fig. 7F). Thus, the effect of immunization with

attenuated mutants was impressive in all groups, leading to consistent reduction in neutrophils influx.

Discussion

B. bronchiseptica has a broad host range and contributes to a number of acute respiratory syndromes in food-producing confinement-reared animals, as well as in pets (Goodnow, 1980; Rajeev et al., 2003). *B. bronchiseptica* also plays a significant role as predisposing factor for secondary infections caused by bacteria or viruses (Sukumar et al., 2009). Although different vaccines are already available, respiratory infections caused by *B. bronchiseptica* are still widespread, since existent vaccines are not completely satisfactory in terms of efficacy and/or safety (King, 2004). Thus, there is still a critical need for more efficient vaccines.

It was previously reported that infected animals are resistant to re-infection by *B. bronchiseptica*, suggesting that infection-induced immunity is more effective than responses stimulated by whole cell or subunit vaccines (Wolfe et al., 2007). Thus, a successful strategy must be directed toward mimicking natural infections. Implementation of mucosal vaccines (e.g. amenable for aerosolization) based on live attenuated and genetically-defined *B. bronchiseptica* mutants possessing all virulent determinants required to trigger protective immunity could represent a breakthrough. This approach would allow triggering protection against both disease (i.e. symptoms) and infection (i.e. colonization). Therefore, we developed attenuated metabolic mutants

of *B. bronchiseptica* based on the inactivation of the genes *aroA* and *dapE*, which encode for enzymes involved in the pre-chorismate and the DAP/lysine pathways, respectively. Studies performed in other bacterial species suggested that inactivation of these genes could result in mutants with an appropriate safety profile (Hess et al., 1996; Gunel-Ozcan et al., 1997; Sizemore et al., 1997).

Thus, metabolic mutants were generated by insertion mutagenesis based on homologous recombination. To improve the safety profile of the strains, the metabolic mutations were combined with a second mutation in the locus encoding the two-component regulatory system RisAS, which is critical for bacterial intracellular survival (Jungnitz et al., 1998). As a result, the risk of reversion to virulence by recombination has been further minimized.

To assess the level of attenuation of the mutants, infection assays were performed. In agreement with previous studies, the BB7865 *aroA* and BB7865 *risA* mutants exhibit impaired survival within HeLa cells (Jungnitz et al., 1998; Stenson et al., 2005). In contrast, the capacity of the single mutant BB7865 *dapE* was much less affected. As expected, when investigating the double mutants, their capacity to persist intracellularly was reduced even more. Similar survival patterns have been observed infecting CB1 cells. However, the survival rates were even more impaired as compared to those observed in HeLa cells. In contrast to what observed for HeLa and CB1 cells, BB7865 *aroA* and BB7865 *dapE* were able to persist within J774A.1 cells to the same extent or even more efficient than their parenteral strain. Only when the *risA* mutation was additionally introduced this capacity was drastically impaired. The higher recovery rates observed for the mutants could be explained by a diminished cytotoxic effect, whereby

reduced cellular damage prevents bacterial killing due to antibiotic influx from the culture medium. In fact, the mutants exhibit a weaker expression of PRN (data not shown), which mediates cytotoxicity on macrophages (Forde et al., 1999). The type III secretion system is another potential factor, since mutants were recovered from J774A.1 cells in significantly higher numbers than the wild type strain (Fennelly et al., 2008).

Next, we investigated the level of attenuation of the *B. bronchiseptica* mutants *in vivo* as well as their potential to stimulate efficient immune responses. Mice infected intranasally with either mutant did not show any signs of toxicity (e.g. weight loss or reduced motility), suggesting an adequate safety profile of the mutants. While all double mutants were cleared from the respiratory tract within 14 to 21 days, the single mutants persisted for at least 46 days in both the lower and upper respiratory tract. This is in contrast to what previously reported for an *aroA* mutant which was cleared from lungs after one week (Stevenson and Roberts, 2004). However, since the number of bacteria in our experimental conditions were very low, this discrepancy might be negligible. Nevertheless, the observed results are consistent with the impaired capacity of the double mutants to survive intracellularly and clearly demonstrate an efficient attenuation of *B. bronchiseptica* by introducing double or even single mutations.

Despite the efficient attenuation of the mutants, immunization of mice by intranasal route stimulated strong humoral and cellular immune responses. A considerable increment in *Bordetella*-specific IgG titers was observed, which is of importance since IgG constitutes a main effector for achieving efficient bacterial clearance. Former studies reported that rises in IgG and IgA titers in dogs vaccinated with an attenuated *B. bronchiseptica* strain correlated with protection (Bey et al., 1981; Stevenson and Roberts, 2002). IgG levels

detected in sera of mice vaccinated with either the single or the double mutants differed only marginally. The mutants containing the *aroA* or *dapE* mutation stimulated similar levels of IgG production in sera, as well as IgA production in local mucosal surfaces. However, BB7865 *risA dapE* protected mice more efficiently against colonization of the lower respiratory tract, but less efficiently against colonization of the upper respiratory tract, as compared to its single mutant counterparts. In contrast, when mice were immunized with the *aroA* based mutants, exactly the opposite was observed. By and large, the immunity stimulated by the mutants was more efficient in the lower than in the upper respiratory tract. This correlates with higher *B. bronchiseptica*-specific IgA titers detected in BALF than in NW of vaccinated animals, and it is consistent with former reports showing that the sIgA titer correlates with the level of protection (Bey et al., 1981; Wolfe et al., 2007).

Protective immunity against *B. bronchiseptica* is also supported by efficient cellular responses (Roberts et al., 1993; McArthur et al., 2003). All *Bordetella* mutants stimulated proliferative responses following intranasal immunization (data not shown). In line with previous reports (Pathak et al., 2010), immunization with our metabolic mutants by intranasal route stimulated only small numbers of IL-4 secreting cells. In contrast, the number of IFN- γ producing cells was significantly increased. Thus, a clear Th1 dominated response pattern with limited stimulation of Th2 cells was promoted, which in turn is known to facilitate *B. bronchiseptica* clearance (Pathak et al., 2010). As reported before, immune responses stimulated by *Bordetella* vaccines differ from those observed following infection (Gopinathan et al., 2007). Interestingly, we observed the same analyzing the ratios of IgG1 and IgG2a subclasses stimulated by *aroA* and *dapE*

mutants following vaccination, which just shifted in favor of IgG2a after mice were challenged with the wild type strain. However, immunization with the mutant strains also promoted a significant increment of IL-17 producing cells, which is consistent with recent findings showing that *Bordetella* redirects adaptive immune responses from a Th1 dominated towards a Th17 response pattern (Siciliano et al., 2006). This might in turn support persistence of *Bordetella* within the respiratory tract. However, the exact role of Th17 helper cells in *B. bronchiseptica* infections is not yet fully understood, since it was suggested that stimulation of a mixed Th1/Th17 response by *B. pertussis* correlates with protection (Fedele et al., 2011).

Protective immunity against *B. bronchiseptica* relies on adaptive immunity. Thus, it is not surprising that clearance of wild type *Bordetella* from the lower respiratory tract of mice vaccinated with either the *dapE* or *aroA* mutants correlates with the immune responses stimulated by them. However, protection against *B. bronchiseptica* also requires an efficient activation of the innate immune system, especially of neutrophils and macrophages, which seems to be TLR4 dependent (Kirimanjeswara et al., 2007; Pathak et al., 2010). Therefore, we evaluated the influx of neutrophils and monocytes following vaccination with our mutant strains and challenge with wild type *Bordetella*. In all immunized mice an early influx of neutrophils and lymphocytes has been observed. This influx is short lived, returning to basal cellular levels already after 2 weeks, when the influx of monocytes reaches its maximum (Pathak et al., 2010). Interestingly, directly after challenge a massive re-influx of neutrophils followed by monocytes occurred. However, basal levels were reached much faster in vaccinated mice, between 3 (neutrophils) and 10 days (macrophages) post challenge, thereby reducing the risk of

tissue damage. Further evaluation of tissue damage using histological analysis of lungs showed almost no pathological changes in the lungs of mice immunized with BB7865 *risA aroA* and BB7865 *risA dapE* on days 14-21 after the challenge with wild type BB7865 strain (data not shown).

Taken together, we identified BB7865 *risA aroA* and BB7865 *risA dapE* as attenuated derivatives of *B. bronchiseptica*, which are able to promote efficient protective immunity against a sub-lethal challenge with virulent bacteria. Thus, they represent promising candidate vaccine strains for further development. However, their safety and efficacy in vaccine target animal species still remains to be established. Furthermore, to allow their implementation in the veterinary field, the generation of defined deletion mutants will be essential. Ideally, the deletion mutants should be combined with a marker mutation, which would allow differentiation between infected and vaccinated animals. It is expected that the resulting strains could be also exploited as a delivery system for heterologous antigens from other respiratory pathogens, such as *Pasteurella multocida*.

Experimental procedures

Generation of attenuated derivatives of *B. bronchiseptica*

B. bronchiseptica strain BB7865, a human nasopharyngeal isolate (Gross and Rappuoli, 1989; Stibitz et al., 1989; Schipper et al., 1994), and its derivative BB7865 *risA*, which harbors a mini-transposon insertion in the *ris* locus (Jungnitz et al., 1998), were selected

to generate attenuated vaccine strains carrying mutations in genes encoding for the 5-enolpyruvyl-shikimate-3-phosphate synthase (*aroA*), and for the N-succinyl-DAP desuccinylase (*dapE*).

Inactivation of the *Bordetella* genes *aroA* and *dapE* was performed using the suicide vector pFUS2 (Antoine et al., 2000). To this end, a 402 bp fragment encompassing positions 460 to 861 of the *aroA* gene was amplified by PCR using chromosomal DNA of the parenteral strain BB7865 as template and the primers AroAFwMd (5'-CCCAAGCTTGGCGGCGGCAGCATTGCG-3') and AroARvMd (5'-GCGGATCCCGCCAGCGTCGCGGCGAACG-3'). A 401 bp fragment encompassing positions 368 to 768 of the *dapE* gene was amplified using the primers DapEFwMd (5'-AAACTGCAGAACCCGGGTTCGATCGCCCTG-3') and DapERvMd (5'-CGCGGATCCGAACAGCGCCACCGCCTCG-3'). The resulting HindIII/BamHI (*aroA*) and PstI/BamHI (*dapE*) fragments were cloned into pFUS2. In order to transfer the resulting pFUS2 derivatives into the *B. bronchiseptica* strains BB7865 and BB7865 *risA* biparental matings were performed using the mobilizing strain *E. coli* SM10 λ pir as donor strain (Simon et al., 1983). Bacterial conjugation was performed on 0.45 μ m sterile filters placed onto Bordet-Gengou agar plates (Difco Detroit, MI, USA) with donor:recipient ratios of 10:1, 4:1 and 1:1. Plates were incubated at 37°C for 4 h or overnight. Homologous recombination events resulted in the integration of the pFUS2 derivatives into the corresponding chromosomal gene, thereby generating the single and double insertion mutants BB7865 *aroA*, BB7865 *dapE*, BB7865 *risA aroA* and BB7865 *risA dapE*, respectively. Transconjugants were selected according to the antibiotic resistance patterns of the recipient strains BB7865 and BB7865 *risA*

(streptomycin and kanamycin), as well as gentamycin as marker for pFUS2 derivative integration.

Infection assay

The eukaryotic cell lines HeLa (ATCC® CCL-2), J774A.1 (ATCC® TIB-67) and CB1 (Paglia et al., 1993) were maintained in complete DMEM and IMDM, respectively. Cells were adjusted to 2×10^5 cells (HeLa and J774A.1) and 5×10^4 cells per well (CB1), respectively, and seeded in 24-well plates (TPP, Switzerland). Prior to bacterial infection the plates were incubated for 12-18 h and then washed twice with complete pre-warmed (37°C) medium. *Bordetella* strains were grown for 24 h on Bordet-Gengou-agar plates, recovered with sterile swabs, suspended in complete medium and adjusted to a MOI of 100 (100 CFU/eukaryotic cell). Bacteria incubated in the absence of eukaryotic cells and uninfected eukaryotic cells were included as controls. Plates were incubated at 37°C, 5% CO₂, 95% humidity under static conditions for 30 min (J774 A.1 and HeLa) and 60-90 min (CB1), respectively. Afterwards, supernatants were discarded and cells were washed twice with warm PBS to remove non-adherent bacteria. To kill remaining extracellular bacteria the medium was replaced by 0.5 ml of medium supplemented with tobramycin and amikacin (100 µg/ml each, Sigma). After incubation, cells were washed 3 times with pre-warmed PBS to remove the antibiotics and then lysed by adding either 450 µl of sterile H₂O (CB1) or 450 µl of 0.025% Triton X-100 in H₂O (HeLa and J774A.1) to each well. The number of CFU recovered from each well was determined by plating 100-fold dilutions onto LB-agar plates supplemented with appropriate antibiotics. Infection assays were performed in triplicates. The values were normalized to infection

dose and shown as percentage of the values obtained for the wild type parental strain BB7865.

Immunization and challenge

B. bronchiseptica strains were grown on supplemented Bordet-Gengou agar. . Bacterial suspensions were recovered with sterile swabs, suspended and diluted in sterile PBS and adjusted to a final concentration of 5×10^7 CFU/ml. Six week old female BALB/c mice were immunized by the intranasal route (3-4 animals per group). In brief, animals were slightly anesthetized with Isoflurane (CuraMED Pharma, Karlsruhe, Germany), and subsequently allowed to inhale 20 μ l (10 μ l per nostril) of the bacterial suspension (approximately 1×10^6 CFU per mouse). Control groups received PBS only.

The non-lethal challenge of immunized mice was performed on day 25 or 26 after immunization using *B. bronchiseptica* wild type strain BB7865, which was administered to the external nostrils of anesthetized mice at a dose of 1×10^6 CFU/20 μ l/per mouse.

Sample collection

Blood samples were collected via the retro-orbital plexus by using 40 μ l glass capillaries after animals were anesthetized with Isoflurane. To collect the BALF, the trachea was intubated and the lung was flushed with 1 ml of sterile ice-cold PBS. In order to obtain NW, the nasal cavity was flushed with 200 μ l of sterile ice-cold PBS and the flow through was collected in sterile 1.5 ml tubes. BALF and NW were immediately placed on ice or stored at -20°C until further processing. Lungs were aseptically removed and placed in 4 ml of sterile ice-cold PBS. Then, lungs were homogenized using a Polytron PT 1200

homogenizer (Kinematica Lucerne, Switzerland). Finally, serial dilutions of the lung homogenates and NW were plated onto LB-agar plates supplemented with appropriate antibiotics to determine viable bacteria in different regions of the respiratory tract.

Cytospin preparations and Pappenheim's staining for differential cytology

BALF were centrifuged for 5 min at 2000x g, supernatants were stored at -20°C for subsequent analysis and cell pellets were resuspended in 500 µl of PBS. Cell numbers were determined and $1-2 \times 10^5$ cells were spun onto a glass slide. Then, cells were centrifuged for 5 min at 500 rpm by using a Shandon Cytofuge. After air-drying the slides, cells were stained according to Pappenheim's protocol. Differential cell counts of 500 cells/slide were performed using a microscope at 400-630x magnification due to morphological criteria. Eosinophils, neutrophils, lymphocytes, epithelia cells and macrophages were assessed taken into account the recovery volume of each sample to calculate the original cell number of every individual animal per ml of BALF. Differences between the groups were expressed as total cell counts.

ELISA

For the detection of *B. bronchiseptica*-specific Ig, 96-well flat-bottom microtiter plates were coated with 50 µl/well of heat-inactivated BB7865 (1×10^9 CFU/ml) in carbonate buffer (pH 9.6 for IgM, IgG and IgG isotypes). Plates were incubated for 30 min at 37°C followed by overnight incubation at 4°C. Then, plates were washed and blocked with 3% bovine serum albumin (BSA) in PBS. To analyze the titers of IgG and IgG-isotypes (IgG1, IgG2a), serum samples were serially diluted (1:2) in 0.1% BSA in PBS and

incubated for 2 h at 37°C. Afterwards, plates were washed six times with 1% BSA/PBS/0.05% Tween 20 and secondary antibodies (biotinylated goat anti-mouse IgG, IgG1 or IgG2a diluted in PBS + 0.1% Tween 20 + 1% BSA) were added. After incubation for 1 h at 37°C followed by six washes, 100 µl of peroxidase-conjugated streptavidin diluted 1:1000 in PBS + 0.1% Tween 20 + 1% BSA were added to each well and plates were further incubated for 30 min at 37°C. After final six washes, peroxidase substrate ABTS supplemented with 0.03% H₂O₂ was added and color reactions were monitored continuously for 30 min. End point titers were expressed as absolute values of the last dilution, which gave an OD_{405nm} of 2 times above the values of the negative controls.

NW, BALF and supernatant fluids of lung homogenates were used to detect total and antigen-specific sIgA. Microtiter plates were coated either with goat anti-mouse IgA for total IgA detection or with specific antigen (heat-inactivated BB7865 in coating carbonate buffer, pH 8.4). After washing and blocking as described above, serial dilutions of NW, BALF and supernatants of lung homogenates in PBS + 3% BSA were added starting with 1:20 and 1:2 dilutions for total and antigen-specific IgA, respectively. After incubation and washes, biotinylated goat anti-mouse IgA antibody was added. The following steps included incubation with peroxidase-conjugated streptavidin and ABTS, and monitoring of color reactions as already described. To compensate for variations in the efficiency of recovery of secretory antibodies among animals, the results were normalized and expressed as end point titers of antigen-specific IgA per µg of total IgA present in the sample.

ELISPOT

To determine the amount of IFN- γ , IL-2, IL-4 and IL-17 secreting cells, murine ELISpot kits (BD Pharmingen) were used according to the manufacturer's instructions. In brief, flat bottomed 96-well plates with a 0.45 μ m hydrophobic High Protein Binding Immobilon-P-Membrane were coated with the corresponding capture antibody (anti-IFN- γ , anti-IL-2, anti-IL-4 or anti-IL-17). Plates were washed and blocked with complete medium. Spleen cells were added at a final concentration of 1×10^6 and 5×10^5 cells/well in triplicates, in the absence (blank, only media added) or presence of heat-inactivated BB7865. To this end, inactivated BB7865 were adjusted with RPMI medium to a concentration of 1×10^7 CFU/50 μ l (2×10^8 CFU/ml) or a ratio bacteria:cell of 10:1, respectively. For positive controls, splenocytes were stimulated with the mitogen concanavalin A. ELISPOT plates were incubated for 16 or 48 h at 37°C in a 5% CO₂ atmosphere to detect IFN- γ , IL-2, IL-4- and IL-17 secreting cells, respectively. Spots were developed according to the manufacturer's instructions, scanned with the ImmunoSpot Series 3A Analyzer and counted using the ImmunoSpot Image Analyzer software v3.2 (C.T.L.). Results were expressed as Spot Forming Units (SFU) obtained from stimulated cells subtracted of background from non-stimulated cells.

Statistical analysis

Statistical analysis was performed using the software Sigma Stat version 3.1 for Windows or GraphPad Prism software (GraphPad Software, Inc., La Jolla, USA). For comparison between two groups the Student's *t*, Mann Whitney, and Two-Way ANOVA Multiple Comparison tests were used. Differences were considered significant at **P* <0.05, ***P* <0.01, and ****P* <0.001.

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Legends to the figures

Fig. 1. Invasion and intracellular persistence of *B. bronchiseptica* mutants in eukaryotic cells. Results are presented as percentage of the values obtained for the wild type parental strain BB7865. Results are representative out of three independent experiments. The SD of triplicates is indicated by vertical lines.

Fig. 2. Kinetic determination of *B. bronchiseptica*-specific serum IgG titers in mice immunized by i.n. route. Blood samples were taken before the immunization and subsequently on days 4, 11, 18, 25 and 35 after immunization. Challenge with the wild type BB7865 strain was performed on day 26. Results are expressed as geometric mean end point titers (GMT).

Fig. 3. Bordetella-specific IgG isotype titers in sera from mice immunized with live attenuated mutants by the i.n. route. IgG subclass pattern detected 21 days after immunization (A), and 10 days after challenge (B). The IgG isotypes titers represent the mean of 3-4 animals per experimental group. Results are expressed as geometric end point titers (GMT). The SD of triplicates is indicated by vertical lines.

Fig. 4. Analysis of antigen-specific secretory IgA in lung homogenates, BALF and NW of immunized mice. Results are presented as *Bordetella*-specific IgA end point titers per μg of total IgA. The SD is indicated by vertical lines.

Fig. 5. IFN- γ -, IL-2-, IL-4- and IL-17-producing cells in mice immunized with *B. bronchiseptica* mutant strains by the i.n. route. Spleen cells (1×10^6 and 5×10^5 cells/well) recovered from vaccinated mice were incubated in the presence of bacterial extracts and the numbers of cytokine producing cells were determined by ELISPOT. Results are presented as spot forming units (SFU) per 10^6 cells, which were subtracted from the values obtained from non-stimulated cells. The SEM of triplicates is indicated by vertical lines.

Fig. 6. Colonization profile in respiratory tract of mice following vaccination and challenge with *B. bronchiseptica*. For each individual animal the number of CFU were

determined in NW (A-D) or homogenized lungs (E-H) plated on LB agar plates supplemented with antibiotics following either vaccination (n=3 per group and time point) or challenge (n=4 per group and time point). The number of CFU for the wild type strain in the PBS control group represents bacterial burden after challenge (performed on day 25) with the BB7865 strain in non immunized mice. *P<0.05; **P<0.01 (loads of the wild type strain in immunized mice versus those from the PBS control group).

Fig. 7. Detection of different subpopulations of immune cells in BALF of mice vaccinated with attenuated derivatives of the *B. bronchiseptica* BB7865 strain by i.n. route. BALF of vaccinated animals were collected: A-D, on days 3, 7, 14 and 21 following immunization (n=3); E-H, on days 1, 3, 10 and 21 after challenge (n=4), respectively. The cellular composition was evaluated by Pappenheim staining of cytopsin preparations. Results are presented as absolute numbers of cells per ml of BALF. P values (*P<0.05; **P<0.01, ***P<0.001) are shown for neutrophil counts of experimental groups compared to control group PBS + challenge.

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