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Bacterial Ghosts Are an Efficient Delivery System for DNA Vaccines[1]

Thomas Ebensen,[2]* Susanne Paukner,[2†‡] Claudia Link,* Pavol Kudela,[‡§] Carola de Domenico,* Werner Lubitz,[†‡] and Carlos A. Guzmán[3]*

Mass implementation of DNA vaccines is hindered by the requirement of high plasmid dosages and poor immunogenicity. We evaluated the capacity of *Mannheimia haemolytica* ghosts as delivery system for DNA vaccines. In vitro studies showed that bacterial ghosts loaded with a plasmid carrying the green fluorescent protein-encoding gene (pEGFP-N1) are efficiently taken up by APC, thereby leading to high transfection rates (52–60%). Vaccination studies demonstrated that ghost-mediated delivery by intradermal or i.m. route of a eukaryotic expression plasmid containing the gene coding for β-galactosidase under the control of the CMV immediate early gene promoter (pCMVβ) stimulates more efficient Ag-specific humoral and cellular ($CD4^+$ and $CD8^+$) immune responses than naked DNA in BALB/c mice. The use of ghosts also allows modulating the major Th response from a mixed Th1/Th2 to a more dominant Th2 pattern. Intravenous immunization with dendritic cells loaded ex vivo with pCMVβ-containing ghosts also resulted in the elicitation of β-galactosidase-specific responses. This suggests that dendritic cells play an important role in the stimulation of immune responses when bacterial ghosts are used as a DNA delivery system. Bacterial ghosts not only target the DNA vaccine construct to APC, but also provide a strong danger signal, acting as natural adjuvants, thereby promoting efficient maturation and activation of dendritic cells. Thus, bacterial ghosts constitute a promising technology platform for the development of more efficient DNA vaccines. ***The Journal of Immunology*, 2004, 172: 6858–6865.**

Nucleic[1] acid vaccination has emerged as a powerful technology, which can be applied for the development of either prophylactic or therapeutic vaccines (1). The genes encoding the vaccine Ags are cloned into a eukaryotic expression plasmid, which is generally administered by i.m. injection or via biolistic skin bombardment with a gene gun. Then the biosynthetic machinery of the vaccinee's cell is responsible for the in vivo expression of the corresponding gene. The presence of immunostimulatory motifs in the DNA further contributes to the elicitation of an immune response. However, routine implementation of this approach in humans still does not seem to be feasible. This is mainly due to poor immunogenicity and the requirement of extremely high plasmid dosages. The low efficiency of traditional naked DNA vaccination can be due, at least in part, to the fact that APC are not specifically targeted and the encoded Ag is not delivered in the context of an adequate danger signal.

Bacterial ghosts are a novel nonliving vaccination technology platform, which is based on the conditional expression of the lethal lysis gene *E* from bacteriophage PhiX174 in Gram negatives (2–6). This leads to the formation of a transmembrane tunnel through the bacterial cellular envelope (Fig. 1*D*) (2). Due to the high internal osmotic pressure, the cytoplasm content is expelled through the tunnel (see Fig. 1*D*), resulting in an empty bacterial cell envelope (7). Bacterial ghosts retain all morphological, structural, and antigenic features of the cell wall and can be used as vaccine candidate per se. Alternatively, they can be exploited as a delivery system for proteins, which are either expressed and anchored to the envelopes before lysis or subsequently loaded (8). Bacterial ghosts can target APC and microvascular endothelial cells (9–11). The envelope components might provide a danger signal through the activation of pattern recognition receptors, thereby acting as natural adjuvants (12). However, the endotoxic effects of free LPS are not observed, because the LPS is associated to the ghost envelopes (13).

In this study, we evaluated the capacity of *Mannheimia haemolytica* ghosts as delivery system for DNA vaccines. In vitro studies demonstrate, for the first time, that ghosts are efficiently taken up by APC, thereby leading to high transfection efficiencies. Ghost-mediated DNA delivery resulted in the elicitation of more efficient immune responses than using naked DNA, allowing also modulation of the obtained immune response from a mixed Th1/Th2 to a more dominant Th2 response pattern. Intravenous immunization with dendritic cells (DC)[4] loaded ex vivo with plasmid-containing ghosts also resulted in the elicitation of specific humoral and cellular immune responses. Further in vitro studies demonstrated that bacterial ghosts promote efficient maturation and activation of DC. Thus, bacterial ghosts act as natural adjuvants, constituting a promising technology for the development of DNA vaccines.

Materials and Methods

Production and loading of M. haemolytica *ghosts*

Bacterial ghosts were produced by controlled expression of the lysis protein E in the *M. haemolytica* strain A23 (14–16). In brief, electrocompetent

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[4] Abbreviations used in this paper: DC, dendritic cells; EGFP/*egfp*, enhanced green fluorescent protein; i.d., intradermal.

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M. haemolytica cells were transformed with the plasmid containing the lysis system (14–16). Recombinant bacteria were grown in liquid medium at 28°C until they reached an OD_{600} of 0.25. Then the bacterial cultures were divided in two batches and further incubated at 28°C (control) and 42°C (induction of the *E*-mediated lysis system). Bacterial lysis was monitored by measuring the OD_{600}, FACS analysis, and determination of viable cell counts. After the lysis was completed, the *M. haemolytica* ghosts were washed, harvested by centrifugation (5000 × *g*, at 4°C, 15 min), and stored lyophilized until further use (14). Lyophilized ghosts (26.2 mg) were then resuspended in 600 μl of HEPES-buffered saline (100 mM NaCl, 10 mM sodium acetate, 10 mM HEPES, pH 7) containing pEGFP (enhanced green fluorescent protein)-N1 or pCMVβ (Clontech Laboratories, Palo Alto, CA), and after $CaCl_2$ supplementation (25 mM), they were incubated at 24°C for 30 min. Scanning electron micrographs were taken with a Hitachi S-800 field emission scanning electron microscope (Hitachi, Tokyo, Japan). Fixation of bacterial cells and sample preparation were performed, as previously described (17).

Real-time PCR

For quantification studies, real-time PCR was performed (Rotor-Gene 2000; Corbett Research, Maria Worth, Austria) with the DNA intercalating dye SYBR Green I (Roche, Grenzach, Germany) and primers (5′-AATG AGTATTCAACATTTCCGTGTC-3′ and 5′-TTACCAATGCTTAATCA GTGAGG-3′) specific for the ampicillin-coding gene. To obtain a standard curve, serial dilutions of the plasmid were prepared (10^{-2} to 10^{-6}).

Fluorescence labeling of bacterial ghosts

Ghosts were labeled by incubation with 10 mM sulforhodamine B (Sigma-Aldrich, St. Louis, MO); dsDNA labeling was performed by PCR amplification of a 400-bp fragment of the phage φCH1 with FITC-labeled primers (5′-CGGCAGGTTTCATCCAGGAG-3′ and 5′-TAACAGCACGCCGG AACTGA-3′). To localize pEGFP-N1 within bacterial ghosts, in situ hybridization with Cy-3-labeled probes obtained by amplifying the *egfp* gene using the oligonucleotides 5′-GGTGAGCAAGGGCGAGGAG-3′ and 5′-TTACTTGTACAGCTCGTCCATG-3′ was performed using ghosts stained with 0.25 μg/ml MitoTracker Green FM (Molecular Probes, Leiden, The Netherlands) (18). Loaded ghosts were analyzed by confocal microscopy (DMIRES; Leica, Solms, Germany), using *z*-axis sections with a maximal distance of 0.122 μm.

Transfection experiments

RAW264.7 macrophages were cultured in RPMI 1640 medium supplemented with L-glutamine, 10% FBS, and 5×10^{-5} M 2-ME Invitrogen, Karlsruhe, Germany). PBMC isolated from buffy coats from healthy donors by density centrifugation were resuspended in RPMI 1640 medium and allowed to adhere to plastic plates for 2 h. Adherent cells were cultured for 6 days in medium supplemented with GM-CSF and IL-4 (1000 U/ml) and harvested on day 7. Macrophages and DC were seeded in 24-well plates at a density of 1×10^5/well and incubated with bacterial ghosts loaded with pEGFP (500 bacteria/cell) for 2 h, washed to remove unbound ghosts, and further incubated for 48 h. Then cells were detached, fixed with 4% paraformaldehyde, and examined for EGFP expression by flow cytometry (Beckman Coulter, Krefeld, Germany). Immunostaining of EGFP was performed using polyclonal rabbit anti-EGFP Abs (1/200; Clontech Laboratories) and R-PE-conjugated polyclonal goat anti-rabbit IgG (1/100; Molecular Probes) (19).

Immunization studies

Six- to 8-wk-old female BALB/c (H-2d) mice were purchased from Harlan-Winkelmann (Borchen, Germany) and maintained under standard conditions according to institutional, local, and European Community guidelines. All experiments were approved by an independent local ethic committee. Groups of animals ($n = 5$) were immunized on days 1, 21, and 42 by either intradermal (i.d.; injection of 20 μl into rear flank) or i.m. (injection of 20 μl into the quadriceps) route with ghost loaded with pCMVβ (10^9 CFU, 210 μg containing 5 μg of DNA), pCMVβ alone (5 μg), or unloaded ghosts. The pCMVβ plasmid contains the β-galactosidase-encoding gene under the control of the CMV immediate early gene promoter. Serum samples were collected on days 0, 21, 42, and 52. Mice were sacrificed on day 52, and spleens were removed and pooled. One representative experiment of three is shown.

Ab assays

Sera from individual mice were assayed for the presence of β-galactosidase- and ghost-specific Abs by ELISA (20), using 96-well plates coated with 100 μl/well β-galactosidase (5 μg/ml; Boehringer Ingelheim, Ingelheim am Rhein, Germany) or ghost lysates (1 μg/ml) in 0.05 M carbonate buffer (pH 9.6). End point titers were expressed as the reciprocal log of the last dilution, which gave an OD at 405 nm of 0.1 U above the values of the negative controls.

Proliferation assays

Splenocytes were adjusted to 1×10^6 cells/ml in complete RPMI 1640 medium, and 100 μl/well was seeded in flat-bottom 96-well microtiter plate (Nunc, Wiesbaden, Germany) in the presence of different concentrations of β-galactosidase. After 3 days, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Buchler, Braunschweig, Germany). Sixteen hours later, cells were harvested and thymidine incorporation was measured in a scintillation counter (Inotech, Wohlen, Switzerland).

IFN-γ ELISPOT assays

$CD8^+$ T cells were negatively enriched by using anti-CD4 beads (Dynal Biotech, Hamburg, Germany). To determine the concentration of IFN-γ-secreting $CD8^+$ T cells, the murine IFN-γ ELISPOT kit (BD Biosciences, San Jose, CA) was used. Cells (5×10^5/well) were incubated 16 h with or without a peptide (10 μg/μl) corresponding to the immunodominant L^d-restricted β-galactosidase epitope (TPHPARIGL), which is specific for MHC class I presentation (21). After 16 h, cells were removed, and locally produced single cell-derived IFN-γ was revealed by an immunoenzymatic reaction into a paper matrix as colored spots, which were counted with an ELISPOT reader.

Preparation and flow cytometric analysis of murine DC

Bone marrow-derived primary DC were prepared from BALB/c mice using murine rGM-CSF (BD PharMingen, San Diego, CA) (22). Ghost-treated DC (1×10^6) were preblocked using anti-mouse CD32/16 Ab for 15 min. Then cells were stained with FITC-labeled monoclonals against mouse MHC class I (SF1-1.1) and class II (AMS-32.1), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), or CD54 (3E2), together with PE-labeled Abs against CD11c (HL3) (BD PharMingen). As negative controls, FITC- or PE-conjugated isotype control Abs were used. The FACS analysis of 20,000 events was performed using a FACSort and the CellQuest software (BD Biosciences) with gating on CD11c-positive cells. For control studies, DC were incubated with LPS from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich) at 1 μg/ml. The levels of IL-1α, IL-6, and IL-12p40 in supernatant fluids after ghost stimulation of DC were quantified by ELISA (OptEIA; BD PharMingen).

Ex vivo DC transfection studies

Bone marrow-derived primary DC were incubated together with ghost loaded with pCMVβ (DC:ghost ratio 1:10) at 37°C (5% CO_2) for 16 h. Then DC were concentrated by centrifugation, washed, resuspended in sterile PBS, and injected by i.v. route into female BALB/c mice (1×10^6 DC per animal, $n = 3$). Serum samples were collected on day 11, and spleens were removed and processed on day 20.

Statistical analysis

The statistical significance of the difference between two groups was determined from the means and SDs by Student's two-tailed *t* test on transformed data ($\log_{10}$) and between more than two groups by the one-way ANOVA. Differences were considered to be not significant with $p > 0.05$.

Results

M. haemolytica *ghosts can be efficiently loaded with plasmid DNA*

M. haemolytica ghosts were loaded with the plasmid pEGFP-N1, which contains a gene coding for *egfp* under the control of the CMV promoter. A linear correlation was observed between the DNA concentration used and the amount of pEGFP recovered from the bacterial ghosts (correlation coefficient = 0.934) over the range of DNA concentrations (0.033–16.5 mg/ml) tested (Fig. 1*A*). The number of bacterial ghosts was then established by flow cytometric analysis, to determine the average plasmid copy number per ghost (~2000 copies), which corresponds to 5 μg of pEGFP/mg bacterial protein.

To investigate whether the plasmid DNA was associated with the inner or outer surface of the ghosts, we compared the capacity of *M. haemolytica* ghosts to be loaded with that of lyophilized and

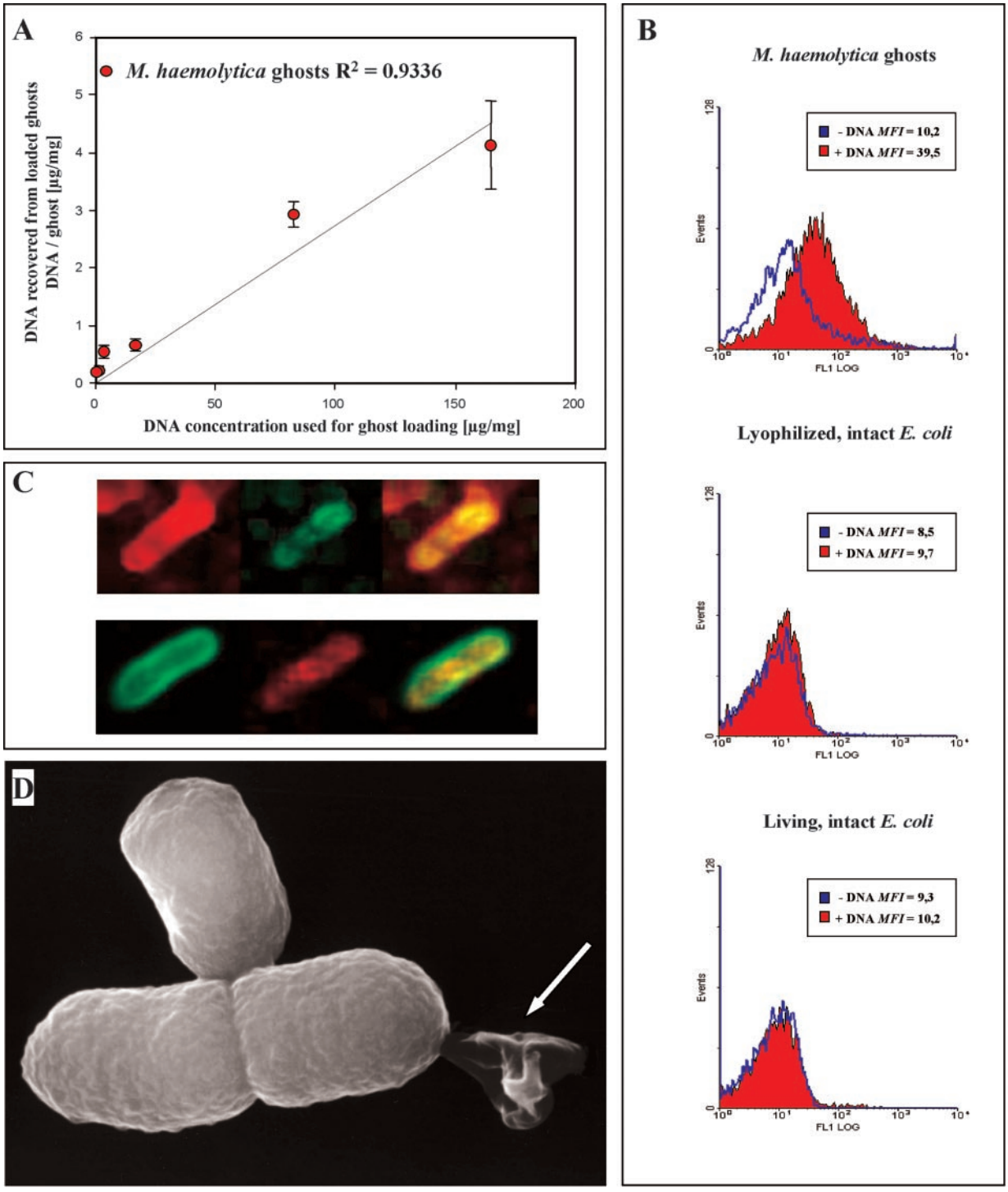

FIGURE 1. Characterization of DNA-loaded *M. haemolytica* ghosts. *A*, Real-time PCR studies allowed to establish that there is a linear correlation between the DNA concentration used for ghost loading and the amount of DNA recovered after washing. Each point is the mean of quadruplicate measurements ± SD; each experiment was repeated at least twice. *B*, The loading capability of bacterial ghosts and intact bacteria was compared using FITC-labeled linear dsDNA (400 bp). The successful loading resulted in a shift in the mean fluorescence intensity. *C*, Colocalization studies of FITC-labeled linear dsDNA in sulforhodamine B-labeled *M. haemolytica* ghosts (*upper panel*) revealed that the loaded DNA (green) was associated with the interior of the bacterial ghosts (red). Furthermore, unlabeled *M. haemolytica* ghosts were loaded with pEGFP-N1 (*lower panel*). The pEGFP-N1 plasmid was detected by in situ hybridization with Cy-3-labeled probes (red) specific for the *egfp*, and ghost membranes were stained with MitoTracker Green FM (green). The fluorescence photomicrographs represent cross sections through the bacterial ghosts. *D*, High resolution field emission scanning electron micrograph of protein E-lysed Gram-negative bacteria. An arrow indicates the efflux of bacterial cytoplasm at the time point of lysis onset through the E-specific lysis tunnel.

nonlyophilized intact live *Escherichia coli*. To this end, FITC-labeled linear dsDNA (400 bp) was used for loading (Fig. 1*B*). A distinct shift in the mean fluorescence intensity of the loaded ghosts was observed in comparison with control empty ghosts. In contrast, the fluorescence of intact *E. coli* cells was not modified (Fig. 1*B*). Then sulforhodamine B-labeled *M. haemolytica* ghosts loaded with FITC-labeled dsDNA were analyzed by confocal microscopy. The FITC-labeled dsDNA (green) was located within

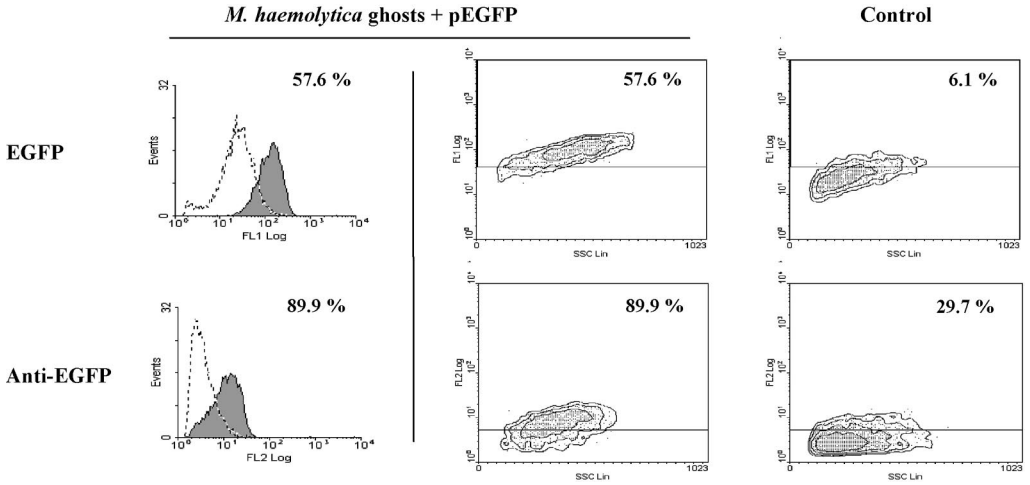

FIGURE 2. EGFP expression by the RAW264.7 macrophages after ingestion of *M. haemolytica* ghosts loaded with pEGFP (~2600 plasmids/ghost). The overlays of the histograms show a distinct shift in the fluorescence of cells treated with pEGFP-loaded ghosts (gray, solid filled) compared with cells treated with the bacterial ghosts alone (black hatched, unfilled), as a result of either the EGFP fluorescence (*upper panels*) or the immunochemical detection of EGFP with polyclonal rabbit Abs and R-PE-conjugated anti-rabbit Abs. Numbers indicate the percentage of the EGFP-expressing cells evaluated by dot plot analysis. One representative experiment of five is shown.

the bacterial ghosts (red), as was demonstrated by the overlay of the fluorescence microphotographs of the *z*-scan sections through the bacterial ghosts (Fig. 1*C*). To rule out the potential influence of the fluorophore on the DNA's binding affinity to the bacterial ghosts, unlabeled ghosts were loaded with pEGFP-N1. The loaded plasmids were subsequently detected by fluorescence in situ hybridization using *egfp*-specific Cy-3-labeled probes, whereas the ghost membranes were stained with MitoTracker Green FM. The *z*-scan sections revealed that pEGFP-N1 (red) was associated with the interior of the ghosts (green), but not to the outside (Fig. 1*C*), thereby confirming the results obtained with FITC-labeled linear dsDNA.

Ghost-mediated transfection of macrophages and DC results in efficient expression of EGFP

Macrophages were incubated with bacterial ghosts loaded with pEGFP-N1 for 2 h, washed, and further incubated for 48 h to allow phenotypic expression of EGFP. The fluorescence shift observed by flow cytometric analysis demonstrated the efficient expression of EGFP by 51.5 ± 1.6% of macrophages (Fig. 2). To rule out potential artifacts arising from macrophage autofluorescence, EGFP was also detected using anti-EGFP primary Abs and PE-conjugated anti-IgG secondary Abs (Fig. 2), thereby confirming that ~60.1 ± 2.4% of the cells express the transgene.

Additional studies were conducted to characterize the capacity of DNA-loaded ghosts to transfect macrophage-derived primary human DC. The obtained results demonstrated that bacterial ghosts are efficiently taken up by DC treated at a ratio of 100 ghosts:DC (Fig. 3). After 10 min, 87% of the DC contains bacterial ghosts, being a plateau reached after 20 min (≥92%). Transfected DC were also able to efficiently express the transgene, as determined by flow cytometry (Fig. 3).

Ghost-mediated DNA vaccination stimulates the elicitation of efficient immune responses

To validate the efficacy of bacterial ghosts as DNA delivery system, groups of mice ($n = 5$) were vaccinated by i.m. or i.d. route with either 5 μg of the plasmid pCMVβ, which contains the β-galactosidase coding gene, ghosts alone, or ghosts loaded with 5 μg of pCMVβ on days 1, 21, and 42. Ghost-vaccinated animals do not show any symptoms of acute or subacute toxicity during the observation time, thereby demonstrating that bacterial ghosts are well tolerated.

Efficient serum Ab responses were stimulated in vaccinated animals with respect to controls, independently of the immunization route. At least one order of magnitude higher anti-β-galactosidase titers was observed in animals receiving DNA-loaded ghosts at the end of the immunization protocol with respect to naked DNA-vaccinated animals ($p < 0.05$, Fig. 4*A*). The use of ghosts stimulated immune responses even after a single dose, whereas at least one additional boost was required when animals were vaccinated with naked DNA. Ghost-specific Abs were also detected in animals receiving the ghost-based formulation by either i.d. or i.m.

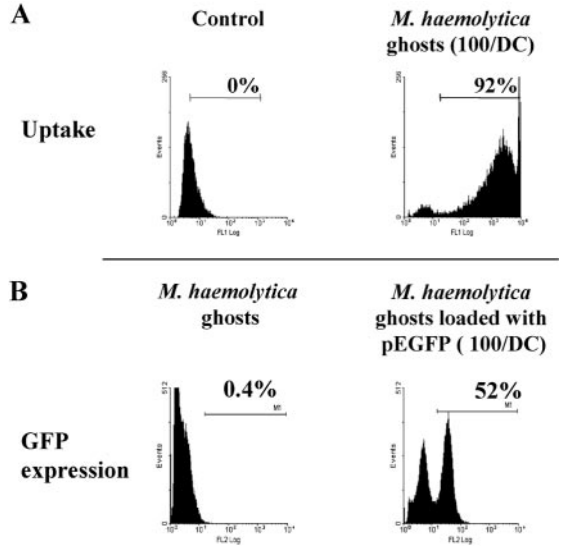

FIGURE 3. Monocyte-macrophage-derived human DC are efficiently transfected using pEGFP-loaded *M. haemolytica* ghosts. *A*, Ghosts are taken up by 92% of the DC when treated for 2 h at a ghost:DC ratio of 100:1. *B*, Up to 52% of the DC present in the preparation was able to express EGFP, as determined by flow cytometry after immune staining. One representative experiment of three, which were performed using cells from different donors, is shown.

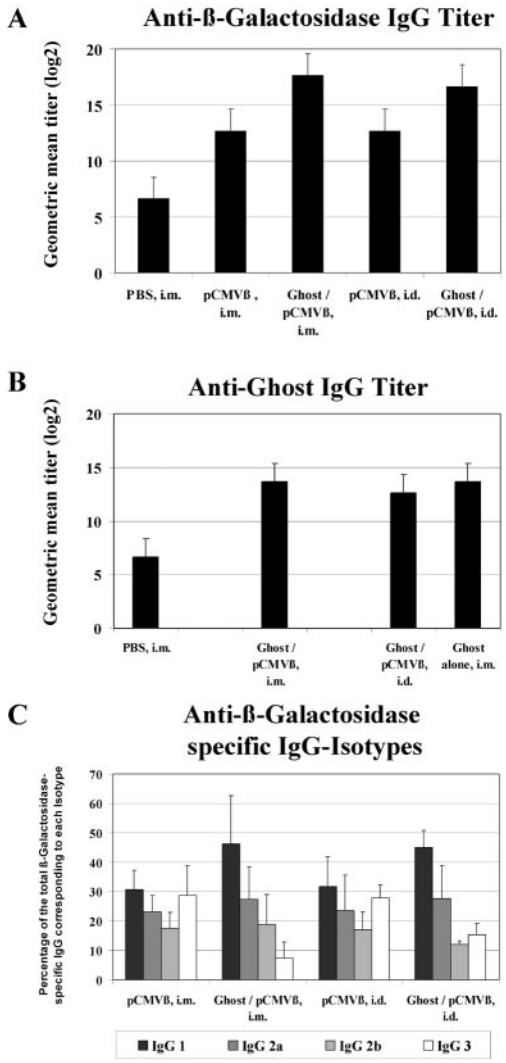

FIGURE 4. Efficient humoral immune responses are stimulated in BALB/c mice ($n = 5$) after vaccination with DNA-loaded *M. haemolytica* ghosts. *A*, β-Galactosidase and *B*, ghost-specific IgG responses in sera from vaccinated mice. Results are expressed as the reciprocal $\log_2$ of the geometric mean end point titer. SEM is indicated by vertical lines. *C*, Isotype profiles of the β-galactosidase-specific IgG Abs present in serum of vaccinated mice. Results are the average of triplicate samples.

route (Fig. 4*B*). This confirms that the ghost-based DNA vaccination approach can also be exploited to develop multivalent vaccines able to protect against diseases caused by the microorganism used for the generation of the ghost.

To evaluate which Th subpopulation was stimulated, the subclass distribution of β-galactosidase-specific IgG Abs was evaluated. The obtained results demonstrated that both IgG1 and IgG2a Abs were stimulated in vaccinated animals (Fig. 4*C*). However, similar levels of both isotypes were detected in mice immunized with naked DNA (mixed Th1/Th2 pattern), whereas a significant ($p < 0.05$) increment in IgG1 together with a reduction in IgG3 was detected in ghost-vaccinated animals. Thus, despite the fact that the Th1 and Th2 components were still present, a shift toward a more dominant Th2 response pattern was obtained using ghosts as a DNA delivery system, suggesting that it is possible to modulate the major Th response by using this approach.

To assess the efficacy of the cellular immune responses stimulated by ghost-mediated DNA vaccination, the proliferative capacity of splenocytes after in vitro restimulation in the presence of β-galactosidase was evaluated. Efficient proliferative responses were only observed in mice immunized by the i.d. route (Fig. 5*A*). Although the observed differences were not statistically significant ($p > 0.05$), slightly better stimulation indexes were detected in animals receiving the ghost preparation. These data demonstrate that also efficient cellular responses can be stimulated by using the ghost-based approach.

To complement the study of the cellular immune responses stimulated by ghost-mediated DNA delivery, we also evaluated the capacity of $CD8^+$ T cells to produce IFN-γ in response to stimulation with a peptide encompassing a MHC class I-restricted immunodominant epitope from β-galactosidase. Higher numbers of IFN-γ-secreting cells were detected using $CD8^+$-enriched T cells recovered from animals vaccinated with pCMVβ-loaded ghosts than by testing T cells from mice vaccinated with naked pCMVβ (Fig. 5*B*).

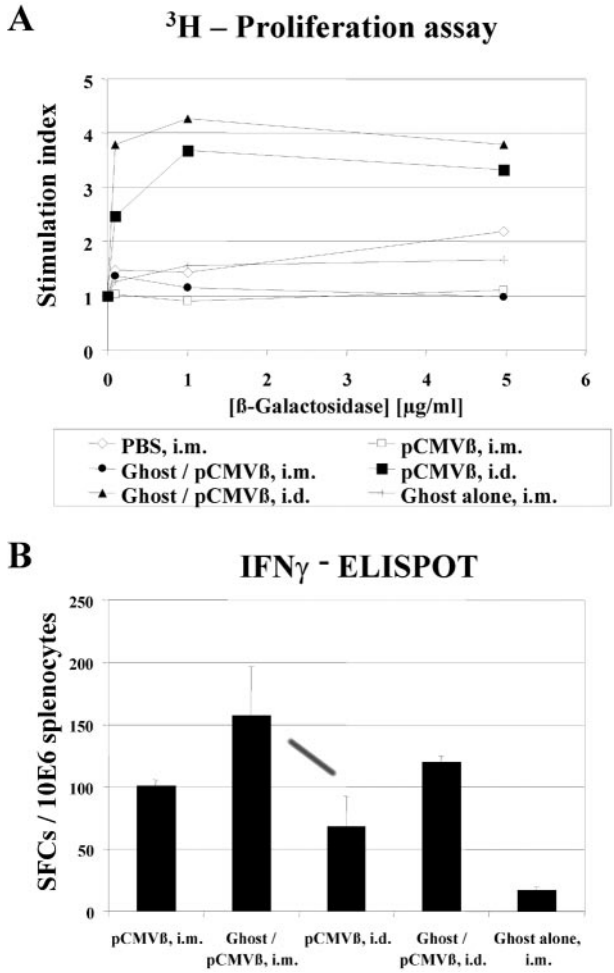

FIGURE 5. Cellular immune responses stimulated in mice after vaccination with DNA-loaded *M. haemolytica* ghosts. *A*, Proliferation was assessed after 4 days of in vitro restimulation of spleen cells in the presence of different concentrations of β-galactosidase, by measuring the incorporation of [^{3}H]thymidine. Results are expressed as the ratio between values (average of triplicates) from stimulated and nonstimulated samples (stimulation index). SEM was lower than 10%. *B*, $CD8^+$ T cell-enriched preparations of spleen cells (5×10^5/well) were incubated for 16 h in the presence of a peptide encompassing a MHC class I-restricted epitope, and the numbers of IFN-γ-producing cells were determined by ELISPOT. The values are arithmetic means of triplicates subtracted from those obtained from nonstimulated cells. Vertical lines indicate SEM.

M. haemolytica *ghosts stimulate maturation and activation of bone marrow-derived murine DC*

The elicitation of improved immune responses following delivery of DNA vaccines by bacterial ghosts, as well as the observed modulation (i.e., altered Th response pattern) suggested that ghost components might affect Ag processing. Because DC are the most powerful APC, we decided to evaluate the effect of *M. haemolytica* ghosts on the maturation and activation of bone marrow-derived murine DC. Immature DC were stimulated in vitro for 16 h either with ghost or, as positive control, with LPS. Then surface markers on CD11c-positive gated DC were investigated by flow cytometry. Preincubation with ghosts (50 ghost/DC) resulted in an increased expression of MHC class I and II molecules, which was similar to that observed using 1 μg/ml LPS (Fig. 6). The expression of the costimulatory molecules CD80 and CD86, as well as that of the adhesion molecules CD40 and CD54 was also up-regulated after ghost treatment. Similar results were obtained using 25 or 10 ghosts/DC (data not shown).

Then the ability of bacterial ghosts to stimulate DC cytokine secretion was evaluated. After stimulation with ghosts, DC production of IL-1α was increased by 2.4-fold in comparison with nonstimulated DC (296 pg/ml). The secretion of IL-6 was also increased by 615-fold (153 ng/ml) in respect to untreated DC (0.25 ng/ml). Finally, the content of IL-12p40 in supernatant fluids of ghost-treated DC was increased 194-fold (69 ng/ml) compared with unstimulated cells (0.35 ng/ml). The obtained results demonstrate that preincubation of immature DC with ghosts results in cellular activation and maturation, thereby explaining the improved immune responses observed using bacterial ghosts as a delivery system for DNA vaccines.

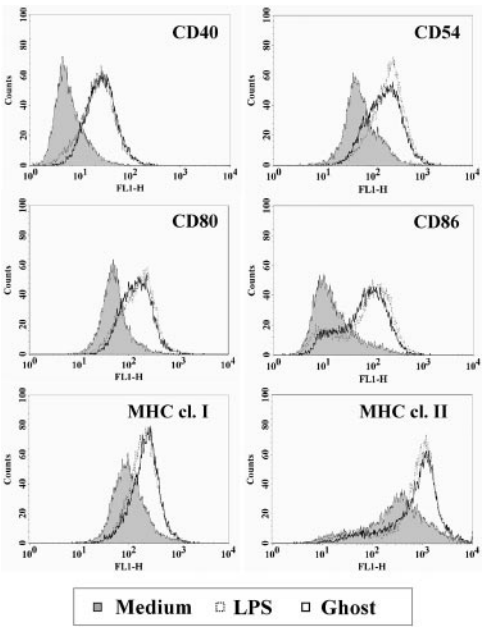

FIGURE 6. Flow cytometric analysis of DC after stimulation with *M. haemolytica* ghosts. Bone marrow-derived murine DC were coincubated with LPS (1 μg/ml) or ghosts (25 ghosts/cell) for 18 h and analyzed by flow cytometry. CD11c$^+$-gated DC showed an enhanced expression of different surface markers after ghost (filled line) or LPS (dotted line) treatment in comparison with nonstimulated DC (shaded area).

Immunization with DC transfected ex vivo using pCMVβ-loaded M. haemolytica *ghosts stimulates β-galactosidase-specific immune responses*

Additional studies were conducted to gain insights on the role played by DC in the immune responses stimulated using ghosts as DNA delivery system. Mice were immunized by i.v. route with 1×10^6 bone marrow-derived DC, which had been transfected ex vivo with the pCMVβ vector by incubating them with plasmid-loaded *M. haemolytica* ghosts. Anti-β-galactosidase Ab responses were detected in all vaccinated mice (Fig. 7*A*). Furthermore, an increment in the number of splenic IFN-γ-producing cells was observed in immunized animals, in response to restimulation with a peptide encompassing the MHC class I-restricted immunodominant epitope from β-galactosidase (Fig. 7*B*). These results suggest that DC play an important role in the elicitation of immune responses when bacterial ghosts are used as a technology platform for the delivery of DNA vaccines.

Discussion

The concept of using naked DNA for immunization purposes constitutes a major breakthrough in the vaccinology field. This new approach can be exploited to design both prophylactic and therapeutic vaccines against a broad range of infectious and noninfectious diseases. However, mass implementation in the field is hampered by the overall poor immunogenicity, which leads to the

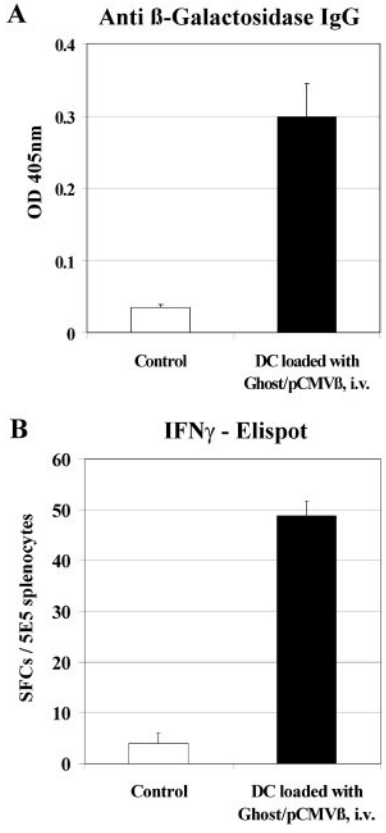

FIGURE 7. Immune responses stimulated by DC transfected ex vivo with pCMVβ-loaded *M. haemolytica* ghosts. *A*, β-Galactosidase-specific serum IgG responses 11 days after vaccination. *B*, Cells (5×10^5/well) were incubated for 16 h in the presence of a peptide encompassing a MHC class I-restricted epitope, and the numbers of IFN-γ-producing cells were determined by ELISPOT. The values are arithmetic means of triplicates subtracted from those obtained from nonstimulated cells. Vertical lines indicate SEM.

requirement of high plasmid dosages. This low efficiency can be explained by the fact that the encoded Ags are not specifically targeted to APC. Using bacterial ghost as DNA delivery system can solve this problem. Our in vitro studies have shown that *M. haemolytica* ghosts are efficiently taken up by macrophages and primary DC, thereby leading to high transfection efficiencies (52–60%).

The fact that the Ags are not delivered in the context of an optimal danger signal may also contribute to the poor immunogenicity of DNA vaccines. In fact, the immune system has evolved to recognize entities causing damage rather than those being foreign (12). Therefore, APC can only be properly activated in the presence of signals received from pathologically altered cells (i.e., infected or necrotic), but not from healthy or apoptotic cells (12). The presentation of Ags by nonactivated DC (i.e., without costimulation) may even promote tolerance, thereby leading to immune escape. These mechanistic events are particularly relevant when self or altered self Ags are considered (e.g., cancer immunotherapy).

When bacterial ghosts are used as a DNA delivery system, they can act as a natural adjuvant due to the presence of envelope components, as suggested by the improved immune responses observed after vaccination with ghost-based candidates (15). In fact, the in vitro studies performed using primary DC demonstrated that *M. haemolytica* ghosts promote DC maturation and activation. This can explain the improved performance of the ghost-based vaccines. In contrast, a potential problem associated with the use of a delivery system derived from Gram-negative bacteria is the presence of LPS (i.e., endotoxin) associated with the cell envelopes. However, previous studies showed that the dosage of bacterial ghosts needed for inducing efficient immune responses can be administered without leading to endotoxin-related side effects (13). At least two orders of magnitude higher LPS concentrations are tolerated when the LPS is associated to bacterial ghosts than when it is in a free form (13). Thus, the LPS content of bacterial ghosts does not limit their use as DNA delivery system.

The nature of the cytokines released during the activation process is also an important parameter to define the type of immune response stimulated. Our data demonstrate that there is an increment in IL-12 secretion by DC in the presence of bacterial ghosts. This is a prototypic Th1-polarizing cytokine, which is subjected to a tight regulation in DC. However, only a marginal effect was observed on IL-1α production, which acts as IL-12 cofactor for Th1 development in mice (23). This, together with the increment in IL-6, explains the shift in the dominant Th response from a mixed Th1/Th2 in animals vaccinated with naked DNA to a dominant Th2 pattern in ghost-vaccinated mice, thereby demonstrating the utility of this approach to fine tune the elicited immune responses. Interestingly, we have also observed an increment in the number of β-galactosidase-specific IFN-γ-producing $CD8^+$ T cells in ghost-vaccinated animals, in response to restimulation with a peptide encompassing the immunodominant MHC class I-restricted epitope. This can be explained, at least in part, by a ghost-dependent improvement in the capacity of APC to process and present MHC class I-restricted Ags. In fact, we have observed an up-regulation in the expression of MHC class I and costimulatory molecules on ghost-treated DC. Furthermore, although reduced, remaining Th1 cells can also contribute to the overall response. The immunization studies performed with ex vivo transfected DC suggest that these cells play an important role in the stimulation of immune responses when bacterial ghosts are used as a DNA delivery system. Ghost-dependent induction of proinflammatory mediators may also facilitate the creation of a local environment conductive to Ag presentation, which favors the recruitment of innate immunity masters, thereby linking innate and adaptive immunity. As a whole, the obtained results highlight the complexity and pleiotropism of the effects triggered by bacterial ghosts during the stimulation of adaptive immune responses.

Previous studies have shown that bacterial ghosts are an effective vaccine delivery system to stimulate strong humoral and cellular immune responses against the ghost itself or heterologous Ags (16, 24). Thus, DNA-loaded ghosts also can be exploited as vaccines against diseases caused by the microorganisms selected for ghost preparation. The use of live bacteria as carriers for DNA vaccine constructs constitutes a valid alternative (25). However, the use of live vectors may be associated with safety concerns, particularly when release under uncontained conditions or use for immunocompromised individuals is considered. In contrast, nonliving bacterial ghosts can be administered at even high doses without safety concerns.

In conclusion, the β-galactosidase-encoding plasmid was efficiently delivered to the target cells, being properly transcribed and translated. The synthesized protein was able to stimulate efficient humoral and cellular immune responses. Thus, bacterial ghosts constitute a promising technology for the development of more efficient DNA vaccines. The easy manufacturing, low production costs, and excellent safety profile of bacterial ghosts constitute additional advantages for mass implementation.

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