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MgtC as a Host-Induced Factor and Vaccine Candidate against *Mycobacterium abscessus* Infection

Vincent Le Moigne,[a] Claudine Belon,[b,c] Céline Goulard,[a] Geoffrey Accard,[b,c] Audrey Bernut,[d] Bruno Pitard,[e] Jean-Louis Gaillard,[a,f] Laurent Kremer,[d,g] Jean-Louis Herrmann,[a,f] Anne-Béatrice Blanc-Potard[b,c]

INSERM, U1173, UFR Simone Veil, Université de Versailles Saint Quentin, Saint Quentin en Yvelines, France[a]; Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier, Montpellier, France[b]; CNRS, UMR5235, Montpellier, France[c]; Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé (CPBS), CNRS FRE 3689, Montpellier, France[d]; IN-CELL-ART, Nantes, France[e]; APHP, Groupe Hospitalo-Universitaire Paris Île-de-France Ouest, Garches et Boulogne-Billancourt, France[f]; INSERM, CPBS, Montpellier, France[g]

***Mycobacterium abscessus* is an emerging pathogenic mycobacterium involved in pulmonary and mucocutaneous infections, presenting a serious threat for patients with cystic fibrosis (CF). The lack of an efficient treatment regimen and the emergence of multidrug resistance in clinical isolates require the development of new therapeutic strategies against this pathogen. Reverse genetics has revealed genes that are present in *M. abscessus* but absent from saprophytic mycobacteria and that are potentially involved in pathogenicity. Among them, *MAB_3593* encodes MgtC, a known virulence factor involved in intramacrophage survival and adaptation to Mg^{2+} deprivation in several major bacterial pathogens. Here, we demonstrated a strong induction of *M. abscessus* MgtC at both the transcriptional and translational levels when bacteria reside inside macrophages or upon Mg^{2+} deprivation. Moreover, we showed that *M. abscessus* MgtC was recognized by sera from *M. abscessus*-infected CF patients. The intramacrophage growth (J774 or THP1 cells) of a *M. abscessus* knockout *mgtC* mutant was, however, not significantly impeded. Importantly, our results indicated that inhibition of MgtC *in vivo* through immunization with *M. abscessus mgtC* DNA, formulated with a tetrafunctional amphiphilic block copolymer, exerted a protective effect against an aerosolized *M. abscessus* challenge in CF (ΔF508 FVB) mice. The formulated DNA immunization was likely associated with the production of specific MgtC antibodies, which may stimulate a protective effect by counteracting MgtC activity during *M. abscessus* infection. These results emphasize the importance of *M. abscessus* MgtC *in vivo* and provide a basis for the development of novel therapeutic tools against pulmonary *M. abscessus* infections in CF patients.**

Mycobacterium abscessus, a rapidly growing mycobacterium (RGM), is the etiological agent of a wide spectrum of infections in humans. *M. abscessus* is responsible for severe and persistent pulmonary infections, disseminated cutaneous diseases, and posttrauma and postsurgery wound infections, mostly in immunocompetent patients and in cystic fibrosis (CF) patients (1–5). Infections by this pathogen lead to a fatality rate that is higher than those seen with other RGM species. Infection of CF patients with *M. abscessus* is a major health-related issue in most CF centers worldwide (1, 2, 6, 7) and is considered a contraindication to lung transplantation, leaving these patients with minimal therapeutic options.

M. abscessus provokes very challenging therapeutic issues because of its natural resistance to most available antibiotics (8–10) and is considered the most drug-resistant mycobacterial species. The lack of optimal therapeutic treatments and the emergence of multidrug resistance in *M. abscessus* stress the need for the discovery of new strategies to combat these infections.

Reverse genetics, based on the comparison between pathogenic and nonpathogenic species, allows the identification of genes missing in nonpathogenic species and encoding potential virulence factors. One major outcome of this comparison relies on (i) an understanding of the impact of the identified genes during infection and (ii) attempts at modulating the virulence phenotype by stimulating an efficient adaptive immune response. As an example, mining the *M. abscessus* genome sequence has revealed several "nonmycobacterial" virulence genes, presumably acquired by horizontal gene transfer (HGT) and absent from the saprophytic RGM *Mycobacterium smegmatis* (11). Interestingly, several of these genes are shared with other major CF pathogens, such as *Pseudomonas aeruginosa* or *Burkholderia cenocepacia*. This is the case with the phospholipase C (PLC)-encoding gene, which not only was important for virulence in an *M. abscessus* mouse model of infection (12) but also exhibited potent vaccine properties in CF (ΔF508 FVB) mice immunized with *plc*-encoding DNA through the production of high anti-PLC antibody titers (13). As such, certain virulence factors may be viewed as relevant antigen targets for the development of protective vaccines against human pathogens (14–16).

In addition to PLC, comparative genomics revealed another *M. abscessus* gene, *MAB_3593*, absent from *M. smegmatis* (11). *MAB_3593* encodes a protein homologous to MgtC, a virulence factor shared by several intracellular pathogens, including *Myco-*

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Address correspondence to Jean-Louis Herrmann, jean-louis.herrmann@aphp.fr, or Anne-Béatrice Blanc-Potard, anne.blanc-potard@univ-montp2.fr.

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bacterium tuberculosis (17, 18). This factor contributes to intramacrophage survival of numerous pathogens as well as to bacterial adaptation in environments with limited Mg^{2+} concentrations (18). MgtC was found to be highly expressed in cellular infection models for several pathogens (19, 20), which emphasizes its importance during infection. MgtC expression in *M. abscessus* has not yet been evaluated in cellular models or in environments with poor Mg^{2+} concentrations, nor has its role during infection been addressed.

As a first step toward the elucidation of the involvement of *MAB_3593* in the virulence of *M. abscessus*, we created a mutant strain in which the *MAB_3593* gene encoding *M. abscessus* MgtC (referred to here as $MgtC_{MAB}$) has been disrupted. We next analyzed the expression of MgtC in a low-Mg^{2+} environment and within infected cells, which prompted us to evaluate the protection against *M. abscessus* conferred to mice after immunization using a formulated $mgtC_{MAB}$ DNA preparation.

MATERIALS AND METHODS

Bacterial strains and growth culture conditions. *M. abscessus* strains (derivatives of CIP 104536^T, smooth variant) were grown at 37°C in Sauton's medium containing 0.025% tyloxapol (Sigma) or on LB plates, in the presence of zeocin (25 μg/ml), when required. Low-magnesium medium was obtained by replacing the magnesium sulfate in the Sauton's medium with a similar concentration of potassium sulfate. *Escherichia coli* (DH5α) was used for cloning and was grown in LB medium with zeocin (25 μg/ml) at 37°C. *Salmonella enterica* serovar Typhimurium strains, derived from wild-type strain 14028s, were grown in LB medium with ampicillin (100 μg/ml) at 37°C.

Cloning of $mgtC_{MAB}$-like genes under the control of the *Salmonella mgtC* promoter and heterologous complementation of a *Salmonella mgtC* mutant strain. The *MAB_3593* and *MAB_0146* genes from *M. abscessus* were amplified by PCR using CIP 104536^T culture as the template and primers indicated in Table S1 in the supplemental material. Each PCR fragment was cloned at the SphI and EcoRI sites of plasmid pNM11, a pBR322 plasmid derivative that harbors the *S.* Typhimurium *mgtC* promoter (21). The resulting plasmids express the MgtC-like proteins from *M. abscessus* with transcriptional and translational regulation sites of the *mgtC* gene of *S.* Typhimurium. Mutation W227A was introduced in a plasmid carrying *MAB_3593* using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions and the primers listed in Table S1. The *S.* Typhimurium NM14 strain (Δ*mgtC* mutant) (21) was transformed by electroporation with plasmids harboring the *MAB_3593* or *MAB_0146* gene. NM14 was also transformed with pNM11, which harbors the *mgtC* promoter only, used as a negative control, with pNM12, which expresses the *S.* Typhimurium *mgtC* gene as positive control (21), and with pNM13, which expresses *M. tuberculosis* MgtC (22). Growth in magnesium-deprived medium was performed in no-carbon essential (NCE) medium supplemented with 10 μM $MgCl_2$, as described previously (23), and the optical density at 600 nm (OD_{600}) was measured after 18 h of growth.

Construction of the *MAB_3593* mutant and the complemented strain. The *mgtC M. abscessus* knockout (KO) mutant was obtained by allelic exchange in *M. abscessus* CIP-S with a strategy using phage recombinase as previously reported (24, 25). A linear DNA fragment (100 ng), carrying the zeocin cassette (from *Streptoalloteichus hindustanus*) as well as 1,000 bp of the region surrounding each side of the *MAB_3593* gene (see primers in Table S1 in the supplemental material), was introduced into electrocompetent *M. abscessus* CIP-S bearing the pJV53 recombineering plasmid (26). Cells with a homologous recombination event were selected on LB agar plates supplemented with zeocin. Proper replacement of the *mgtC* gene by the zeocin cassette was checked by a PCR screen using forward and reverse primers outside the deleted region (see Table S1).

To construct a complemented strain, *mgtC* with its upstream region (500 bp) was first amplified with primers MgtC-reg-PvuII and MgtC-reg-HindIII (see Table S1 in the supplemental material) and cloned into the integrative pMVH361-hygromycin plasmid. This plasmid was then transformed in the *mgtC M. abscessus* KO mutant.

Macrophage infection assays. J774 cells were maintained at 37°C in 5% CO_2 using Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). J774 cells were allowed to adhere in a 24-well plate at a density of 5×10^4 cells/well for 24 h at 37°C in 5% CO_2.

For infection, *M. abscessus* cultures grown exponentially in Sauton's medium (OD_{600} between 0.8 and 1) were centrifuged and washed in phosphate-buffered saline (PBS) and bacterial clumps were disrupted by 6 successive passages through a 26-gauge syringe needle. The remaining aggregates were then eliminated with a short spin procedure (1 min at 1,100 rpm). Macrophages were infected at a multiplicity of infection (MOI) of approximately 2. The infection was allowed to proceed for 3 h before four PBS washes were performed to remove extracellular bacteria. Cells were then lysed with 0.1% Triton X-100–PBS, and the number of intracellular mycobacteria was counted by plating appropriate dilutions onto LB agar plates. To evaluate the multiplication rate, infected cells were incubated for 48 h in DMEM prior to cell lysis and bacterial enumeration. The ratio between the numbers of internalized bacteria at 24 h or 48 h and the numbers of internalized bacteria at 3 h after infection was calculated.

THP1 cells were maintained at 37°C in 5% CO_2 using RPMI medium (Gibco) supplemented with 10% FBS and were differentiated into adherent macrophages by adding 60 nM phorbol myristate acetate (PMA) for 72 h. Infection was carried out as described above in a 24-well plate at a density of 2×10^5 cells/well.

RNA extraction, qRT-PCR, and analysis of protein expression by Western blotting. RNA and cDNA were prepared from 5 ml of mid-logarithmic-phase bacterial cultures grown in Sauton's medium containing or not containing magnesium as previously described (27). Control experiments without reverse transcriptase were performed for each RNA sample to rule out DNA contamination. The 16S rRNA (*rrs*) gene was used as an internal control (28). The sequences of primers used for quantitative real-time PCR (qRT-PCR) are listed in Table S1 in the supplemental material. For isolation of bacterial RNA from *M. abscessus*-infected J774 macrophages, cells were seeded into a 100-cm^2 tissue culture dish and infected at an MOI of 5. After a 2-h incubation period, cells were washed with phosphate-buffered saline (PBS) medium and then incubated for 1 h in DMEM–200 μg/ml amikacin. After 24 h, cells were harvested, washed with PBS, and lysed with PBS containing 0.1% Triton X-100. Bacteria were then pelleted by centrifugation at 13,000 rpm for 10 min at 15°C. Bacteria were finally resuspended by adding 500 μl PBS, and total RNA was isolated as described above.

To prepare bacterial lysates, *M. abscessus* cultures grown in Sauton's medium with or without magnesium were centrifuged at 4,000 × *g* and resuspended in Tris-buffered saline (TBS) (the resuspension volume was normalized according to OD_{600}). *M. abscessus* lysates from macrophage cocultures were prepared following J774 infection by pelleting the cells by centrifugation at 900 × *g* for 5 min and resuspending the pellet in 5 ml cooled PBS–1% Triton X-100 for 5 min at room temperature. After the addition of 2.5 ml of PBS, the samples were centrifuged at 4,000 × *g* for 15 min at 4°C and resuspended in TBS and processed as described for the classical *M. abscessus* cultures. Samples were sonicated on ice three times for 30 s each time and centrifuged at 14,000 × *g*, and protein concentrations in the lysates were determined using the Bradford assay. The crude extracts were then separated using 12.5% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with mouse serum collected after DNA immunization (see below). The specificity of the mouse serum was checked using a crude lysate from *M. smegmatis* expressing the recombinant His-tagged $MgtC_{MAB}$ protein. A peroxidase-conjugated goat anti-mouse IgG (H + L, Southern Biotech) was then added (dilution, 1/5,000)

prior to detection using the peroxidase substrate. Rat anti-KasA antibodies (29) were used as a loading control.

Serum samples from CF patients enrolled in the French study cohort "OMA" (4) were diluted 1:1,000 for Western blot analysis.

Immunization protocol in mice. Plasmid pVAX1-MAB_3593 (3,654 bp) contains a kanamycin resistance gene and a *MAB_3593* DNA insertion of 723 bp under the control of a previously reported cytomegalovirus (CMV) promoter (13). The $mgtC_{MAB}$ gene sequence was optimized for expression in eukaryotic cells by substitution of rare codons. The control plasmid pCMV β-galactosidase carries an ampicillin resistance gene and a DNA-*E. coli* β-galactosidase gene (3,141 bp) under the control of a CMV promoter (13). The immunization scheme shown in Fig. S1 in the supplemental material was followed because it was successfully used with other antigens as reported earlier (13). We used a synthetic delivery system composed of a tetrafunctional 704 block copolymer that has been shown to increase dramatically the vaccination efficiency over that seen with naked DNA in mouse model (30). The polymer was administered by intramuscular injection. For the aerosol challenge, mycobacterial inocula of *M. abscessus* CIP 104536^T were prepared as described previously (31, 32). Five to seven 6-week-old homozygote ΔF508 FVB female mice (33) were aerosolized with *M. abscessus* on day 56, i.e., 14 days after the last immunization as previously described (13). Mice were sacrificed on days 1, 7, 14, and 21 after infection. Lungs, spleen, and liver were removed aseptically, and CFU counts were determined as described previously (34).

Statistical analysis. Student's *t* test and Fisher's exact test were used. A *P* value of <0.05 was considered significant.

RESULTS

***M. abscessus* encodes two MgtC-like proteins that belong to different phylogenetic clusters.** The presence of an MgtC-encoding gene (*MAB_3593*) in *M. abscessus* was recently reported (11). MAB_3593 exhibits 43% identity with and 59% similarity (at the protein level) to *M. tuberculosis* MgtC (Rv1811; here referred to as $MgtC_{MTB}$) (Fig. 1A). However, the genomic organization around *mgtC* seen with *MAB_3593* is distinct from that seen with *Rv1811* (Fig. 1B), and *MAB_3593* was probably selected by HGT acquisition in *M. abscessus* together with *MAB_3592c* (11) (Fig. 1B). MAB_3593 belongs to the same phylogenetic cluster as $MgtC_{MTB}$, considered the cluster of MgtC proteins with an intracellular function (11, 35). This cluster includes MgtC members in other important CF pathogens, such as *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Mycobacterium avium*, and *Stenotrophomonas maltophilia* (see Fig. S2 in the supplemental material). Intriguingly, a second MgtC-like protein is encoded by the *M. abscessus* genome (*MAB_0146*) (http://bigsdb.web.pasteur.fr/mycoabscessus/mycoabscessus.html). MAB_0146 is more divergent than Rv1811, especially in the C-terminal domain (Fig. 1A), and belongs to a different phylogenetic clade that includes an MgtC-like protein from *M. avium* and one from another CF pathogen, the fungus *Aspergillus fumigatus* (36) (see Fig. S2).

MAB_3593 ($mgtC_{MAB}$) complements partially a *Salmonella mgtC* mutant. Heterologous complementation studies were carried out using a *Salmonella mgtC* mutant with both *M. abscessus mgtC*-like genes, which provide a simple and rapid way to test for the functionality and activity of each copy (21, 22). In fact, the *Salmonella mgtC* mutant is defective for growth in low-magnesium (Mg^{2+}) medium and we have previously shown that heterologous complementation with $MgtC_{MTB}$ significantly improves the growth of the *Salmonella mgtC* mutant in such a medium (22) (Fig. 1C). A partial complementation was also seen with *MAB_3593* (Fig. 1C), to levels similar to those obtained with $mgtC_{MTB}$, suggesting a conserved function with regard to adaptation to Mg^{2+} deprivation. In contrast, no *in vitro* complementation was noticed with *MAB_0146* in low-Mg^{2+} medium. Interestingly, a tryptophan residue near the C-terminal end of MgtC that is important for MgtC function in low-Mg^{2+} medium (21) is conserved in MAB_3593 but not in MAB_0146 (Fig. 1A). Replacement of Trp227 residue by an Ala (W227A) in MAB_3593 suppressed the partial complementation (Fig. 1C), supporting the idea of the functional importance of this residue, as reported for other MgtC proteins. As MAB_3593 appears to be the sole *M. abscessus* MgtC-like protein that shares functional similarity with the *Salmonella* and *M. tuberculosis* MgtC proteins, it is referred to here as $MgtC_{MAB}$.

An $mgtC_{MAB}$ mutant is defective for optimal growth in Mg^{2+}-deprived medium. A *M. abscessus mgtC* KO mutant was generated by allelic exchange using the recombineering system (25) with insertion of a zeocin cassette in *MAB_3593* (Fig. 2A). PCR screening was performed to check for the proper replacement of $mgtC_{MAB}$ by a zeocin cassette (Fig. 2B). We then investigated the phenotype of the Δ$mgtC_{MAB}$ mutant in low-Mg^{2+} medium and in macrophages. The Δ$mgtC_{MAB}$ mutant exhibited a growth defect compared to the wild-type strain in Mg^{2+}-deprived medium (Fig. 2C) but not in medium supplemented with Mg^{2+} (Fig. 2D). Complementation of the mutation, with an integrative plasmid carrying the *mgtC* gene and its upstream promoter region, suppressed the growth defect in Mg^{2+}-deprived medium (see Fig. S3 in the supplemental material). These results emphasize the contribution of $MgtC_{MAB}$ in the adaptive response of *M. abscessus* to low-Mg^{2+} concentrations. Similarly, the Δ$mgtC_{MAB}$ mutant exhibited a replication rate that was slightly lower than those seen with the parental strain in J774 murine macrophages (Fig. 2E) and THP1 human macrophages (see Fig. S4) after 2 days of infection. There was a similar finding at a shorter infection time (replication rates of 2.4 ± 0.44 for the wild-type strain and 2.1 ± 0.52 for mutant strain at 24 h postinfection). However, for both macrophage cell lines, the differences in the intracellular behaviors between the Δ$mgtC_{MAB}$ mutant and the control strain were not significant.

$mgtC_{MAB}$ expression is induced in low-Mg^{2+} medium and within macrophages. The observed defect of the Δ$mgtC_{MAB}$ mutant in Mg^{2+}-deprived medium raises the issue of *mgtC* regulation as a consequence of the use of various Mg^{2+} concentrations. To address this issue, the expression of $mgtC_{MAB}$ was investigated in the wild-type *M. abscessus* strain at both the transcriptional and translational levels. Bacteria were grown in Sauton's medium supplemented with Mg^{2+} or not supplemented prior to mRNA extraction. Expression of the $mgtC_{MAB}$ transcripts was determined along with that of two other genes: *MAB_3592c*, located immediately upstream of $mgtC_{MAB}$ (Fig. 1B), and the *mgtC*-like *MAB_0146* gene. Quantitative RT-PCR performed using the 16S rRNA gene as an internal control indicated that expression of *mgtC* was highly (about 50-fold) induced by Mg^{2+} deprivation. In sharp contrast, minor (2- to 3-fold) transcriptional induction was noticed for *MAB_3592c* or *MAB_0146* in the absence of Mg^{2+} (Fig. 3A).

Western blotting was subsequently performed to analyze the expression of $MgtC_{MAB}$ at the protein level in cultures grown in Sauton's medium with or without Mg^{2+}. Crude bacterial lysates were probed using anti-$MgtC_{MAB}$ antibodies and hyperimmune sera obtained after DNA immunization of mice (see below). Be-

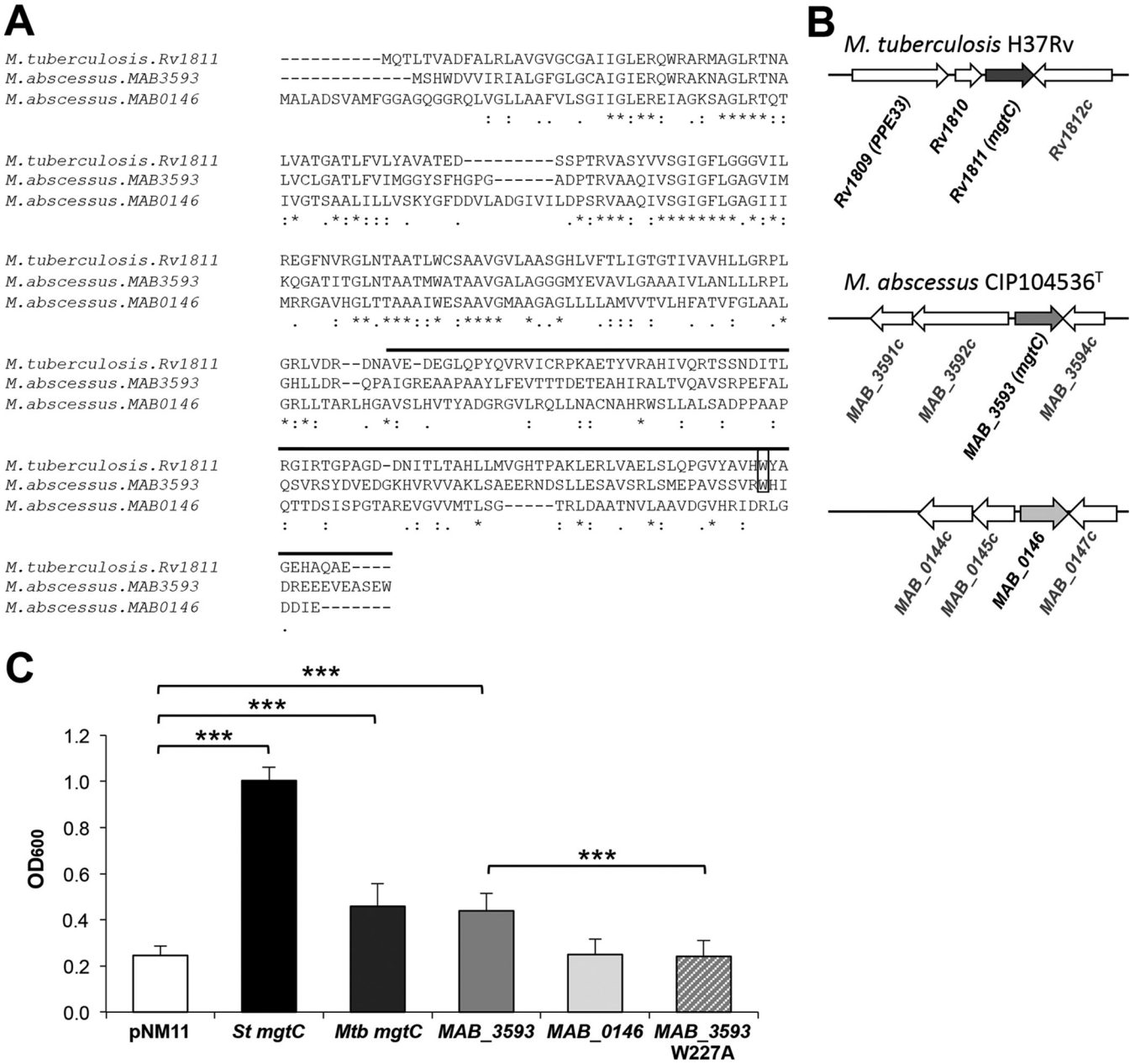

FIG 1 MgtC-like proteins encoded by *M. abscessus* genome. (A) Alignment of *M. tuberculosis* MgtC ($MgtC_{MTB}$) and *M. abscessus* MgtC-like proteins (MAB_3593 and MAB_0146) was done with the T-COFFEE Multiple Sequence Alignment Server (tcoffee.crg.cat/). The upper line indicates the predicted soluble C-terminal part. Near the C terminus, a conserved tryptophan residue identified as important for MgtC function in *Salmonella* in Mg^{2+}-deprived medium is indicated by a black rectangle. Conservation of residues is indicated below the sequences as follows: asterisk (*), residues that are identical in all sequences in the alignment; colon (:), conserved substitutions; period (.), semiconserved. (B) Genomic organization around *mgtC*-like genes. (C) Functional analysis of *M. abscessus* MgtC-like proteins by heterologous complementation of a *Salmonella mgtC* mutant strain compared to $MgtC_{MTB}$. A *Salmonella* Typhimurium (*St*) *mgtC* mutant was transformed with plasmids harboring *MAB_3593*, *MAB_0146*, or $mgtC_{MTB}$ (*Rv1811*). A mutation was introduced in *MAB_3593* to test the role of functional relevance of the W227 residue. The OD_{600} was measured after growth of complemented strains in medium with a low concentration of Mg^{2+} for 18 h. A negative control was provided with a strain harboring an empty vector (pNM11), whereas a positive control was provided with a strain expressing the *Salmonella mgtC* gene ($mgtC_{ST}$). Data represent the mean values plus standard errors of results from at least three independent experiments. *Mtb*, *M. tuberculosis*; ***, $P < 0.001$ (Student *t* test).

fore testing *M. abscessus* lysates, the specificity of the serum was first verified on a lysate from a *M. smegmatis* strain overexpressing a recombinant His-tagged $MgtC_{MAB}$ protein (see Fig. S5 in the supplemental material). As shown in Fig. 3B, $MgtC_{MAB}$ was not detected in lysates from bacilli grown in medium containing Mg^{2+}. However, expression of $MgtC_{MAB}$ was readily detected when *M. abscessus* was grown in medium without Mg^{2+}, further supporting the hypothesis of strong induction at the protein level mediated by Mg^{2+} depletion. The KasA protein used as a control was similarly detected in the two media. As anticipated, no immu-

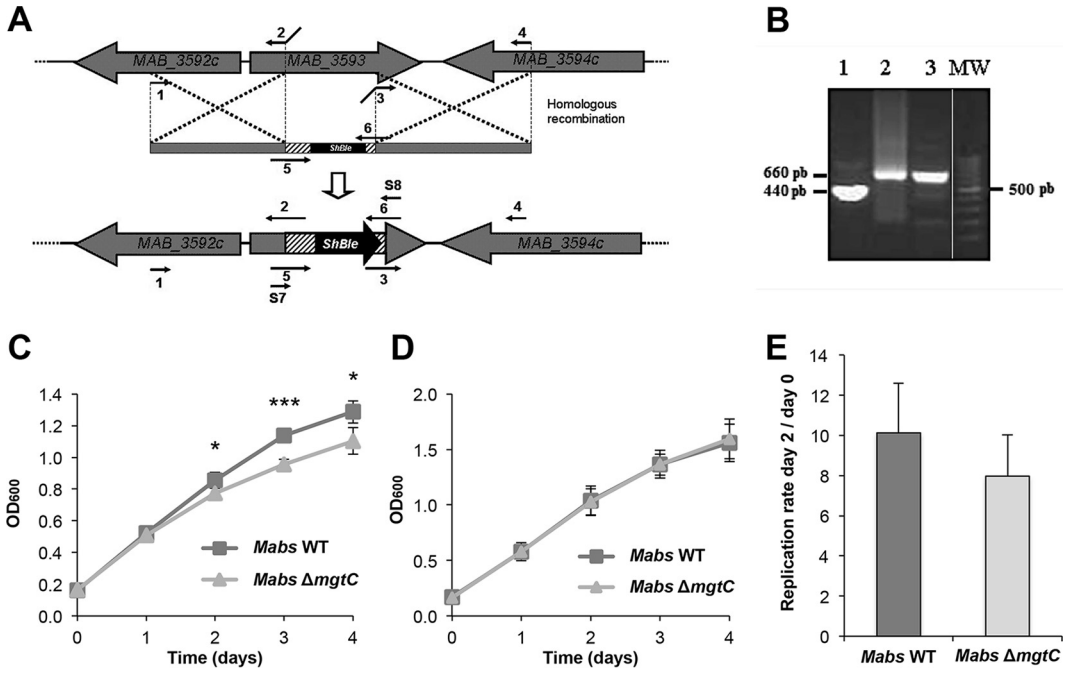

FIG 2 Role of $MgtC_{MAB}$ in adaptation to magnesium (Mg) deprivation and intramacrophage environment. (A) Construction of the Δ*mgtC*$_{MAB}$ mutant by homologous recombination (HR). Primers were designed for amplification of nearly 1,000 bp of two *M. abscessus* regions encompassing *mgtC* and containing the initial and the final sequences of this gene and for cloning between them and the zeocin resistance gene (*Streptoalloteichus hindustanus* [*ShBle*]). The entire fragment was then electroporated into *M. abscessus* CIP-S containing the pJV53 plasmid for homologous recombination and integration into the *M. abscessus* chromosome. Numbers 1 to 6 correspond to primers AM-MgtC F, AM-MgtC R, AV-MgtC F, AV-MgtC R, Zeo-MgtC F, and Zeo-MgtC R, which are described in Table S1 in the supplemental material, respectively. Details of primers Screening 7 (S7) and Screening 8 (S8), used for mutant screening, are given also in Table S1. (B) Validation of the Δ*mgtC*$_{MAB}$ mutant by PCR using primers Screening 7 and Screening 8. PCR was performed on the *M. abscessus* CIP wild-type strain (lane 1); on the PCR product used for allelic exchange (lane 2); or on a zeocin-resistant clone selected after the recombination event (lane 3). Lane MW, DNA ladder (lane taken from a different area of the same gel); pb, base pairs. (C and D) Growth of the Δ*mgtC*$_{MAB}$ mutant in Mg^{2+}-deprived liquid medium. The growth curves represent wild-type (WT) and Δ*mgtC*$_{MAB}$ strains grown in Sauton's medium without magnesium (C) or in regular Sauton's medium supplemented with magnesium (D). OD_{600} data over the growth period are indicated. The experiment was independently repeated three times (*, $P < 0.05$; ***, $P < 0.001$). *Mabs*, *M. abscessus*. (E) Replication of the *mgtC* mutant in the J774 macrophage cell line after 48 h. Results are expressed as means + standard deviations (SD) of the results from four independent experiments. The difference in replication rates between the two strains is not significant ($P = 0.1$, Student's *t* test).

noreactive band was detected in the lysate of the Δ*mgtC*$_{MAB}$ mutant grown under inducing and noninducing conditions.

We next monitored gene expression in bacteria internalized in J774 macrophages. After 24 h of macrophage infection, the *mgtC*$_{MAB}$-specific transcripts were found to be strongly induced (by about 15-fold) compared to the expression level in plankton-growing cultures (Fig. 3A). Comparatively, *MAB_0146* was mildly induced (about 4-fold) in macrophages (Fig. 3A). Consistent with this gene expression pattern, the MgtC protein was detected in bacterial lysate recovered from macrophages (Fig. 3B).

To further confirm the *in vivo* $MgtC_{MAB}$ expression results, we evaluated the antibody responses of a *M. abscessus*-infected CF patient during infection. Sera from infected CF patients recognized an $MgtC_{MAB}$ recombinant protein expressed in *M. smegmatis* (Fig. 4). The specificity of the hybridization signal was shown using, as negative control, a *M. smegmatis* lysate that did not express $MgtC_{MAB}$ (Fig. 4, lanes C). Moreover, sera from *M. abscessus*-negative patients did not recognize $MgtC_{MAB}$ (Fig. 4).

Taken together, these results indicate that $MgtC_{MAB}$ is not expressed under standard *in vitro* growth conditions but that its expression is highly induced under conditions of Mg^{2+} deprivation, within infected macrophages, and during infection in CF patients as shown by the presence of $MgtC_{MAB}$ antibodies. We therefore reasoned that neutralizing the activity of MgtC through the production of specific antibodies may protect against *M. abscessus* infection in mice and would allow us to arrest the MgtC contribution *in vivo*.

Formulated *mgtC*$_{MAB}$ DNA immunization protects CF mice against *M. abscessus* infection. To further evaluate the impact of MgtC expression *in vivo* by an alternative approach to the *ex vivo* macrophage infection experiment, an MgtC immunotargeting approach was considered. Indeed, the pronounced induction of $MgtC_{MAB}$ expression in infected host cells is an important element in considering *MgtC*$_{MAB}$ as a potent immune target. Our recent success using a DNA-based immunization approach (13) prompted us to develop a plasmid DNA with a codon-optimized *mgtC*$_{MAB}$ gene transcribed under the control of a strong CMV promoter in formulation with a tetrafunctional amphiphilic block copolymer. This preparation was used to immunize CF mice bearing the cystic fibrosis transmembrane conductance regulator (CFTR) ΔF508 mutation, currently the most frequently encountered CFTR mutation in CF patients, as well as their wild-type FVB littermates. In addition to formulated *mgtC*$_{MAB}$ DNA, the corresponding β-galactosidase gene DNA was included as a control. Following immunization, mice were challenged using the aerosol route with 4×10^8 CFU of *M. abscessus*, as illustrated in Fig. S1 in the supplemental material (13). As shown in Fig. 5A, infection with *M. abscessus* was cleared more rapidly from the lungs of

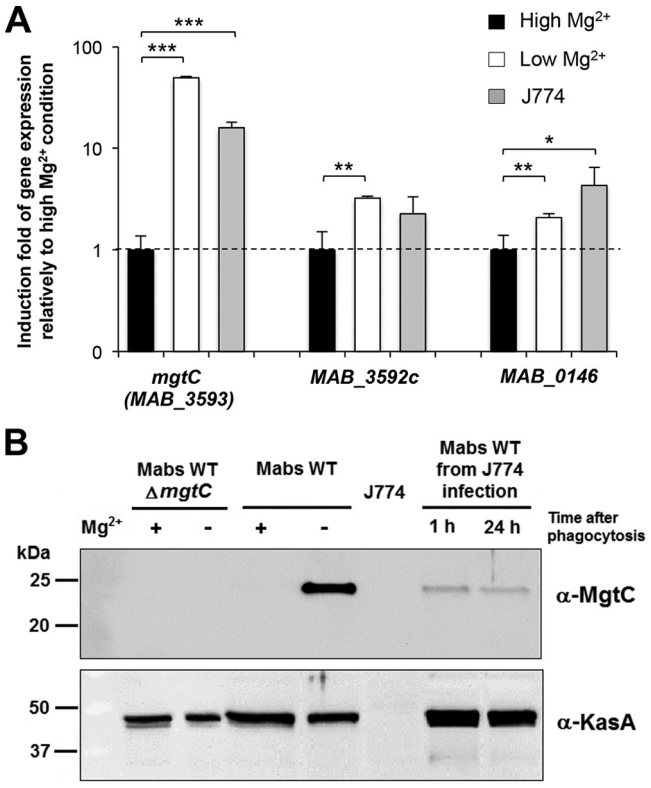

FIG 3 Expression of $mgtC_{MAB}$ in Sauton's medium and inside macrophages. (A) Quantification of $mgtC_{MAB}$ (*MAB_3593*), *MAB_3592c*, and *MAB_0146* RNA expression by qRT-PCR using RNA isolated from the wild-type strain grown in medium with a high or low Mg^{2+} concentration or internalized for 24 h inside J774 macrophages. The 16S rRNA gene was used as an internal standard. Results are expressed as means + standard deviations of the data from three experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test). (B) Expression of *M. abscessus* MgtC protein from wild-type *M. abscessus* grown under conditions of a high or low Mg^{2+} concentration or internalized for 1 or 24 h inside J774 macrophages. Lysate of *M. abscessus mgtC* mutant grown with or without Mg^{2+} was loaded as a negative control. Bacterial lysates equivalent to 4 μg (lysates from bacterial cultures) or 20 μg (lysates from infected macrophages) of total proteins were separated using 12.5% SDS-PAGE. For the detection of $MgtC_{MAB}$, the membrane was immunostained using a hyperimmune serum specific for $MgtC_{MAB}$ (see Fig. S5 in the supplemental material), washed, and probed with peroxidase-conjugated goat anti-mouse (IgG; 1/5,000). As a control, the samples were immunostained with an immune serum specific for the KasA protein of *M. tuberculosis*.

ΔF508 (CF) mice immunized with formulated $mgtC_{MAB}$ than from those of mice that had received the β-galactosidase-encoding DNA at 7 ($P = 0.0178$), 14 ($P = 0.0145$), and 21 ($P = 0.0001$) days postinfection. CFU counts also decreased significantly faster in the spleen (day 7; $P = 0.0159$) of immunized ΔF508 mice than in those of the control mice. Furthermore, only two (33%) of six *mgtC*-immunized ΔF508 mice were still culture positive in the lungs at 21 days postchallenge (Table 1) whereas seven (100%) of seven mice receiving the β-galactosidase control plasmid remained culture positive ($P = 0.02$). Only one (20%) of five *mgtC*-immunized ΔF508 mice remained culture positive in the spleen 7 days postchallenge, whereas five (83%) of six mice receiving the β-galactosidase control plasmid were culture positive at that time point. It is, however, noteworthy that the formulated *mgtC* DNA failed to protect the infected parental FVB littermates, thus highlighting a potent effect of the CFTR ΔF508 mutation in unmasking the protective effect against an aerosol challenge in CF mice (Fig. 5B and Table 1).

DISCUSSION

Genome sequence comparison between pathogenic and nonpathogenic microorganisms has revealed the potential of key virulence determinants as vaccine candidates. *MAB_3593*, uncovered from whole-genome sequencing of *M. abscessus* (11), has emerged as an attractive gene candidate as it encodes MgtC, a well-known virulence factor that is present in various bacteria and shared by several CF opportunistic pathogens but absent from *M. smegmatis*, a nonpathogenic RGM.

MgtC has been shown to promote bacterial growth in magnesium-depleted medium in all bacteria tested so far, including *M. tuberculosis* and *M. marinum* (17, 20, 37). In addition, expression of *mgtC* was shown to be highly induced by low Mg^{2+} concentrations in *M. marinum* (37), as well as in the nonrelated bacterial species *S.* Typhimurium (38) and *P. aeruginosa* (20). Despite its unique genomic location and organization (differing, for example, from that in *M. marinum* [37]), a similar result was observed in *M. abscessus*, with a 50-fold induction rate for the $mgtC_{MAB}$ transcripts found upon Mg^{2+} deprivation. That Mg^{2+}-dependent expression of MgtC is conserved in phylogenetically distantly related organisms is very likely linked to the conserved function of MgtC in the adaptation to Mg^{2+} fluctuations, which is supported by the contribution of $MgtC_{MAB}$ to optimal growth in Mg^{2+}-deprived broth medium. A second MgtC-like protein is encoded by the *M. abscessus* genome, which belongs to a phylogenic cluster distinct from *MAB_3593*, is poorly regulated by Mg^{2+} (2-fold induction), and fails to promote growth in low Mg^{2+} concentrations in a heterologous experimental system. The presence of two MgtC-like proteins has also been reported earlier in *P. aeruginosa*, where MgtC (PA4535) is regulated by Mg^{2+} deprivation and promotes growth in low-Mg^{2+} medium whereas the second MgtC-like protein (PA2558) does not (20, 21). However, the precise function(s) of these MgtC-like components remains unknown and whether they can compensate for a potential MgtC defect in macrophages remains to be established.

$MgtC_{MAB}$ is not expressed in standard planktonic cultures, but, importantly, both the transcription of $mgtC_{MAB}$ and the production of $MgtC_{MAB}$ are significantly induced within infected macrophages. This publication presents the first report of the detection of an MgtC protein in infected macrophages. An induction by the intramacrophage environment has also been shown at the transcriptional level for *S.* Typhimurium *mgtC* (19) and *P. aeruginosa mgtC* (20). It can be inferred that, despite different genomic organizations, a conserved regulatory mechanism facilitates *mgtC* induction within host cells. The host signal that drives *mgtC* expression is unlikely to be associated with Mg^{2+} deficiency and remains to be clearly identified. Phagosome acidification has been proposed to contribute to the optimal expression of *P. aeruginosa mgtC* (20), and a combination of acidification and antimicrobial peptides has been reported to activate the *Salmonella* sensor that regulates *mgtC* within macrophages (39).

Although MgtC contributes to intramacrophage survival in several bacterial pathogens (18), the present findings suggest that $MgtC_{MAB}$ does not significantly contribute to *M. abscessus* intramacrophage survival under our experimental conditions, even though a slight replication defect was observed for the $\Delta mgtC_{MAB}$ mutant. A similar behavior has been described for a *M. marinum*

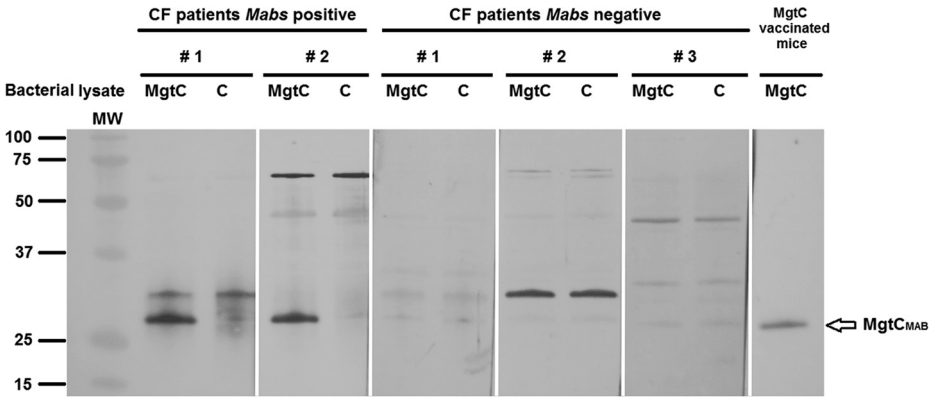

FIG 4 Presence of specific MgtC antibodies in the serum of CF patients infected with *M. abscessus*. Sera (diluted 1:1,000) from two CF patients infected with *M. abscessus* and three CF patients negative for infection with *M. abscessus* were tested using Western blotting against a total lysate of *M. smegmatis* overexpressing the recombinant $MgtC_{MAB}$ protein tagged with a C-terminal polyhistidine (lanes MgtC) or control *M. smegmatis* lysate (lanes C). Serum from mice immunized with $mgtC_{MAB}$ DNA against the *M. smegmatis* lysate overexpressing the recombinant $MgtC_{MAB}$ protein is included as a positive control (right lane). The blots are issued from a single membrane and hybridized with different sera. MW, molecular weight.

mgtC mutant and was correlated, potentially, to fast and important escape of the bacteria to the cytoplasm (37). The lack of a strong phenotype of the $\Delta mgtC_{MAB}$ mutant in macrophages may also be linked to a similar phenomenon, supported by the view that *M. abscessus* is not a strict intracellular pathogen *per se* (A. L. Roux, A. Viljoen, A. Bah, R. Simeone, A. Bernut, T. Deramaudt, M. Rottman, J. L. Gaillard, L. Majlessi, R. Brosch, I. Vergne, C. de Chastellier, L. Kremer, and J. L. Herrmann, submitted for publication). In addition, we cannot exclude the possibility that the loss of $MgtC_{MAB}$ function inside macrophages is at least partially com-

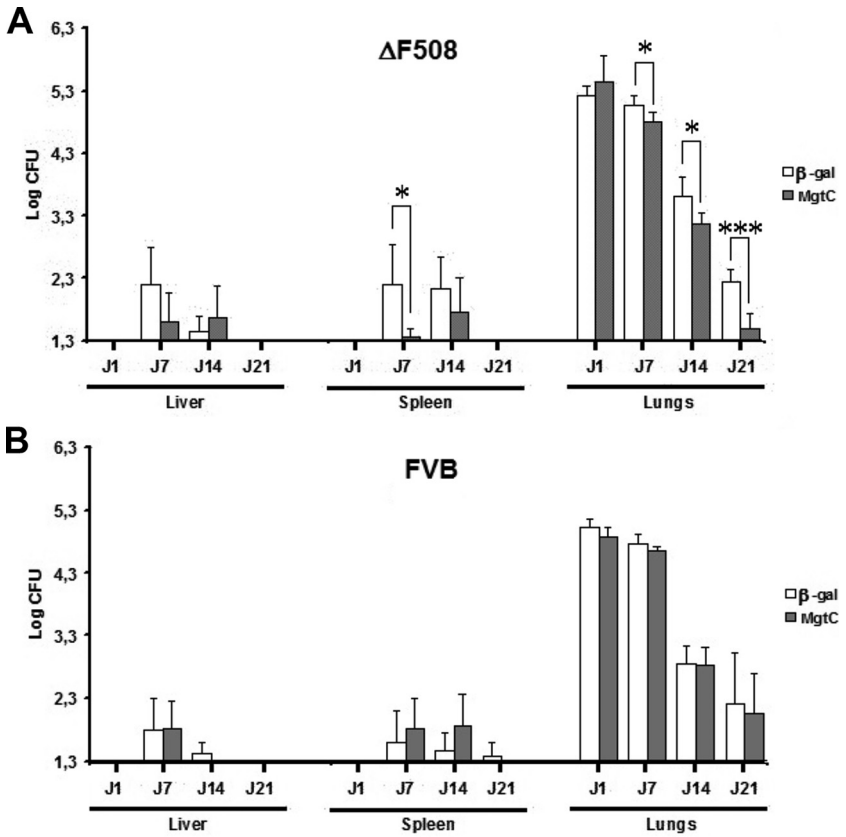

FIG 5 Bacterial persistence of *M. abscessus* CIP in liver, spleen, and lungs of tetrafunctional amphiphilic block copolymer formulating $MgtC_{MAB}$ DNA-vaccinated or β-galactosidase DNA-vaccinated ΔF508 FVB mice (A) or the wild-type FVB littermates (B) after an aerosol infection challenge. Lungs, spleen, and liver of mice infected with an aerosolized solution containing 4×10^8 bacterial cells/ml of CIP S were collected and homogenized by dislocation. The homogenates of lungs, spleen, and liver were serially diluted and plated for CFU counts. Results are expressed as the log units of CFU for formulated $mgtC_{MAB}$ DNA-vaccinated mice (gray bars) or formulated β-galactosidase (β-gal) DNA-vaccinated mice (control group, white bars) at days 1, 7, 14, and 21 postinfection (*, $P < 0.05$; ***, $P < 0.001$).

TABLE 1 Number of mice with residual liver, spleen, and lung infection on day 7 (liver and spleen) or 21 (lungs) post-aerosol challenge compared to the total number of immunized mice

Organ(s) and day post-aerosol challenge[a]	Mouse strain	No. of infected mice/total no. of mice tested		*P* value
		β-Gal DNA[b]	$mgtC_{MAB}$ DNA	
Liver				
7	ΔF508	5/6	2/5	0.24
7	WT FVB	4/6	5/6	1
Spleen				
7	ΔF508	5/6	1/5	0.08
7	WT FVB	2/6	4/6	0.56
Lungs				
21	ΔF508	7/7	2/6	0.02
21	WT FVB	6/8	7/8	1

[a] Days with significant results based on the CFU analysis (Fig. 5) are taken into account.
[b] β-Gal, β-galactosidase.

pensated by the MgtC-like protein encoded by the *MAB_0146* gene, whose expression is also induced intracellularly, although at a lower level. Moreover, our results do not preclude the possibility of a contribution of MgtC to *M. abscessus* virulence in other infection models, and experiments should be conducted in animal models to further investigate the role of $MgtC_{MAB}$ during infection.

MgtC expression was observed *in vivo*, indirectly demonstrated by antibody production in *M. abscessus*-vaccinated mice and in CF patients infected with *M. abscessus* (Fig. 4). Similar results were observed with *M. abscessus* PLC expression *in vivo* (13). Reactive T cells and production of antibodies against PLC were observed after PLC DNA vaccination, both responses being required for effective protection of PLC-vaccinated CF mice against an *M. abscessus* aerosol challenge (13). Although we did not look at the T cell response, we hypothesized that the production of antibodies against MgtC induced by the DNA vaccination using a similar synthetic delivery system comprising a tetrafunctional block copolymer (13) might be an important component of the protection conferred to animals with CF that were vaccinated against an aerosol challenge. This protective efficacy was judged for its ability to reduce the bacterial burden in the lungs of infected mice and the more rapid lung clearance in the context of a CF mutation, validating the potential of our vaccine-based approach. Unexpectedly, this protection occurred in the context of a CFTR defect (ΔF508 mutation) but not in the parental mice and thus supports the idea of the importance of MgtC in *M. abscessus* pathogenicity in CF mice.

Vaccination represents a useful approach in the fight against multidrug-resistant bacteria, of which *M. abscessus* remains a notorious example. The search for vaccine targets has largely benefited from reverse vaccinology, primarily based on *in silico* analyses of annotated genomes to select the most appropriate proteins as vaccine antigens (14). However, information in addition to that obtained from *in silico* genome analyses, such as the selection of proteins upregulated during infection, can be used to further prioritize antigens for assessment of their vaccine properties. Our findings using the $mgtC_{MAB}$ DNA formulation confirm this hypothesis. The development of a relevant murine model for *M. abscessus* vaccination is still a challenge. Despite the natural clearance occurring around day 21 to day 30 of infection, we were able to see a more rapid decrease in bacterial burden in $mgtC_{MAB}$-vaccinated mice, compared to beta-galactosidase-vaccinated mice. In addition, the protective effect triggered by the formulated $mgtC_{MAB}$ DNA vaccine appears even greater than that triggered by our previously described *plc*-based vaccine candidate, considering the percentage of culture-positive lungs at 21 days postinfection (13).

In conclusion, among all RGM species, *M. abscessus* is the only one that is a recognized respiratory pathogen in CF patients possessing several virulence factors acquired by HGT, such as MgtC (this work) or PLC (13). Since MgtC is also expressed in *P. aeruginosa* and *B. cenocepacia*, two major opportunistic pathogens in CF patients, we speculate that with regard to immune cross-reactivity, the $mgtC_{MAB}$ vaccine might also be beneficial in protection against both mycobacterial and Gram-negative infections in CF patients.

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