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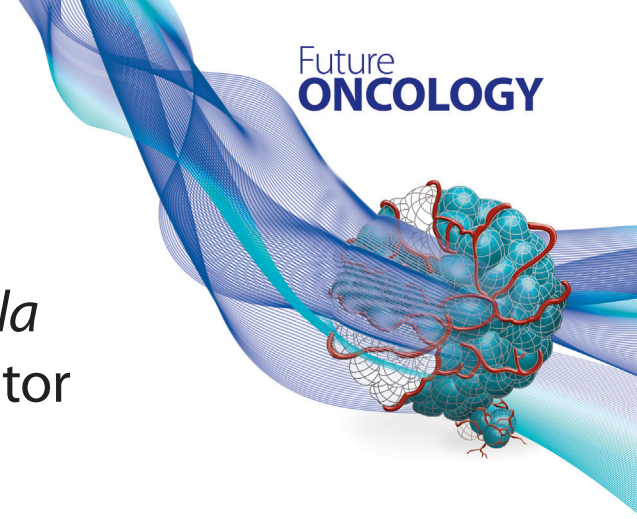

Antimelanoma effect of *Salmonella* Typhimurium integration host factor mutant in murine model

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Aim: This study aimed to evaluate an attenuated *Salmonella ihfA*-null mutant strain as therapeutic agent to control tumor growth. **Materials & methods:** After bacterial toxicity evaluation, C57BL/6JUnib mice were inoculated with B16F10 cells and treated with two *Salmonella* strains (LGBM 1.1 and LGBM 1.41). **Results:** LGBM 1.1 can reduce tumor mass, but it exerts some toxic effects. Although LGBM 1.41 is less toxic than LGBM 1.1, it does not reduce tumor mass significantly. Indeed, animals treated with LGBM 1.41 present only slightly initial delay in tumor progression and increased survival rate as compared with the control. **Conclusion:** The null-mutants of *ihfA* gene of *Salmonella* Typhimurium could be a promising candidate for melanoma treatment.

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KEYWORDS
• cancer treatment • *Salmonella enterica* Typhimurium • skin (melanoma)

Malignant melanoma is one of the most aggressive types of skin cancer. It originates in pigment-producing melanocytes present in the basal layer of the epidermis and in the eye. Melanoma has high metastatic potential and is extraordinarily resistant to anticancer agents. In the USA, the number of malignant melanoma cases has increased by 2.8% every year since 1981 [1–3]. Most patients with cancer undergo surgery, radiation therapy, chemotherapy or a combination of these treatments. However, cancer regression is hard to achieve because the region available for surgical procedures is restricted, patients may develop drug resistance, and harmful side effects may arise [1–3]. Effective tumor targeting and treatment toxicity are the major concerns in current cancer therapy. In solid tumors, hypoxic regions pose a further problem – they are resistant to many treatments [4].

The first time bacteria were intentionally applied in cancer treatment was at the end of the 19th century [5]. In the beginning, anaerobic bacteria such as species belonging to the genera *Bifidobacterium* and *Clostridium* were employed. However, these species were not able to grow in viable tumor tissues, which limited their efficacy [6]. In this context, *Salmonella enterica* serovar Typhimurium stands out: it is a facultative anaerobe that can target both small and large tumors at densities as high as 1:10,000 as compared with normal organs [7,8]; can be genetically engineered to grow selectively in tumor tissues [9]; survive and replicate in host cells; and induce the desired immune response, reducing tumor size [10].

The mechanisms through which *S. enterica* exerts its antitumor activity probably constitute a multifactorial process that involves both direct toxicity of the bacterium to the tumor cell and induction of an inflammatory response with participation of B and T cells, among other process [11]. Studies have demonstrated the roles played by INF-λ and TNF-α in *S. enterica* antitumor activity [12,13]. A

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recent work has also demonstrated that, in contrast to *E. coli* MG1655, an attenuated *S. enterica* Typhimurium strain can activate inflammasome signaling molecules such as IPAF, NLRP3 and P2X7. The latter strain is also associated with increased induction of inflammatory cytokine IL-1β in tumors, which suppresses tumor growth [14]. Moreover, researchers have demonstrated that cell death induction by apoptosis and autophagy are related to *S. enterica* antitumor activity [11].

Although wild-type bacterial strains can target tumors, their virulence may result in host disease and death. Fortunately, it is possible to modify bacteria genetically, to reduce their virulence which makes them safer to the host [4,15]. The tumor invasion process of systemically inoculated *S. enterica* is not clear yet, but this process apparently involves nitric oxide metabolism and bacterial motility [16–19]. In addition, different bacterial pathogen-associated molecular patterns (PAMPs; e.g., lipopolysaccharide, flagellin, and CpG) trigger an innate proinflammatory antitumor response [20] as discussed above.

Strains with attenuated virulence have been used in human clinical trials, but not as successfully as in assays involving animals [21]. To promote selection of safer and more efficient strains, some standards have been characterized *in vitro* and/or *in vivo* [15].

Considering the need to develop new *S. enterica* antitumor candidates, in this work our group decided to investigate the therapeutic effect of an *ihfA*-mutant strain (LGBM 1.1) on tumor growth. The *ihfA* gene underlies the expression of the A subunit of the integration host factor (IHF). IHF is a nucleoid-associated protein that participates in the modulation of the nucleoid structure; it affects many cellular functions and directly influences *S. enterica* virulence gene expression [22]. We have found that after gastric inoculation of the *S.enterica ihfA*-mutant strain in the mouse model, the bacterium has attenuated virulence [da Silva das Neves M, Martines Teixeira Mendes G, Unpublished Data]. In the present study, we will demonstrate that inoculation of the *S.enterica ihfA*-mutant strain by the intraperitoneal route also reduces the virulence of this bacterial strain, and we will show that *S.enterica ihfA*-mutant inhibits melanoma tumor growth in the mouse model. In addition, we will report the ability of an *S. enterica* Δ*ihfA*Δ*asd* strain (LGBM 1.41) to impair *in vivo* tumor replication.

Materials & methods

• Growth conditions & bacterial strains

One *S.enterica* Typhimurium strain [Lopes Sales AI *et al.*, Flagellar monophasic Salmonella enterica 1,4,[5],12:i:- isolated in Brazil share some characteristics with the U.S. and differ from European and Spanish clones regarding fljBA operon deletion (2016), Manuscript in preparation] previously isolated from human stool cultures (ethics committee approved the study protocol, number 2997/2005) was used as recipient of the plasmid and linear DNA required for Lambda Red recombination [23]. The wild-type strain was named LGBM 1. The mutants 662StmΔ*ihfA* (LGBM1.1) and 662StmΔ*ihfA*Δ*asd* (LGBM 1.41) had been constructed previously [da Silva das Neves M, Martines Teixeira Mendes G, Unpublished Data] and in the present study, respectively. LGBM 1.41 consists of a *S. enterica* serotype Typhimurium mutant strain that carries deletion of the *asd* gene (Δ*asd*) which is the gene encoding an enzyme that is necessary for the synthesis of Diaminopimelic acid. Therefore, LGBM1.41 growth requires diaminopimelic acid, which is an essential constituent of the peptidoglycan of the cell wall of gram negative bacteria [24].

Bacterial cultures were grown in Luria Bertani broth (LB) and Luria Bertani agar (LBA) plates prepared according to Sambrook and Russell [25] under vigorous aeration at 37°C. Diaminopimelic acid was added at a concentration of 50 µg/ml to promote growth of the Δ*asd* mutant strain. When necessary, the antibiotics Ampicillin (50 µg/ml; Sigma®, Spain) and Chloramphenicol (25 µg/ml; USB, UK) [25] were added.

• Experiments on animals

Animals were female C57BL/6JUnib mice aged between 6 and 8 weeks, obtained from the Multidisciplinary Center for Biological Research (CEMIB – UNICAMP). The animals were housed under specific pathogen-free conditions in our Animal Research Facility and maintained at 24 ± 2°C, in a 12 h light/dark cycle, under controlled humidity. The Ethics Committee on Animal Research of the University of Campinas (protocol number 3369-1) approved all the procedures performed in this study.

• Determination of virulence in mice

6-week-old female C57BL/6JUnib mice were acclimatized for 7 days after arrival at the local animal facility, before the start of the experiments. To determine LD_{50}, the bacterial strains

were grown under aeration (180 rpm) in LB broth at 37°C. For LGBM 1.41 growth, 50 µg/ml diaminopimelic acid was added. When the cultures reached OD_{600} between 0.8 and 0.9, they were harvested by centrifugation at 2057 *g*, at room temperature, and suspended in phosphate-buffered saline (PBS) to a final concentration of approximately 10^9 CFU/ml. Groups of mice (five per group) were intraperitoneally inoculated with 100 µl of serial dilutions of LGBM 1.1 (10^2, 10^3, 10^4, 10^5 and 10^6 CFU/ml), LGBM 1 (10^2, 10^3 and 10^4 CFU/ml), or LGBM 1.41 (10^3, 10^4, 10^5, 10^6 and 10^7 CFU/ml). The number of CFU used here was based on our previous results on the attenuation profile of each bacterial strain by the oral route. Mice inoculated with PBS [25] were used as negative control. The animals were observed for 30 days [26].

• Eukaryotic cell invasion & toxicity assays

The murine melanoma cell line B16-F10 was grown in RPMI 1640 medium (Sigma, USA) containing 10% fetal bovine serum, supplemented with penicillin G (10,000 U/ml). The cells were washed with PBS, centrifuged at 200 *g* and 4°C for 10 min, counted, and suspended in antibiotic-antimycotic-free RPMI 1640 supplemented with 10% fetal bovine serum. A total of 2×10^5 cells/ml were placed in 24-well plates (Costar 3524 Corning Incorporated). The cells were incubated in humidified atmosphere containing 5% CO_2 at 37°C for 24 h, for adhesion. Nonadherent cells were removed by washing the wells three-times with PBS. The wild-type strain LGBM 1 and the mutant strains LGBM 1.1 and LGBM 1.41 were then added to the cells at a multiplicity of infection of 10:1. For the invasion assay, the plates were incubated for 2 h. The cells were then washed twice in PBS to remove nonadherent/invasive bacteria. Next, the cells were reincubated in culture medium containing Gentamicin (10 µg/ml; Sigma, China) for 1 h to kill any remaining extracellular bacteria. After that, the cells were washed again with PBS, immediately lysed in 0.5% Triton X-100, and plated for colony counting. The neutral red protocol [27] was used to determine cell viability. In this case, the eukaryotic B16-F10 cells were treated with the wild-type or the mutant bacterial strains for 10 h.

• *S. enterica* antitumor activity in a mouse model

A suspension of 5×10^5 B16-F10 cells was subcutaneously inoculated in the right flank of 6-week-old C57BL/6JUnib mice. When the tumors grew to about 100–200 mm^3 (10–13 days), the mice were distributed into groups of five animals for the treatments. The animals were treated with intratumoral injection of about 10^5 CFU/ml of *S. enterica* LGBM 1.1 or 10^7 CFU/ml of *S. enterica* LGBM 1.41 (the doses were calculated based on LD_{50} values). The number of CFU used here was based on our previous results on the attenuation profile of each bacterial strain by the intraperitoneal route of inoculation. Tumor size was measured with the aid of calipers (Starret® 799) every 2–3 days as described previously [20]. The negative control group was treated with PBS. The tumor volume was calculated by using the formula: $h \times w^2 \times 0.52$, where h = height and w = width. The initial measure was considered 100% [28]. If tumors reached 20 mm in any dimensions or 4000 mm^3 in volume, the mouse was euthanized.

• Tissues & tumor colonization

After 24, 48 or 72 h, the mice were euthanized, and the tumors, spleen and liver were extracted, weighed, mechanically disrupted with scissors (Tissue master 125 OMNI), and suspended in sterile PBS. The mixed organ suspension was serially diluted and plated on MacConkey agar (Oxoid Limited, Thermo Fisher Scientific Inc.). Blood samples were also collected and plated in LB agar. Bacterial colonies were counted after overnight incubation at 37°C.

• Biochemical assay

Blood samples were collected at 24, 48 and 72 h after treatment to analyze total protein (TP), albumin (ALB), glucose (GLU), alkaline phosphatase (PHOS) and catalase (CAT) in the serum. The activity of CAT was determined by using a spectrophotometric method with UV light and expressed as IU/mg of protein [29]. The other dosages were performed according to guidelines given by the kits GLU, ALB and TP (Laborclin) as well as PHOS (Laborlab, Sao Paulo, Brazil).

• Statistical analysis

The survival rate data were compared by the log-rank (Mantel–Cox) and the Gehan–Breslow–Wilcoxon test. The other assays were compared by one-way analysis of variance (ANOVA); Tukey's test was applied, and all the calculations were carried out by using the Graph Pad Prism 5.0 statistic program. Differences at

a confidence level of 95% were considered significant (*$p < 0.05$).

Results

• Administration by the intraperitoneal route also indicates attenuation of *S. enterica* mutant strains in mice

In this work, we determined the LD_{50} of LGBM 1, LGBM 1.1 and LGBM 1.41 after their intraperitoneal inoculation in mice (Table 1). The intraperitoneal LD_{50} of the corresponding wild-type bacterium was less than 100 cells. The LD_{50} of LGBM 1.1 was higher than 10^3 CFU, which indicated that this strain had attenuated pathogenicity when compared with the wild-type. For LGBM 1.41, calculation of the LD_{50} was not possible because none of the mice died at the highest inoculated bacterium dose (10^6 CFU/ml). Subcutaneous inoculation of different LGBM 1.1 CFUs (10^6, 10^7, 10^8 and 10^9) aided study of its virulence. None of the mice treated with 10^6 or 10^7 CFU/ml died, but mouse death occurred at higher doses (10^8 and 10^9 CFU/ml; data not shown). LGBM 1.41 was not tested because it was greatly attenuated upon intraperitoneal inoculation in mice. The doses used to treat the mice were chosen on the basis of these data.

• Eukaryotic cell invasion & toxicity assays

Infection of B16-F10 cell cultures with the *S. enterica* strains *in vitro* helped to check the bacterial invasion capacity and toxicity toward a tumor cell lineage. To measure the *in vitro* cell invasion efficiency of *S. enterica* mutant strains, bacteria in the logarithmic growth phase were co-cultured with B16-F10 cells for 2 h, and the number of intracellular bacteria was determined. Figure 1A attests to the ability of the strains to invade melanoma cells. LGBM 1.1 displayed the same invasion profile as wild-type strains, whereas LGBM 1.41 had significantly ($p < 0.05$) lower invasion/multiplication capacity.

The neutral red assay enabled determination of bacterial toxicity to B16-F10 cells (Figure 1B). B16-F10 cell cultures treated with the wild-type strain (LGBM 1) constituted the positive control; B16-F10 cultures without added bacteria served as the negative control. The LGBM 1.1 toxicity profile resembled the toxicity profile of the wild-type strain (Figure 1B). In both cases, the percentage of living cells was lower as compared with the negative control ($p < 0.05$). However, LGBM 1.41 showed poor toxicity to B16-F10 cells without statistical differences as compared with the negative control. These results accounted for the very low LGBM 1.41 toxicity toward melanoma cells.

Table 1. Determination of the LD_{50} of *Salmonella enterica* Typhimurium strains after intraperitoneal inoculation of C57BL/6JUnib mice.

Salmonella enterica Typhimurium[†]	LD_{50} (CFU/ml)
LGBM 1	<100
LGBM 1.1	5.4×10^3
LGBM 1.41	$>10^6$

Results are representative of three independent experiments with five mice per group (LGBM 1 and LGBM 1.1) and one experiment with five mice per group (LGBM 1.41). Mice treated with phosphate-buffered saline were used as negative control.

• *S. enterica* mutants have antitumor activity in mice

After the preliminary tests *in vitro*, mice with melanoma were treated with 1×10^5 CFU of LGBM 1.1 and with 1×10^7 CFU of LGBM 1.41 by intratumoral inoculation to evaluate whether these bacterial cells affected tumor growth *in vivo*.

Although LGBM 1.41 was the most attenuated strain among the tested *S. enterica* strains (it exhibited less aggressive profile and lower toxicity), its therapeutic efficacy demanded improvement. According to Figure 2A, the volumes of the melanoma tumors treated with LGBM 1.41 did not decrease at the same rate observed upon treatment of the mice with LGBM 1.1 (Figure 2B). However, LGBM 1.41 retarded tumor growth and maintained lower mortality rate ($p < 0.05$) when compared with the negative control group (PBS treatment) (Figure 2C) and the mice treated with LGBM 1.1 (Figure 2D). Tumor growth in the mice treated with LGBM 1.1 decreased markedly (Figure 2B), but the mortality rate was similar to the PBS control group (Figure 2D).

• Quantification of bacteria in mouse tissues & organs

To check the presence of bacteria in mouse tissues including tumors, C57BL/6JUnib mice were infected with LGBM 1.1 and LGBM 1.41 by intratumoral injection of 10^5 and 10^7 CFU, respectively. Blood, spleen, liver and tumor were homogenized and suspended in sterile PBS as indicated above. They were then plated in LBA and MacConkey Agar after 24, 48 and 72 h post-infection (Figure 3).

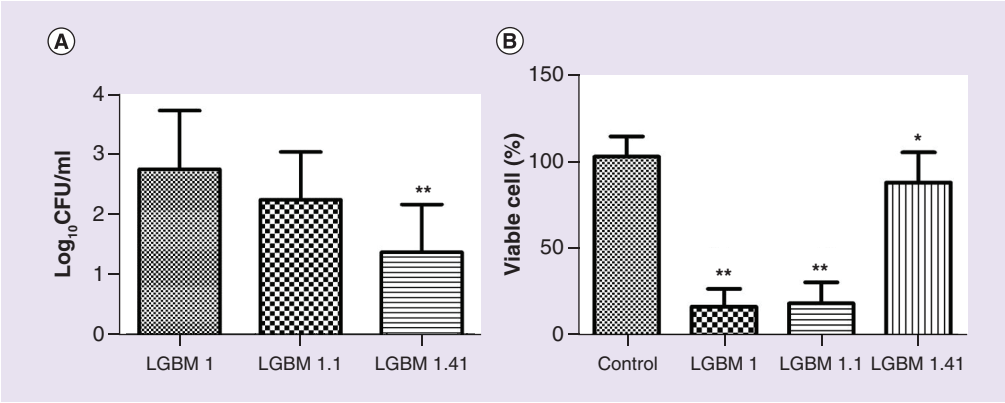

Figure 1. Toxicity of *Salmonella enterica* LGBM 1.1 and LGBM 1.41 strains to B16-F10 melanoma cells (B16-F10). Approximately 5×10^5 melanoma cells were incubated in each well, and then they were infected with 5×10^6 CFU/ml (MOI of 10) of *S. enterica* mutant strains. The wild-type strain, LGBM 1, was used as positive control. Results are representative of three independent experiments with three replicates each. **(A)** Intracellular CFU (log_{10}) of *S. enterica* strains after 2 h of incubation. **p < 0.05 when compared to LGBM 1. **(B)** Percentage of viable cells after 10 h of *S. enterica* infections. **p < 0.05 when compared to the control and *p < 0.05 when LGBM 1.41 is compared to LGBM 1. Tukey's Multiple comparison test was related with the control in **(A)** and with the wild-type in **(B)**.

The mutants exhibited distinct colonization. LGBM 1.41 had reduced persistence *in vivo* as compared with LGBM 1.1. As expected, the LGBM 1.41 burden decreased in the tumor, spleen and liver **(Figure 3A–C)**; the bacteria were completely cleared from the blood within 24 h (data not shown). These results corroborated the *in vitro* tests and the lower tumor reduction observed for LGBM1.41. In contrast, tissue and organ colonization by LGBM 1.1 remained relatively high in the liver, spleen and tumor **(Figure 3A–C)** as compared with LGBM 1.41, not to mention that complete LGBM 1.1 clearance from the blood only occurred after 72 h (data not shown).

• Biochemical evaluations

Evaluation of biochemical parameters (serum levels of GLU, TP, ALB, CAT and PHOS) in treated mice helped to assess the overall nutritional status as well as the possible liver damage and oxidative stress response, possibly influenced by tumor growth and/or treatment. The mice treated with the LGBM 1.1 strain presented lower ($p < 0.05$) ALB and PHOS **(Figure 4A & B)** as compared with the healthy mice (mice without tumor) and the negative control (animals with tumor, but without treatment), which can indicate liver toxicity. Mice treated with LGBM 1.41 exhibited the ALB dosage not statistically different **(Figure 4A)** as compared with the negative control, but had significantly lower ($p < 0.05$) PHOS dosage **(Figure 4B)**. This pointed to the lower liver toxicity of LGBM 1.41 as compared with LGBM 1.1. The biochemical serum analysis of GLU **(Figure 4C)**, TP **(Figure 4D)** and CAT **(Figure 4E)** after mouse treatment with one of the evaluated bacterial strains revealed no statistical differences as compared with the positive and negative controls.

Discussion

In recent years, researchers have explored different types of bacteria that can target cancer cells [10,30–31]. Among these bacteria, *S. enterica* serovar Typhimurium strains have proven to be promising anticancer agents, which has led scientists to modify these bacteria to obtain strains with fewer side effects and improved therapeutic action [4,7,10–11,20,32–33]. Several studies have demonstrated that leu-arg auxotrophic *S. enterica* strain (A1-R) can effectively inhibit and in some cases even eradicate different types of primary and metastatic tumors when used as monotherapy in mouse models of prostate cancer [33–35], breast cancer [36–38], lung cancer [39,40], ovarian cancer [41,42], cervical cancer [43], pancreatic cancer [44–48], sarcoma [49–51] and glioma [52,53]. Recently, researchers have demonstrated that the *S. enterica* Typhimurium strain (STM) deficient for a zinc transporter operon can invade and proliferate in tumor cells, exerting therapeutic effect in the mammary adenocarcinoma mouse

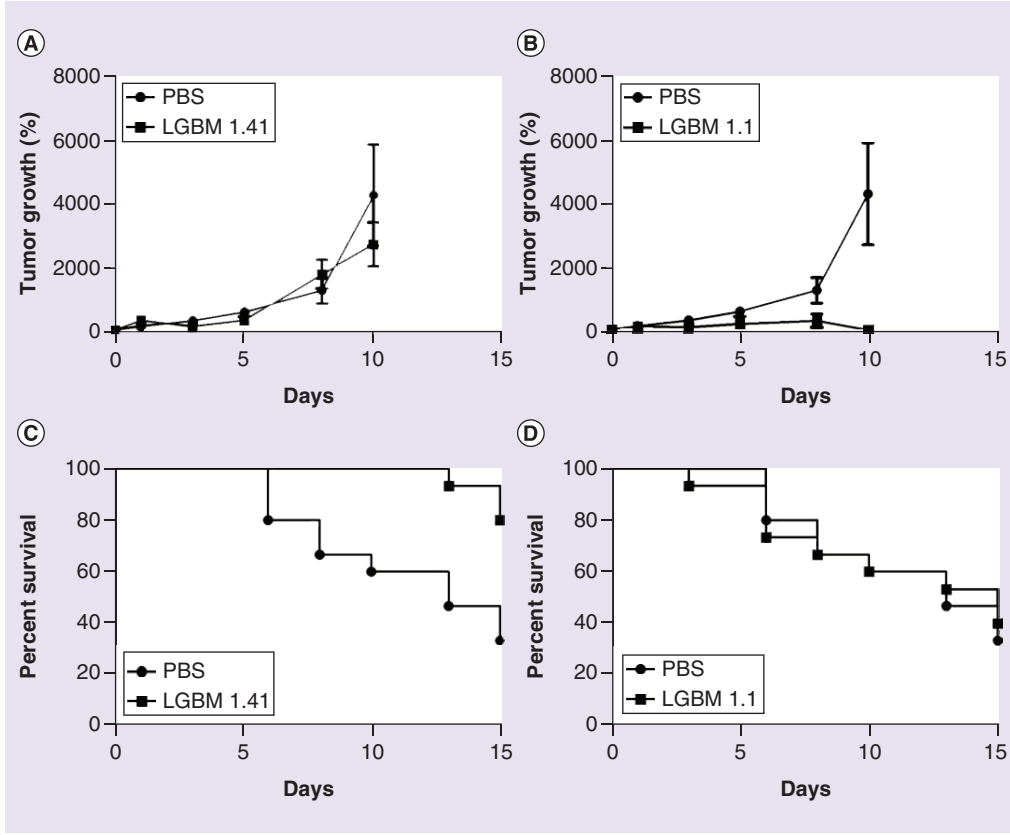

Figure 2. Tumor volumes in mice treated with *Salmonella enterica* mutant strains. Results are representative of three independent experiments with five mice per group. PBS served as negative control. Tumor volumes were measured with calipers. **(A)** Tumor development after infection with LGBM 1.41. **(B)** Tumor development after infection with LGBM 1.1. **(C)** Survival of mice after treatment with *S. enterica* Typhimurium LGBM 1.41 (10^7 CFU/ml). **(D)** Survival of mice after treatment with *S. enterica* Typhimurium LGBM 1.1 (10^5 CFU/ml) . $p < 0.05$ when PBS control group is compared to LGBM 1.41 treated mice (log-rank Mantel–Cox test).
PBS: Phosphate-buffered saline.

model by promoting an antitumor immune response [54].

Evaluation of VNP20009, an attenuated *S. enterica* Typhimurium strain (*msbB*$^-$ and *purA*$^-$), against many types of tumors [7,28] has shown that this strain provided good results in animal models, but it failed in terms of tumor colonization efficacy and antitumor activity in human patients during Phase I clinical trials [21]. This result highlighted that the delicate balance between host protection and bacterial virulence is essential [20].

S. enterica induces potent proinflammatory response via bacterial components (PAMPs) that include lipopolysaccharide and flagellin. This happens not only upon oral *S. enterica* delivery, but also upon local *S. enterica* administration via the vagina or intratumorally, for example [55]. Increased INF-λ, TNFα and IL-1β production and significantly enhanced tumor infiltration by leukocytes such as macrophages, dendritic cells, neutrophils, CD8$^+$ T cells and B cells occur in *Salmonella*-treated tumor. This implies that immunological system induction is an essential mechanism of *S. enterica* antitumor activity [14,56]. In addition, it has been demonstrated that *Salmonella*-induced cell death of tumor cells is mediated by a mechanism involving apoptosis and autophagy through modulation of the caspase and AKT/mTOR pathways, respectively [11].

Here, we have demonstrated that two mutant attenuated *Salmonella* Typhimurium strains reduced melanoma growth in the mouse model: LGBM1.1, mutated in the *ihfA* gene, which underlies expression of a nucleoid-associated protein involved in many cellular functions and

virulence genes expression [22], and LGBM1.41, a double-mutant Δ*ihfA* and Δ*asd*, which presents deficient replication in the absence of diaminopimelic acid [24].

IHF consists of two types of subunits (IHFα and IHFβ) and can exist as homo (IHFαα or IHFββ) or heterodimer (IHFαβ) [57]. Transcriptomic analyses have indicated IHF has an important role in the regulation of virulence genes and genes related to the stationary growth phase [22]. These observations have prompted us to investigate whether attenuation of an *S. enterica ihfA* mutant occurs in the mouse model and whether this mutant, which cannot express the homodimer (IHFαα) or heterodimer (IHFαβ), displays antitumoral activity. Indeed, LGBM 1.1 possesses some characteristics that are important for a potential antitumor live agent: it is virulence attenuated by the oral and intraperitoneal route of inoculation, it can replicate in tumor tissues, and *in vitro* assays have confirmed its high toxicity to melanoma cells. The main LGBM 1.1 feature is that it can reduce tumors, a property that only a few *S. enterica* mutants have been shown to display so far. However, further studies on different mutant strains are still necessary to obtain more effective agents because trials conducted on human volunteers have demonstrated that none of the strains developed to date can effectively eliminate tumors in humans [21,58–59].

Our biochemical analyses indicated that mice submitted to therapy with LGBM 1.1 developed liver toxicity. Besides that, some animals died during therapy. These deaths could have

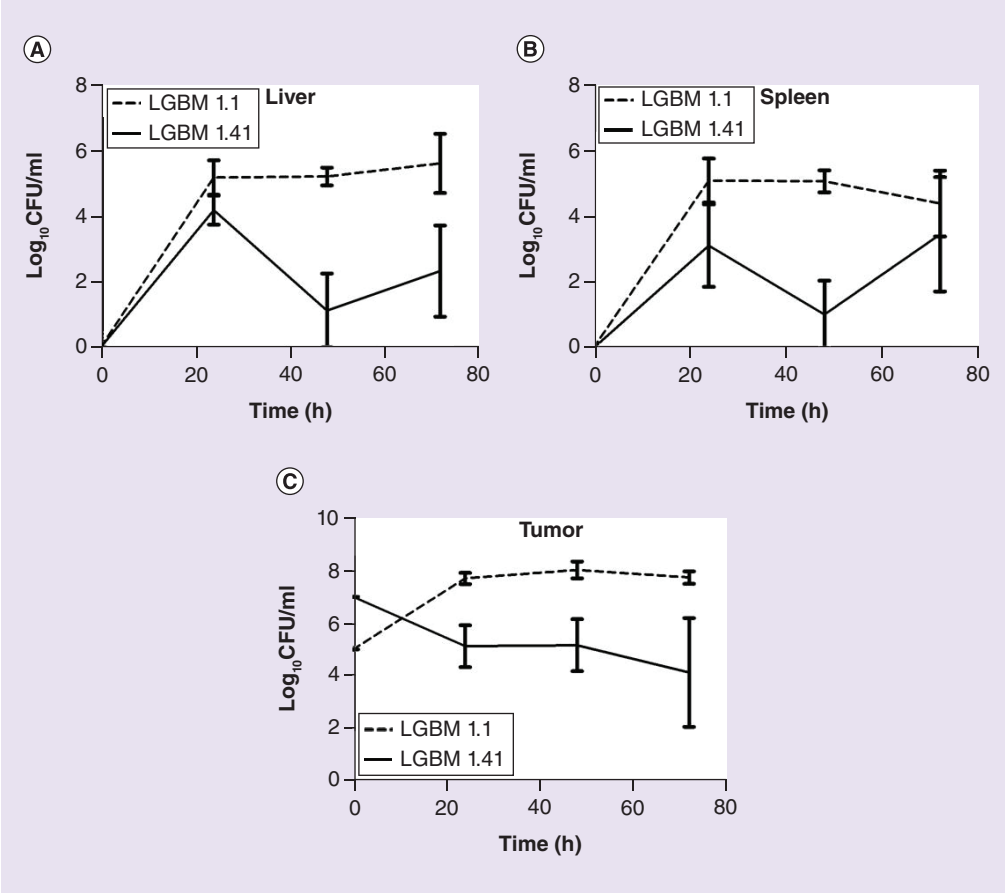

Figure 3. Tumor and organ colonization of C57BL/6JUnib mice by *Salmonella enterica* nucleoid-associated protein mutants. Mice were treated intratumorally with 10^5 CFU of LGBM 1.1 or 10^7CFU of LGBM 1.41 *S. enterica* strains. **(A)** CFU counts in liver. **(B)** CFU counts in spleen **(C)** CFU counts in tumor. Bacterial burdens were determined by plating serial dilutions of tissue homogenates for three different times (24, 48 and 72 h). Phosphate-buffered saline was the negative control of mice with tumor. In addition, the organs of healthy nontreated mice were used as control. Results are representative of two independent experiments with two mice per group.

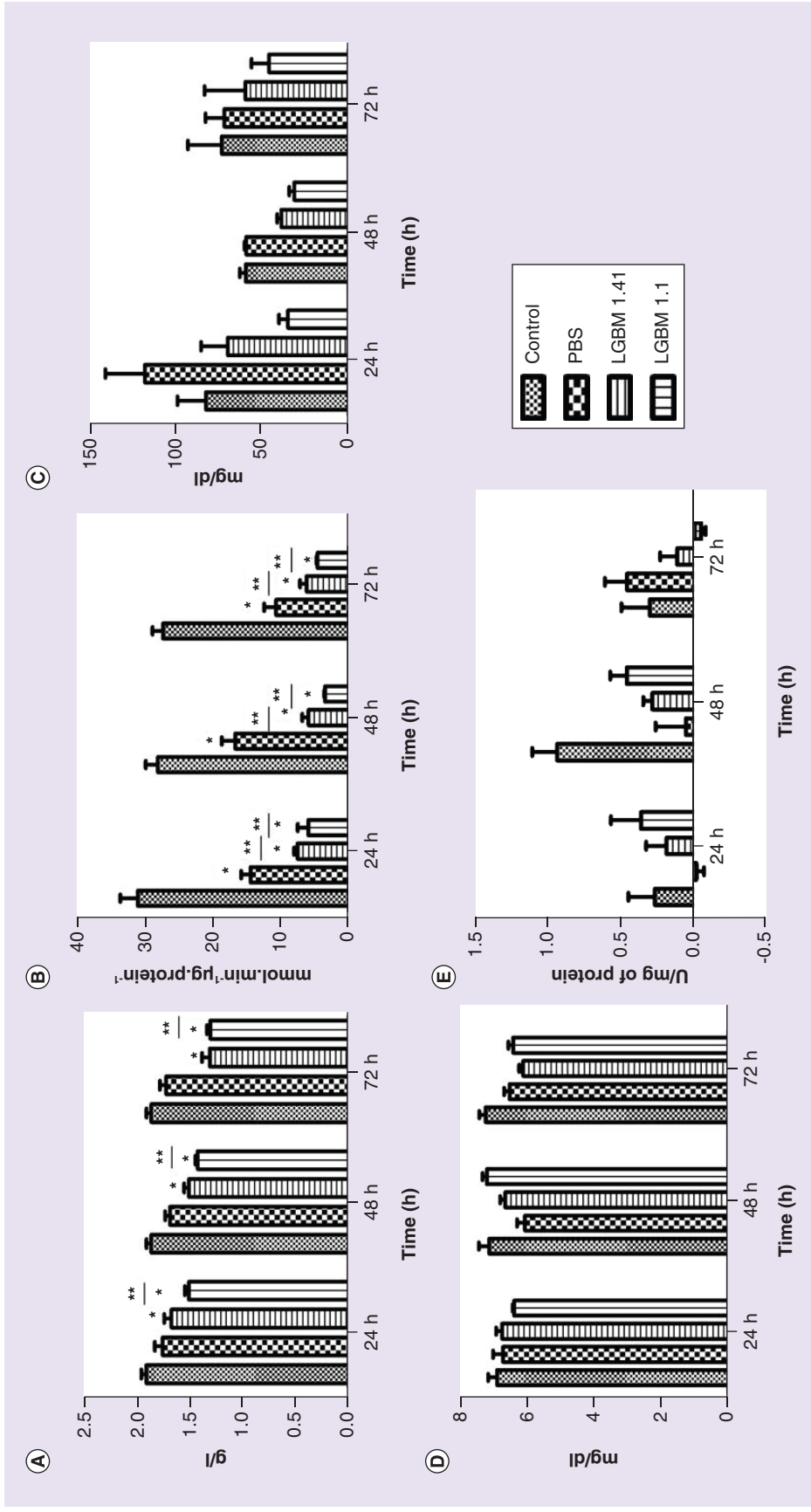

Figure 4. Biochemical serum analysis of mice treated with *Salmonella enterica* strains for 24, 48 and 72 h shown as mean ± standard deviation. Results are representative of two independent experiments with a serum pool of two mice per group. **(A)** Concentration of albumin (g/l). **(B)** Activity of phosphatase (mmol.min^{-1}µg.protein^{-1}). **(C)** Concentration of glucose (mg/dl). **(D)** Concentration of total protein (mg/dl). **(E)** Activity of catalase (U/mg of protein). *p < 0.05 versus control; **p < 0.05 versus PBS. Nontreated mice were used as control.
PBS: Phosphate-buffered saline.

resulted from the amount of bacteria used in the experiments (about of 10^5 UFC), which caused a residual pathogenic effect that is particularly important in debilitated tumor-treated animals, to toxicity mediated by bacterial PAMPs, a rapid tumor regression (tumor lysis syndrome) [60], or to a combination of these factors. In this sense, future studies should explore the development of more attenuated strains. *S. enterica* Δ*ihfA* mutants express genes related to flagellar biogenesis to a lesser extent, which could consequently reduce their motility [22] [da Silva das Neves M, Unpublished Data]. In fact, recent studies have pointed out that motility plays an important role in antitumoral activity [18,19], and this characteristic could influence the antitumor effect of *ihf* mutants. However, *ihf*-mutants do not present completely impaired motility [22, unpublished results], which calls for further investigations to clarify this issue.

Absence of diaminopimelic acid impaired LGBM 1.41 replication, a condition that also occurred *in vivo*. The highly attenuated phenotype of this strain and the importance of bacterial replication in antitumor activity motivated us to assay the antitumoral action of LGBM 1.41. Even at higher doses, LGBM 1.41 exhibited more attenuated profile than LGBM 1.1, and none of the mice died. LGBM 1.41 persisted in the tumor for 72 h, but these bacteria were found in lower number and did not have the same efficiency as LGBM 1.1. Indeed, LGBM 1.41 was totally cleared from blood less than 24 h after inoculation. Clairmont and colleagues reported similar results when they tested the VNP20009 strain in C57BL/6 mice and in nonhuman primates [7]. Another characteristic of LGBM 1.41 was its lower liver toxicity as compared with LGBM 1.1, which could also explain the extended survival of the mice treated with this strain as compared with the control group. Zhang and colleagues described similar findings for *Salmonella* A1-R during treatment of ovarian cancer in mice [38]. Finally, although treatment with LGBM 1.41 did not reduce tumor mass significantly, tumor progression was initially delayed as compared with the negative control group.

Our data for LGBM 1.41 suggested that bacterial replication is important for effective antitumor activity at least in the case of the melanoma model employed here. Absence of diaminopimelic acid impairs LGBM 1.41 replication *in vivo*, which is associated with lower persistence of this strain in tumors and with its lower capacity to induce tumor regression as compared with LGBM 1.1. Nevertheless, LGBM 1.41 can still retard tumor development and prolong mice survival. Yoon and colleagues [61] have also shown that a modified *Salmonell*a *(*Δ*aroA*Δ*aroD*) expressing E7 fusion protein can suppress tumor growth and prolong animal survival, but it cannot eliminate tumors. An alternative to circumvent this problem is to engineer *S. enterica* to express the *asd* gene only in the conditions found in tumor tissues. For instance, in elegant studies, the *asd* gene was placed under the control of a promoter activated in the anaerobic environment found in tumors [4,62]. The *S. enterica* Typhimurium YB1 is an SL2707-derived strain containing the *asd* gene under the control of PpepT, an anaerobic regulated promoter. This strain also contains a genetic modification that impairs *asd* expression in the aerobic conditions. YB1 efficiently and safely treats tumor-bearing mice [4,62].

Together, the results of the present study have shown that *ihfA*-null mutants of *S. enterica* Typhimurium have potential application in melanoma cancer treatment. Probably, a combination of *S. enterica* LGBM 1.41 with traditional therapies like antitumor drugs could extend mouse survival and improve tumor regression. A recent study combining *Salmonella* A1-R with trastuzuma B showed that this combination was effective against patient-derived cervical cancer growing in nude mice [63]. Additional LGBM 1.1 attenuation or even a combination of lower LGBM 1.1 doses with LGBM 1.41 could improve cancer therapy.

Conclusion & future perspective

Based on the mouse model used herein, *S. enterica* Typhimurium Δ*ihfA* mutants can reduce the melanoma growth in this model. The strain LGBM 1.1 exerted some toxic effects, but it reduced tumor volume or even cleared tumor in some mice. On the other hand, LGBM 1.41 presented fewer toxic effects. Although the latter strain did not reduce the tumor volume significantly; it delayed tumor progression and increased the survival rate. Our data pave the way for further studies investigating the use of nucleoid-associated protein mutants in cancer treatment. If not alone, mutants can be used in combination with drugs but at a lower dose.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Administration by the intraperitoneal route

- The LD_{50} of LGBM 1.1 increased by 100-fold as compared with LGBM 1.
- Calculation of the LD_{50} of LGBM 1.41 was not possible because no mouse died at the highest inoculated bacterium dose (10^6 CFU/ml).

Bacterial invasion capacity & toxicity

- The toxicity and invasion profile of LGBM 1.1 resembled the profile of the wild-type strain.
- LGBM 1.41 showed poor toxicity and invasion profile toward B16-F10 cells.

Antitumor activity

- Tumor growth in the mice treated LGBM 1.1 reduced markedly.
- Tumor growth in the mice treated with LGBM 1.41 did not decrease at the same rate; however, LGBM 1.41 retarded it.

Presence of bacteria in mouse tissues

- Tissue and organ colonization by LGBM 1.1 remained relatively high in the liver, spleen and tumor.
- LGBM 1.41 had reduced organ colonization when compared with LGBM 1.1.

Biochemical evaluations

- Serum analyses showed that LGBM 1.1 can have liver toxicity.
- LGBM 1.41 showed a lower liver toxicity as compared with LGBM 1.1.

Conclusion

- LGBM 1.1 can reduce or even clear the tumor.
- The mutants have potential antimelanoma effect.

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