



Inflammasome activation, NLRP3 engagement and macrophage recruitment to tumor microenvironment are all required for *Salmonella* antitumor effect

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Abstract

Salmonella-based cancer therapies show great potential in preclinical models, but for most cases the observed antitumor effect is transient. Understanding the basis of the antitumor efficacy might guide the design of improved strains that elicit long-lasting effects, paving the way for clinical use. Here, we deepened into the role of macrophages and inflammasome activation in the context of *Salmonella* anti-melanoma effect. We showed inflammasome activation in melanoma cells upon infection, which correlated with cell surface exposure of gasdermin-D (GSDM-D) and calreticulin (CRT) and High mobility group box 1 protein (HMGB-1) release, suggesting immunogenic cell death, particularly pyroptosis. *Salmonella* infection upregulated levels of *Caspase-11* (*Casp11*) mRNA, but not *Nlrp3* or *Nlrc4* mRNA, the only described inflammasome receptors engaged by *Salmonella*, suggesting that non-canonical inflammasome activation could be occurring in melanoma cells. Intratumoral administration of *Salmonella* to melanoma-bearing mice elicited local inflammasome activation and interleukin-1 β (IL-1 β) production together with tumor growth retardation and extended survival in wild type but not *Caspase-1/11* (*Casp1/11*) knockout mice despite similar levels of intratumoral IL-1 β in the later. *Salmonella* antitumor activity was also suppressed in melanoma bearing *Nlrp3* knockout mice. *Salmonella* induced macrophage recruitment to the tumor site and infiltrating cells exhibited inflammasome activation. Depletion experiments confirmed that macrophages are also essential for *Salmonella* anti-melanoma effect. Intratumoral macrophages showed a marked M2/M1 shift soon after treatment but this inflammatory profile is then lost, which could explain the transient effect of therapy. All in all, our results highlight CASP-1/11 axis and macrophages as essential players in *Salmonella*-based cancer immunotherapy and suggest a possible target for future interventions.

Keywords *Salmonella* immunotherapy · Melanoma · Inflammasome · Macrophage recruitment · M2/M1 shift

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Précis: *Salmonella* antitumor activity requires functional CASP-1/11 activation, NLRP3 engagement, and recruitment of M1 macrophages to the tumor site. Absence of CASP-1/11 prevents macrophage intratumoral recruitment and hence the antitumor activity.

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Introduction

For many years, facultative anaerobic bacteria have been evaluated for the treatment of different types of cancer, showing highly effective in delaying tumor growth and prolonging survival in tumor-bearing mice [1–3]. Particularly, *Salmonella* has several features that make it an excellent candidate for immunotherapies. These include ability to grow easily under lab conditions, short replication cycles, ability to reach and replicate preferentially in tumor sites, and being facultative anaerobic, which enables them to reach both small oxyc tumors or big hypoxic ones; amongst others ([4, 5], also reviewed in [6]). We have previously demonstrated that *Salmonella* Typhimurium LVR01 treatment induces the infiltration of immune cells as neutrophils and CD8+ T lymphocytes, correlating with a better outcome in

both lymphoma and melanoma [7, 8]. Nevertheless, *Salmonella* antitumor effect is modest yet significant, and since the underlying mechanisms are not fully elucidated, additional evidence in this regard is imperative.

Inflammasome activation has emerged as an interesting topic since its discovery almost two decades ago. *Salmonella* can induce inflammasome assembly and activation in myeloid cells by at least two independent -but redundant- pathways. Direct recognition of intracellular flagellin by NLRC4/NAIP5, or NLRP3 indirect activation by the detection of common signals related to cell damage induced by infection such as changes in potassium levels or membrane disruption [9, 10]. In addition, the NLRP3 inflammasome may also be engaged by cytosolic LPS through direct binding to CASP-11 [11]. These events lead to the secretion of active IL-1 β and IL-18 and may also promote a lytic type of caspase-dependent cell death named pyroptosis [12], which amplifies the inflammatory response through the release of DAMPs to the extracellular environment [13]. Gasdermin D (GSDM-D), the pore-forming effector protein of pyroptosis has also shown to be crucial in the release of IL-1 β and IL-18 since they do not have a secretion signal [14]. The relationship between pyroptosis and cancer is controversial: on one hand, GSDM-D has been described as a tumor-suppressing molecule and on the other hand, a proinflammatory microenvironment can be tumor-promoting (reviewed in [15]).

Salmonella upregulates transcript levels of core molecules of inflammasome signaling when used as immunotherapy in colon carcinoma models [16] as well as triggers the production of dendritic cells-derived IL-1 β , which leads to tumor growth suppression [17]. In this work, we pursued to establish the mechanisms behind *Salmonella* antitumor activity in a murine melanoma model deepening into the role of *Salmonella*-induced inflammasome activation. Our results show that upon infection, macrophages are recruited to the tumor site and both tumor and immune cells show rapid inflammasome activation. We also demonstrate that these events are indispensable for *Salmonella* to exert its antitumor effect. Loss over time of this acute inflammation profile could be a plausible explanation for the transient effect of *Salmonella*-based therapy.

Materials and methods

Bacterial strains

Salmonella enterica serovar Typhimurium LVR01, an avirulent *aroC*-mutant [18], was used in the present study. A LVR01 flagellin double knockout mutant (LVR01flag-) was constructed by phage P22 transduction of a *fliC::KanR* cassette from *S. Enteritidis* SEFK32 [19] and a *fliB::CmR* cassette from *S. Typhimurium* TH2795 [20], both strains kindly

provided by Dr. Jean-Claude Sirard (Institut Pasteur of Lille, France). Correct insertion of each cassette was confirmed by PCR (primers available under request). Bacteria were routinely grown aerobically in an orbital shaking incubator (200 rpm) at 37 °C in LB broth (Difco, Detroit, MI), supplemented with kanamycin (50 μ g/ml) and chloramphenicol (25 μ g/ml) (both from Sigma-Aldrich, St. Louis, MO) when needed. Absence of flagellin expression was confirmed using agglutination tests for flagellar antigens with commercially available flagellar polyvalent sera (polyHA and polyHB, Difco, Detroit, MI). Additionally, phase-contrast microscope visualization of mid-log-phase bacterial cultures and motility tests were also performed. The latter consisted of plating bacteria in LB soft agar (0.3%) and measuring halo after incubating for 6 h at 37 °C.

Cell lines

B16F1, a C57BL/6 skin melanoma cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA). Tumor cells were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (DMEMsup) at 37 °C in 5% CO₂.

Infection assays, caspase-1 activation and cell death assessment

B16F1 tumor cells were seeded in 24-well plates (Greiner bio-one, Germany) to a density of 5×10^5 cells per well. *Salmonella* LVR01 and LVR01flag- were diluted in the appropriate culture medium and added to the tumor cells at a multiplicity of infection (MOI) 100:1, and then incubated at 37 °C in 5% CO₂ atmosphere. After 1 h, cell cultures were rinsed and incubated in medium containing gentamicin sulfate (100 μ g/ml) (Sigma-Aldrich, St. Louis, MO) to kill extracellular, but not intracellular, bacteria. After 20 h cells were collected, washed twice with cold PBS, and then resuspended in 1X Binding Buffer (BD Pharmingen) at a final concentration of 1×10^6 cells/ml prior to staining with 5 μ l of FITC-Annexin V (BD Pharmingen). Cells were then incubated for 15 min at room temperature (RT) in the dark. Ten minutes before flow cytometry analysis (FACS Canto II, BD Pharmingen), 5 μ l of Propidium Iodide (300 ng/ml) (Sigma-Aldrich, St. Louis, MO) was added to the cells.

Inflammasome activation was measured using FAM-FLICA® Caspase-1 Assay Kit (ImmunoChemistry Technologies, MN) following manufacturer's instructions.

For cell death assessment, 20 h after gentamicin addition cells were stained with either anti-GSDM-D antibody (ab209845, abcam) followed by secondary antibody (anti-rabbit IgG-FITC, Thermo Fisher Scientific); or anti-CRT antibody—Alexa Fluor® 647 (ab196159, abcam). Cell

staining was analyzed by flow cytometry using FACS Canto II Flow cytometer equipped with six lasers (Becton–Dickinson, Oxford, UK). For data acquisition and analysis FACS Diva software (Becton–Dickinson) was used. Cell culture supernatants were assessed for HMGB-1 release, using HMGB1 ELISA Kit (IBL International, Germany) following manufacturer's instructions.

Quantitative RT-PCR

B16F1 cells were seeded and infected as described above. After 20 h of incubation, cells were resuspended in TRIzol (Invitrogen) and frozen at -80°C to preserve RNA until extraction. In case of tumor homogenates, tumors were excised at different time points and preserved the same way in 500 μl TRIzol. RNA quality and quantity was assessed by spectrophotometric measurements at 260/280 nm using NanoDrop 2000 (Thermo Scientific). Prior to cDNA synthesis, 1 μg of total RNA was treated with DNase-I (Invitrogen) according to manufacturer's instructions. Retrotranscription was performed in a final volume of 20 μl in the presence of Random primers (200 ng), dNTPs (0.5 mM), DTT (0.01 M), RNaseOUT (40 U), and M-MLV Reverse Transcriptase (Invitrogen). Quantitative RT-PCRs for *Nlrp3*, *Nlr4*, *Asc*, *Casp1*, *Casp11*, *Il1b*, *Il18*, *Cxcl9*, *Cxcl10*, *Nos2*, and *Il12* mRNA was conducted using QuantiTect® SYBR® Green PCR Kit (Qiagen) in a Rotor-Gene 6000 (Corbett), primer sequences are available under request. Beta-Actin encoding gene *Actb* was used as house-keeping gene. The relative mRNA amount in each sample was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method as previously described [21] where $\Delta\text{Ct} = \text{Ct}_{\text{gene of interest}} - \text{Ct}_{\text{Actb}}$, and expressed as relative mRNA levels in the test groups compared to the control group.

Animals

Female C57BL/6 (DILAVE, Uruguay), and *Casp1/Il1^{-/-}* or *Nlrp3^{-/-}* mice (The Jackson Laboratories, ME, USA and bred at the Institut Pasteur Montevideo, Uruguay), 6–8 weeks old were used for in vivo experiments. All protocols for animal experimentation were carried out in accordance with procedures authorized by the University's Ethical Committee for Animal Experimentation, Uruguay, to whom this project was previously submitted (CNEA No 0011/11).

Tumor model and immunization schedule

Tumor cells were grown in culture and harvested in log phase, washed twice with PBS, and resuspended in saline. Mice were injected into the right flank with 2.5×10^5 B16F1 cells. When tumors became palpable (average size 100 mm^3), all mice were inoculated the same day directly into

the tumor central area (intratumoral (i.t.) injections) with 1×10^6 cfu of *Salmonella* diluted in 100 μl of saline and then monitored for tumor growth every other day. Tumors were measured and volumes were calculated as length x width x depth x $\pi/6$. Mice were euthanized when tumors reached 4,000 mm^3 or before if they showed any sign of distress. These time points were defined as survival time.

Tumor-Infiltrating cells analysis

In a different set of experiments, animals were sacrificed at different time points and each tumor was removed for further analysis. Analysis of tumor-infiltrating cells was conducted by flow cytometry on tumor homogenates by mechanical disruption. Cells were stained with FAM-FLICA® Caspase-1 Assay Kit (ImmunoChemistry Technologies, MN) for 30 min at 37°C and after that they were immunostained for additional 30 min, 4°C , with the following panel of antibodies: PE-conjugated anti-Ly6G, PercP/Cy5.5-conjugated anti-CD11b and APC-conjugated anti-F4/80 antibodies. For macrophage phenotyping, Fc block step was performed for 10 min and then the following panel of antibodies was used: PE/Cy7-conjugated anti-CD11c, APC-conjugated anti-CD206, APC/Cy7-conjugated CD11b, and Pacific Blue-conjugated anti-F4/80 (all reagents from BD Pharmingen or Biolegend, CA, USA). Cells were finally washed and analyzed by FACS as stated above.

Cytokine production assessment

IL-1 β and IL-18 levels were quantified in tumor homogenates using ELISA MAX Deluxe Set Mouse IL-1 β (Biolegend, CA, USA) and Platinum ELISA Mouse IL-18, Ready-To-Use Sandwich ELISA kit (eBioscience, CA, USA) according to manufacturer's instructions.

Macrophage depletion

Systemic macrophage depletion was carried out with Clophosome® clodronate liposomes (Neutral) (FormuMax Scientific, CA, USA). The regime consisted of 3 intraperitoneal doses of 700 $\mu\text{g}/\text{ml}$ each separated by 5 days as previously described [22] and adapted to our model. PBMC was assessed for macrophage depletion over time. We obtained more than 70% of depletion 3 days after the third clodronate dose.

Statistical analysis

Differences in survival times were determined using Kaplan–Meier and log-rank test using GraphPad Prism 5 software. For the in vitro assays, the statistical significance of differences between study groups was analyzed using two

ways ANOVA or Student T-test. In every case, a value of $p < 0.05$ was considered statistically significant.

Results

Salmonella induces inflammasome activation and cell death in melanoma cells

We have previously shown that *Salmonella* LVR01 infection resulted in decreased viability of melanoma B16F1-infected cells [8]. Thus, we assessed for different hallmarks of cell death. Twenty hours after *Salmonella* infection, B16F1 cells showed increased Annexin V expression in cell membranes (Fig. 1a). Besides, melanoma cells displayed GSDM-D expression which could be indicative of pyroptosis (Fig. 1b). Further, we found increased CRT membrane exposure as well as HMGB-1 release to the extracellular milieu both of which suggest immunogenic cell death (Fig. 1c, d).

We then evaluated inflammasome activation in *Salmonella*-infected melanoma cells. We found a marked increase in *Casp11*, but not *Casp1*, transcripts 20 h after *Salmonella* infection (Fig. 1e), while *Nlrp3* and *Nlrc4* transcripts were not detected (data not shown). Besides, FLICA staining showed that *Salmonella* infection induces CASP-1 activation in melanoma cells (Fig. 1f).

Salmonella induces inflammasome activation in the tumor microenvironment in melanoma-bearing mice

B16F1 melanoma-bearing mice received a single intratumoral injection of LVR01 and 72 h later tumors were assessed for expression of inflammasome-associated molecules. Figure 2 shows that *Salmonella* treatment induces increased levels of IL-1 β , but not IL-18 protein in tumor homogenates (Fig. 2a). In addition, we observed increased levels of mRNA of inflammasome-related genes, such as *Nlrp3* and *Nlrc4* (Fig. 2b), both probably expressed by immune cells as we did not find these transcripts on melanoma infected cells. At later time points, this inflammatory expression profile decreases until most of the genes are not detected anymore, except for IL-1 β that remains high (yet non-significant).

Salmonella antitumor effect depends on Caspase-1/11 activity

We have previously shown that intratumorally delivered LVR01 slows tumor growth and improves survival in melanoma-bearing mice [8]. We now demonstrate that this antitumor effect is dependent on the CASP-1/11 axis, inasmuch *Salmonella* antitumoral activity is lost in melanoma-bearing

Casp1/11 ko mice (overall survival Control vs LVR01-treated $p = 0.0038$ in wt mice, $p = 0.3489$ in ko mice) (Fig. 3). Of note, intratumoral levels of IL-1 β (around 2000 pg/ml) were similar in *Salmonella*-treated tumors in both wt and *Casp1/11* ko mice (Fig. 2a).

NLRP3 inflammasome activation is essential for antitumor effect while NLRC4 is dispensable

We found that *Salmonella* induces overexpression of NLRP3 at the tumor site (Fig. 2b), indicating local activation of NLRP3 inflammasome. Thus, we assessed whether this is involved in the antitumor activity. LVR01 treatment to melanoma-bearing *Nlrp3* ko mice still induces tumor growth retardation although to a lesser extent than in wt mice and it does not extend overall survival (Fig. 4), suggesting that NLRP3 inflammasome engagement is central in *Salmonella* mediated antitumor activity.

Flagellin, the main building block of *Salmonella* flagella, is an NLRC4 agonist and it has been described as the main effector for CASP-1 activation upon *Salmonella* infection [23]. Thus, we tested whether aflagellated *Salmonella* still retains anti-melanoma effect. For that, we constructed a *Salmonella* mutant lacking *FliC* and *FljB* (LVR01flag-). Melanoma cells infected with LVR01flag- still activate CASP-1, though to a much less extent than flagellin expressing LVR01 (Fig. 5a, Control vs LVR01 $p = 4.33 \times 10^{-10}$, Control vs LVR01flag- $p = 9.20 \times 10^{-6}$, LVR01 vs LVR01flag- $p = 1.78 \times 10^{-7}$). However, in the absence of flagellin *Salmonella* still retains antitumor effect as shown in Fig. 5b (overall survival Control vs LVR01flag- $p = 0.0121$), with no evident difference between strains (overall survival LVR01 vs LVR01flag- $p = 0.8999$).

Salmonella treatment recruits macrophages to the tumor site and activates their inflammasome

We found that *Salmonella* recruits macrophages to the melanoma tumor site and also induces CASP-1 activation in a percentage of those tumor-infiltrating cells, but this recruitment is lost in *Casp1/11* knockout mice (Fig. 6a and b). We also found *Salmonella*-induced upregulation of molecules that have been linked to the M1 phenotype, particularly *Cxcl9*, *Cxcl10*, *Nos2*, and *Il12*, in tumor microenvironment (Fig. 6c), suggesting a shift of intratumoral macrophages towards pro-inflammatory antitumoral phenotype. Indeed, we confirmed this phenomenon by determining the percentage of M2 (defined as CD206+ macrophages) and calculating the ratio M1/M2 within tumor-infiltrating macrophages. As seen in Fig. 6d, soon after *Salmonella* administration, we observed on average a 15/1 ratio of M1/M2 macrophages in *Salmonella*-treated mice, whereas the ratio was 4/1 in control mice. Increased M1/M2 ratio was maintained for a few

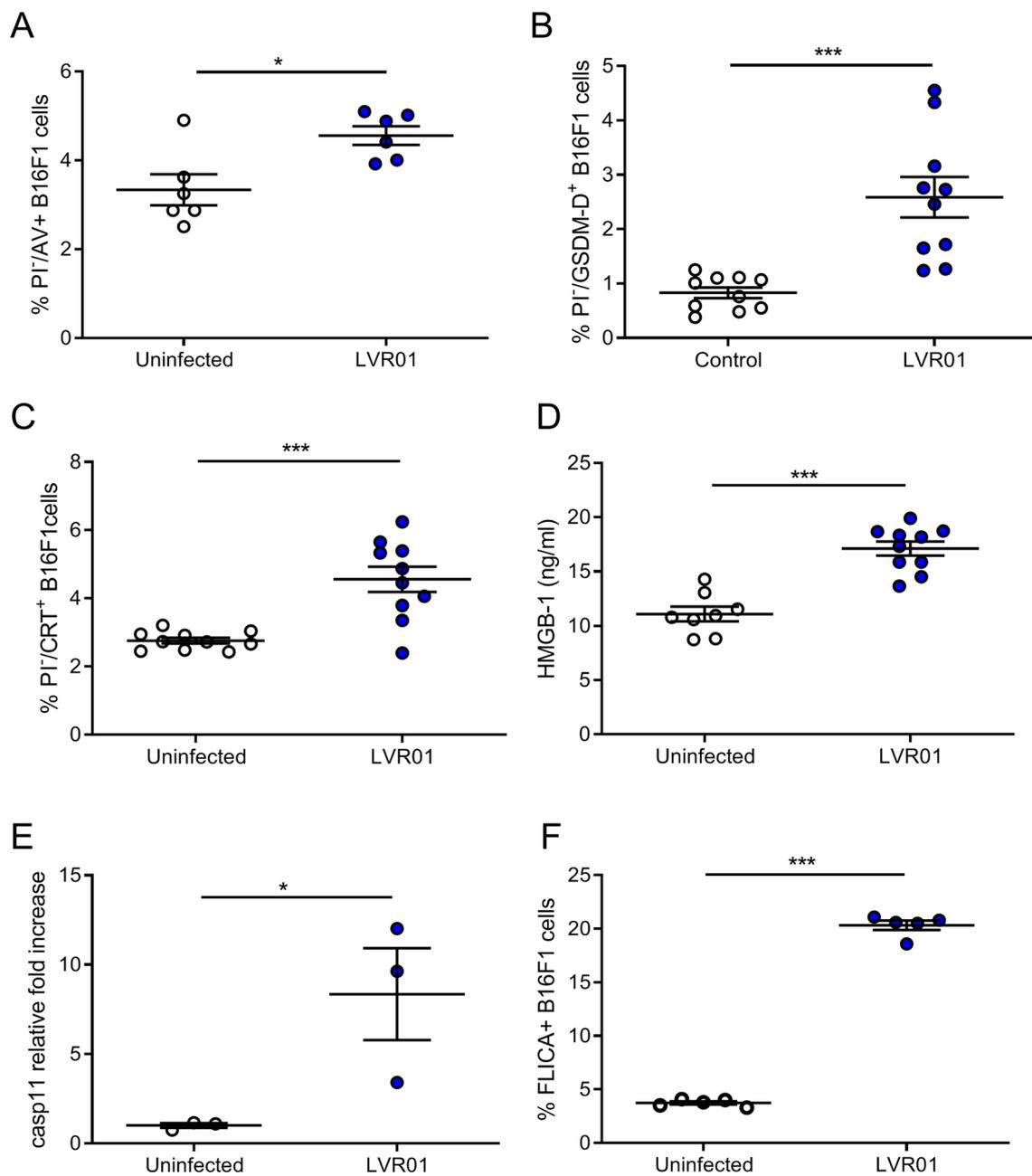


Fig. 1 B161 cells infected for 1 h with *Salmonella* resulted positive after 20 h for **a** Annexin V, **b** gasdermin-D, and **c** calreticulin expression. **d** HMGB-1 concentration in cell supernatants. **e** Relative *Casp11* mRNA

expression in B16F1 cells; **f** FLICA positive B16F1 cells. Results are shown as mean \pm SEM. * $p < 0.05$, *** $p \leq 0.001$. Representative dot plots for A-C and F are available in Suppl. Figure 1

days but significantly diminishes by day 7 post *Salmonella* until it becomes similar to that of control mice.

Macrophages are essential for *Salmonella* mediated anti-tumor activity

Since we found that *Salmonella* i.t. treatment results in CASP-1 activation in tumor-infiltrating macrophages

(Fig. 6b) and that *Salmonella* antitumor activity is dependent on the CASP-1/11 axis (Fig. 3), we then wanted to assess whether macrophages are involved in *Salmonella* anticancer effectiveness. Melanoma-bearing mice in a macrophage depletion regime with clodronate liposomes (Fig. 6e) received i.t. *Salmonella* as previously. As shown in Fig. 6f, depletion of macrophages completely abrogates *Salmonella* antitumor activity.

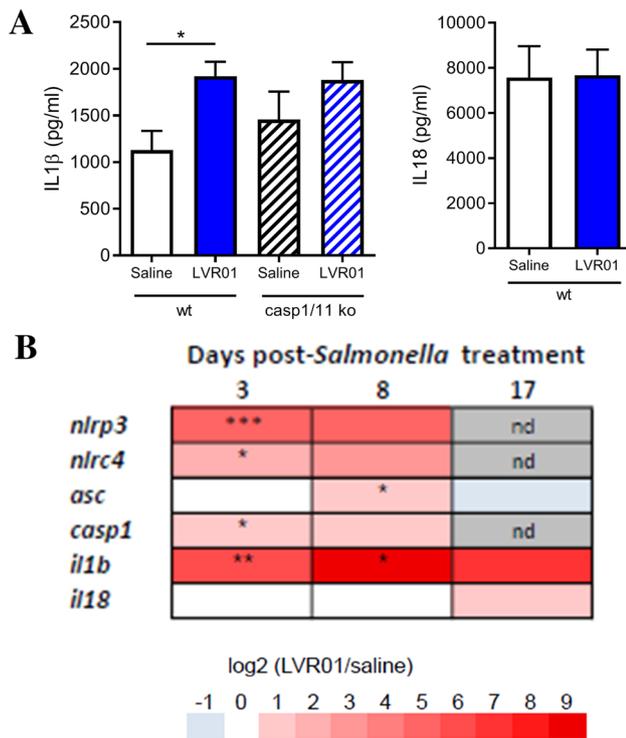


Fig. 2 **a** IL1 β and IL18 concentration in tumor homogenates 72 h post-*Salmonella* intratumoral injection. **b** Heatmap representing relative fold increase of inflammasome associated molecules on days 3, 7, and 18 post *Salmonella* intratumoral injection, nd: not detected. Data representative of two independent experiments. Results are shown as mean \pm SEM. $n = 12$ – 13 /group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Discussion

Salmonella has shown great potential for the development of new immunotherapies for cancer but the number of clinical trials is scarce and so far, they have not reproduced the effects observed in the preclinical models. Deciphering the molecular and cellular basis of the antitumor effectiveness of *Salmonella* might allow the designing of new strains

with improved therapeutic efficacy and help to predict clinical responses.

In this work, we investigated *Salmonella* LVR01 anti-tumor activity in a melanoma preclinical model. We found that LVR01 induces GSDM-D expression, CRT membrane exposure as well as HMGB-1 release to the extracellular milieu all of which suggest immunogenic cell death [24], particularly pyroptosis [25]. Immunogenic cell death (ICD) drives adaptive immunity and the establishment of long-term immunological memory, influencing the cancer-immunity balance by skewing it towards antitumor immunity. Indeed, many agents used in cancer immunotherapies induce ICD [26]. Therefore, therapeutic engagement of ICD through *Salmonella* may represent an interesting approach to elicit more effective antitumor immunity [27]. Cleavage of GSDM-D protein is an important mediator of pyroptosis, a process that was originally described as a form of cell death mediated by CASP-1 cleavage [12] that ended up in the release of the inflammatory cytokine IL-1 β [28]. We found that LVR01 infection significantly upregulated *Casp11* transcripts in melanoma cells and induced CASP-1 activation as well as IL-1 β release to the extracellular milieu in melanoma-bearing mice, suggesting that *Salmonella*-induced cell death at the tumor site is, at least in part, responsible for the antitumor activity. However, cleavage of inflammatory caspases as CASP-1 or CASP-11 does not always end up in cell death. Inflammatory caspases activation and GSDM-D cleavage have proven to have other physiological roles, inasmuch they are required to release IL-1 β from living “hyperactive” cells [14]. We cannot exclude the possibility that this effect is taking place.

It has been previously reported that intravenous *Salmonella* administration upregulates transcript levels of core molecules of inflammasome signaling and triggers production of dendritic cells-derived IL-1 β in tumors [16, 17]. It was also suggested that *Salmonella* antitumor activity is mediated by IL-1 β since administration of this pro-inflammatory cytokine partially restored intrinsic antitumoral

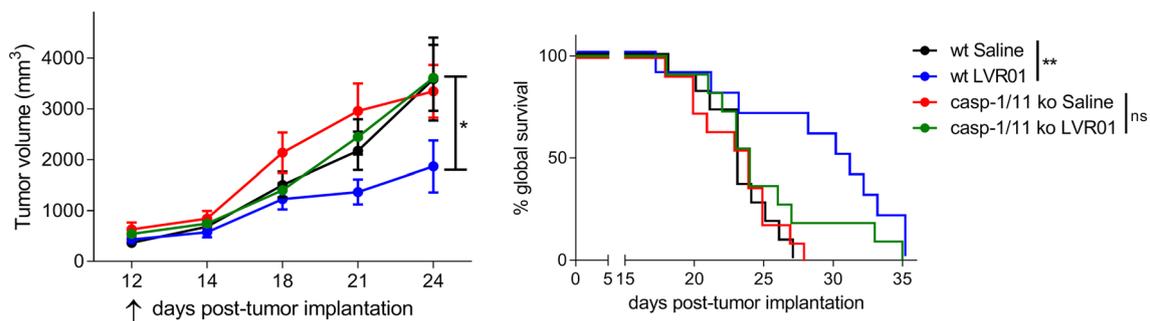


Fig. 3 Tumor growth (left) and survival (right) of LVR01 treated melanoma-bearing wt or *Casp1/11* ko mice. Data representative of three independent experiments. Results are shown as mean \pm SEM. $n = 12$ – 13 /group. * $p < 0.05$, ** $p < 0.01$. Arrow indicates *Salmonella* i.t. administration

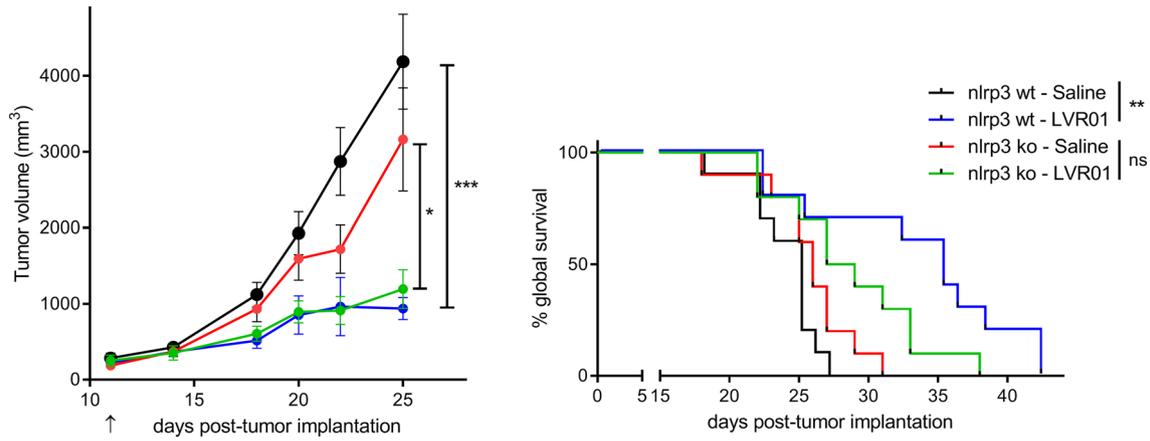


Fig. 4 Tumor growth (left) and survival (right) of LVR01-treated melanoma-bearing wt or *nlrp3* ko mice, $n=12-13$ /group. Data representative of two independent experiments. Results are shown as

mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Arrow indicates *Salmonella* i.t. administration

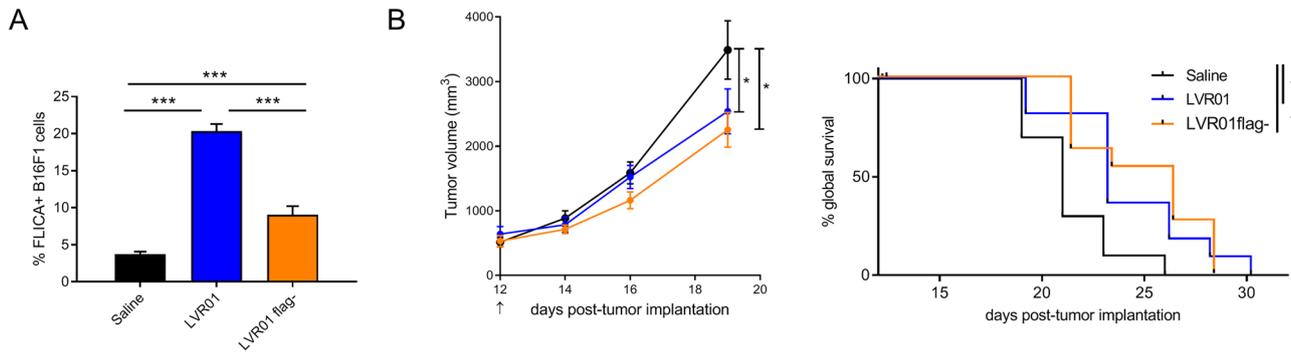


Fig. 5 **a** Percentage of FLICA positive B16F1 cells 20 h after infection with *Salmonella*, $n=3$ /condition. *** $p < 0.001$. **b** Tumor growth (left) and survival (right) of B16F1 tumor-bearing mice treated with

different *Salmonella*. Results are shown as mean \pm SEM. $n=12$ per group. * $p < 0.05$. Arrow indicates *Salmonella* i.t. administration

activity [17]. Here, we report that LVR01-induced CASP-1/11 activation is essential for *Salmonella* antitumor activity in melanoma-bearing mice since antitumor effect is lost in *Casp1/11* deficient mice. However, we also found that high intratumoral production of IL-1 β could still be found in melanoma bearing *Casp1/11* ko mice (Fig. 2a), most likely due to inflammasome activation of B16F1 implanted cells, suggesting that this cytokine may contribute but not be essential for *Salmonella* antitumoral activity. Inflammasomes have been proposed as a target for cancer immunotherapies, particularly for melanoma which is highly plastic and therefore rapidly displays mechanisms for immunotherapy resistance [29]. Still, the role of inflammasome activation in tumor progression is controversial, and several reports have attributed it a double-edged sword effect [30, 31]. On one hand, increased expression of NLRP3 has been associated with multiple cancer types, melanoma included [32]. Conversely, IL-18 secretion induced by NLRP3 inflammasome

activation in colorectal cancer has demonstrated to be protective, through IFN- γ production [33]. It is important to remark that despite IL-18 is considered an inflammasome-regulated cytokine, its modulation has proven to be different from that of IL-1 β [34], and it has even been suggested that they may have opposite effects [35]. This could explain our results that showed no differences between *Il18* mRNA expression and secretion after infection with *Salmonella*, while *Il1 β* mRNA expression and protein production does increase upon treatment (Fig. 2). Another explanation for the IL-18 unchanged levels after therapy is that cancers, including melanoma, exhibit high basal amounts of IL-18 secretion [36], possibly masking a discrete effect induced by *Salmonella*.

The key defining event to predict the results from inflammasome activation in cancer could be the nature of the inflammation (acute vs chronic) since it is required to fight cancer cells but also represents a hallmark of cancer [37].

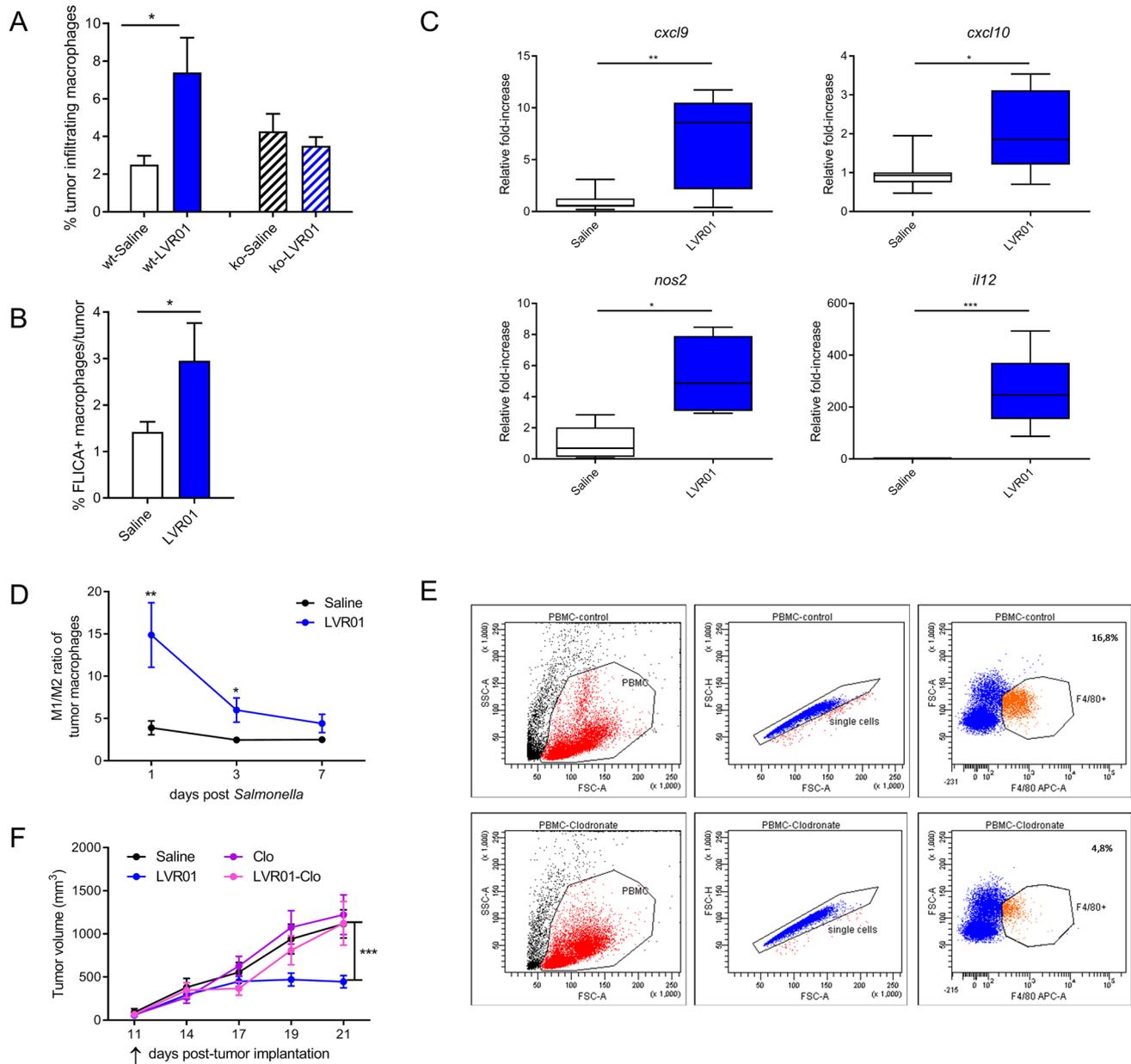


Fig. 6 **a** *Salmonella* induced percentage of tumor-infiltrating macrophages and **b** FLICA positive macrophages, $n=3-5$ /group. **c** Relative *Cxcl9*, *Cxcl10*, *Nos2*, and *Il12* mRNA expression in tumor homogenates 3 days post LVR01 i.t. treatment, $n=5-7$ /group. **d** M1/M2 ratio of tumor macrophages over time, $n=4-5$ /group. **e** Dot plots showing macrophage depletion in PBMC 3 days after finishing

clodronate treatment. **f** Tumor growth of LVR01 treated melanoma-bearing mice with or without macrophage depletion, Clo: clodronate, $n=12-13$ /group. In every case, results are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Arrow indicates *Salmonella* i.t. administration

Our results show that *Salmonella*-induced inflammation in the tumor microenvironment is acute since inflammasome-related gene expression upon treatment dissipates over time (Fig. 2b). However other aspects like cancer type, stage, experimental conditions, microbiota, etc. are all additional factors that need to be taken into account when considering different outcomes. In line with this, it has been proposed that different subpopulations of macrophages (M1 or M2

phenotypes) exhibit differential inflammasome activation in response to stimuli [34], pinpointing that polarization is fundamental for shaping tumor microenvironment, which in turn is essential for the treatment outcome. Here, we found that *Salmonella* intratumoral administration induces a rapid phenotype shift of tumor-infiltrating macrophages towards classic M1, which together with the demonstration that macrophages are essential for the antitumor activity, suggests

that this event is responsible for the antitumor activity of *Salmonella*. Nevertheless, by day 7 after treatment the phenotype shift is lost, coinciding with a progressive decrease in inflammasome-related gene expression upon treatment, being tempting to speculate that all these findings explain why *Salmonella* antitumor effect is only transient. In turn, this reinforces the requirement for strategies that can boost and/or maintain this antitumor phenotype. In this regard, it has been described that forced activation of Notch signaling increases M1 macrophages enhancing their antitumor capacity [38], suggesting that a mixed strategy including *Salmonella* to induce M1 profile and Notch signaling agent to maintain phenotype could be more beneficial in the long term.

We also found that *Salmonella* activates inflammasomes in tumor-infiltrating macrophages and that macrophages, *Salmonella*-mediated CASP-1 activation, and NLRP3 engagement are all essential for antitumor activity. The expression of inflammasome-related CASP-1 is up-regulated in M1 but not in M2 cells particularly in NLRP3 inflammasome engagement [34], suggesting that *Salmonella* mediated NLRP3 assembly resulted in M1 polarization of tumor-infiltrating macrophages and that this cell is at the center of its antitumor activity. This bacteria-induced phenotype shift has already been reported in human macrophages infected in vitro with an obligatory anaerobic *Salmonella* Typhimurium strain [39]. In addition, an engineered FlaB-secreting *Salmonella* strain also proved to be effective in reducing tumor size and prolonging survival in a colon cancer model, inducing a reduction in the percentage of M2-type macrophages as well as an increase in M1-type macrophages [40], pinpointing the antitumoral role of the latter.

In macrophages, the activation of CASP-1 induced by *Salmonella* is mainly mediated by the NLR family member NLRC4 that senses cytosolic flagellin. It has been proposed that *Salmonella* first engages NLRC4 and subsequently NLRP3, maximizing IL-1 β and IL-18 production [9]. Nevertheless, the group of Tourlomousis recently showed that in absence of NLRC4, macrophages produce IL-1 β via NLRP3 and because these cells do not rapidly die, they secrete large amounts of IL-1 β between 6 and 24 h [41]. In this scenario NLRC4 restricts NLRP3 dependent production of inflammasome related proteins as IL-18 and IFN- γ [41], so not engaging NLRC4 would be an advantage. In line with this is the compelling recent finding that NLRC4 recognition suppresses, rather than facilitates, CD4+ T cell responses against *Salmonella* [41]. The authors suggest that flagellin modification could lead to stronger protective immunity to *Salmonella*, but in the context of our studies the lack of protective immunity could be a positive aspect to be further investigated. Our results here point out that regardless of the presence of flagellin, LVR01 is able to activate CASP-1 in melanoma cells (Fig. 5A) and to exert its antitumor effect

(Fig. 5B) so in this case, NLRC4 engagement would be neither beneficial nor detrimental for the immunotherapy outcome.

In summary, our results demonstrate that *Salmonella* antitumor activity in melanoma is dependent on tumor-infiltrating M1 macrophages and CASP-1/11 inflammasome activation. This data may inform the development of new strains with improved antitumor effectiveness and will help to move forward the application of *Salmonella*-based immunotherapies in clinical setup.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00262-022-03148-x>.

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