



## Salmonella-induced immune response reduces recurrence and tumor dissemination in preclinical melanoma model

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### ABSTRACT

Localized melanoma is easy to remove by surgery, resulting in a high five-year relative survival rate. However, when disseminated the disease management is challenging. The use of immunotherapies, such as anti-checkpoint monoclonal antibodies, has improved treatment options but still only a small percentage of patients responds to these expensive treatments. In this work, we apply a bacteria-based immunotherapy using LVR01, an attenuated *Salmonella enterica* serovar Typhimurium, as neoadjuvant therapy one week before surgery in a preclinical disseminated murine melanoma model. LVR01 administration resulted in tumor growth retardation prior to tumor resection, due to a rapid upregulation of inflammatory genes in the tumor microenvironment. As a consequence, cell infiltration increased, particularly neutrophils, macrophages and NK cells, being the latter involved in *Salmonella* anti-tumor activity. Besides, tumor-draining lymph node infiltration is characterized by reinvigorated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes. Induced immune response could account for the prevention or delay of tumor recurrence and appearance of metastasis, resulting in a prolonged overall survival after surgery. Furthermore, upon rechallenge mice show partial protection, suggesting the existence of specific memory against melanoma. We propose that neoadjuvant LVR01 treatment could represent an interesting inexpensive alternative that may ease tumor resection, while preventing tumor recurrence in patients with melanoma.

### 1. Introduction

Melanoma is the most lethal type of skin cancer with an increasing incidence in young population worldwide (Matthews et al., 2018). The use of BRAF and MEK inhibitors has shown some benefit, but its use is restricted to approximately half of the patients who carry BRAF mutations (Chapman et al., 2011; Grimaldi et al., 2017). In the last years, new therapies have been approved for the management of advanced disease, as is the case of antibodies against immune checkpoints CTLA-4, PD-1 and PD-L1, alone or combined (Hodi et al., 2010; Robert et al., 2014; Gutzmer et al., 2020). However, these approaches present major side effects, induce resistance to treatment and exhibit escalating costs, making them available for a reduced number of patients, mainly from high income countries (reviewed in (Shah and Dronca, 2014)). The recent FDA approval of the first microbe-based therapy for advanced melanoma patients, Talimogene Laherparepvec (Andtbacka et al., 2015), while also expensive, has paved the road for new

immunotherapies based on the use of live attenuated agents, as virus or bacteria.

New strategies promoting tumor cell death and/or inducing protective host antitumor immunity are of high priority. In this regard, *Salmonella*-based immunotherapies meet both conditions. *Salmonella* displays tumor tropism (Forbes et al., 2003), and once it reaches the tumor microenvironment, it can invade and replicate within tumor cells, leading to cell death (Mi et al., 2019). Besides, *Salmonella* triggers inflammation (reviewed in (Zhou et al., 2018)), promoting the development of antitumor immunity. Indeed, *Salmonella* has been shown to be an effective immunotherapy in many cancer models (Hernandez-Luna and Luria-Perez, 2018) and safe, even in oncologic patients (Cunningham and Nemunaitis, 2001; Toso et al., 2002).

We have previously developed LVR01, an attenuated *Salmonella enterica* serovar Typhimurium, that showed to be safe as vaccine vector in many animal models (Chabalgoity et al., 2000; Petavy et al., 2008; Goni et al., 2015; Grille et al., 2014; Kramer et al., 2015; Bascuas et al.,

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2018; Vola et al., 2018; Chilibröste et al., 2021; Mónaco et al., 2022). Besides, it holds great potential as a vaccine vector and tool for cancer immunotherapies (Chabalgoity et al., 2000). Using that strain, we demonstrated that administered intratumorally, it induces inflammatory activation in both tumor cells and infiltrating macrophages, and that this phenomenon is central for the exertion of the antitumor effect (Mónaco et al., 2022). Furthermore, intratumoral injection of LVR01 together with topical administration of the TLR7 agonist imiquimod retards tumor growth and dissemination and prolongs overall survival through the induction of the expression of pro-inflammatory Th1 cytokines and chemokines in the tumor microenvironment (TME), that resulted in a broad antitumor response in local and distant masses (Vola et al., 2018). Moreover, when using neoadjuvant *Salmonella* combined with intraperitoneal dacarbazine (DTIC) chemotherapy we found a strong synergistic anti-tumor effect, driven by combination of the DTIC-induced reduction of secondary lymphoid organ cellularity and the activation of cytotoxic cell compartments (Chilibröste et al., 2021). Analogously, lymphoma-bearing mice undergoing CHOP chemotherapy benefit from LVR01 administration, resulting in prolonged overall survival and an improved health status (Bascuas et al., 2018). These results highlight the potential of *Salmonella* as immunotherapy to control low tumor burden. In this work, we evaluated the benefit of LVR01 administration prior to surgery in melanoma-bearing mice and sought to explain the mechanisms involved in this prolonged antitumor effect.

## 2. Material and methods

### 2.1. Tumor cell lines

B16F1 and B16F10 melanoma cells were purchased from American Type Culture Collection (ATCC, CRL-6323 and CRL-6475, respectively) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO<sub>2</sub> atmosphere.

### 2.2. Bacterial strain

*Salmonella enterica* serovar Typhimurium LVR01, an attenuated strain constructed by introducing a null deletion into the *aroC* gene of the parental canine *S. Typhimurium* isolate P228067 (Chabalgoity et al., 2000) was used in this study. Bacteria were grown at 37 °C in Luria-Bertani (LB) media shaking at 200 rpm ON and stored at –80 °C in 15% glycerol stocks until used. For *in vivo* experiments, bacteria were thawed and resuspended in saline solution to a final concentration of  $1 \times 10^7$  CFU/ml. Bacterial inoculum was confirmed by serial dilution and plating in LB agar.

### 2.3. Animal experiments

Female C57BL/6 mice (Dilave, Uruguay), 6–8 weeks old, were used for *in vivo* experiments. Animals were housed on 12:12 h light/dark cycles with controlled temperature ( $22 \pm 2$  °C) and humidity (60%), with water and food *ad libitum*. All animal experimentation protocols were approved by the University's Ethical Committee for Animal Experimentation, Uruguay.

#### 2.3.1. Neoadjuvant and adjuvant setting

*Salmonella* LVR01 neoadjuvant melanoma model was performed as previously described (Vola et al., 2018). Briefly, C57BL/6 mice were intradermally inoculated with  $1 \times 10^5$  melanoma cells in the right flank, giving rise to only one primary tumor per mice. When tumors were palpable (day 11 post-tumor inoculation, pti), mice were divided into 2 groups: control (treated with 100 µl of saline solution) and LVR01. Animals were treated with one single dose of intratumoral (i.t.) LVR01 ( $1 \times 10^6$  CFU). Primary tumor resection was performed on day 18–19 pti, and relapse and/or occurrence of metastasis were followed up for 90–100 days pti. Affected lymph nodes and tumor relapse were

measured with a caliper and volume was calculated as: (length x width x depth) x  $\pi/6$ . Mice were sacrificed by cervical dislocation when tumor volume exceeded 4000 mm<sup>3</sup> or before if they showed any sign of distress. Disease-free survival (DFS) corresponds to the day that tumor recurrence was detected for the first time, while overall survival (OS) corresponds to the day that mice died.

Rechallenge was performed on the opposite flank (left flank) of tumor-free surviving mice, no further treatment received. Animals were followed up as described above.

*Salmonella* LVR01 adjuvant melanoma model was performed as a modification of the above described neoadjuvant model. B16F1 melanoma was implanted on day 0 and primary tumor resection was performed on day 18–19 pti, without receiving any previous treatment. Nine days after surgery, animals were divided into 2 groups, control and LVR01, and were treated with a weekly LVR01 injection ( $1 \times 10^6$  CFU) for the next 4 weeks within the tumor if it was palpable or otherwise subcutaneously in the same area. Affected lymph nodes and tumor relapse were measured with a caliper and sizes were calculated using the above-mentioned formula.

#### 2.3.2. In vivo melanoma tumor models and treatment

C57BL/6 mice were subcutaneously inoculated with  $2.5 \times 10^5$  B16F1 melanoma cells. Animals were divided into 2 or 3 groups: control, *Salmonella* LVR01 and *Salmonella* plus depletion when needed. At day 11 pti, *Salmonella* ( $1 \times 10^6$  CFU) were intratumorally injected. Mice were followed up every 2–3 days, and tumor sizes were calculated using the above-mentioned formula.

#### 2.3.3. Depletion regimes

C57BL/6 mice were subcutaneously inoculated with  $2.5 \times 10^5$  B16F1 melanoma cells as stated above. Animals were divided into 3 groups: control, LVR01 and depletion + LVR01. At day 11 pti, *Salmonella* ( $1 \times 10^6$  CFU) were intratumorally injected. Mice were followed up as described above. Rat  $\alpha$ -CD8 (clone 2.43) and  $\alpha$ -Gr1 (clone RB6-8C5), and mouse  $\alpha$ -NK1.1 (clone PK136) antibodies were purified by affinity chromatography (Hi-trap G protein column) from hybridoma supernatants and amount of each was quantified using Bradford protein assay. All protocols were based on previous reports (Stern et al., 2015; Cook and Whitmire, 2013; Westphal et al., 2008) and adapted to our model. For CD8 depletion, a single dose of 100 µg of antibody was administered, for NK cell depletion two consecutive daily doses of 75 µg and for the depletion of neutrophils, three successive daily doses of 100 µg. In every case, the depletion was performed by intraperitoneal injection and started one day prior to LVR01 treatment. Depletions were followed up by flow cytometry using PBMC from blood samples taken overtime.

#### 2.3.4. Gene expression analysis

In independent experiments, which did not include surgery, mice were sacrificed at day 3 or 8 post LVR01-treatment (days 14 or 19 pti) and tumors were removed, collected in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at –80 °C until processed as previously described (Vola et al., 2018). Following retrotranscription, quantitative RT-PCR for *Ifng*, *Tnfa*, *Il6*, *Il12*, *Il18*, *Cxcl1*, *Cxcl2*, *Cxcl9*, *Cxcl10*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5* and *Ccl20* mRNA were conducted using QuantiTect® SYBR® Green PCR Kit (Qiagen) in a Rotor-Gene 6000 (Corbett), primer sequences are available under request. This panel was based in our previous work, with modifications (Chilibröste et al., 2021). GAPDH encoding gene *Gapdh* was used as house-keeping gene. The relative mRNA amount in each sample was calculated using the  $2^{-\Delta\Delta Ct}$  method as previously described (Livak and Schmittgen, 2001) where  $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{gapdh}}$ , and expressed as relative mRNA levels in the test groups compared to the control group.

#### 2.3.5. Absolute numbers

In independent experiments, which did not include surgery, tumor-bearing mice were sacrificed at day 16 post LVR01-treatment (day 27

pti) and tumor draining lymph nodes (TDLN) were removed to prepare a single-cell suspension. Cell numbers were determined using Cellometer K2 automatic cell counter (Nexcelom Bioscience LLC, Lawrence, MA, USA).

### 2.3.6. Flow cytometry analysis

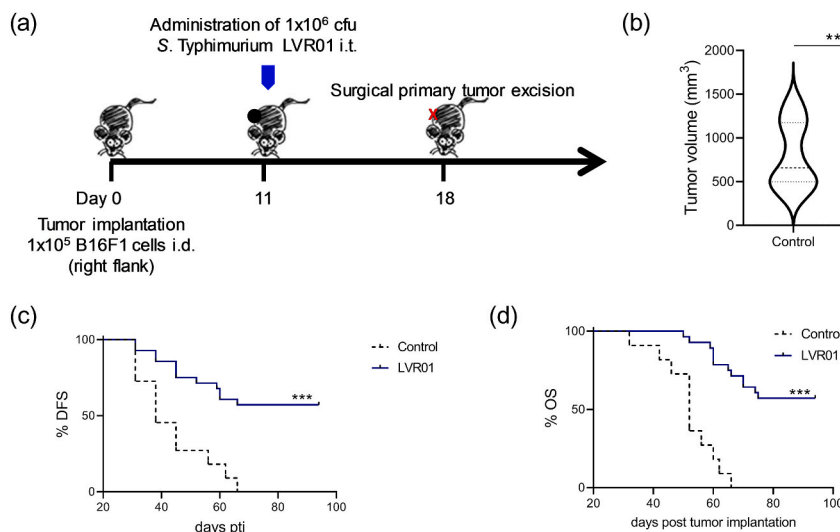
In independent experiments, which did not include surgery, mice were sacrificed at day 3, 8 or 16 post LVR01-treatment (days 14, 19 or 27 pti) and tumors or TDLN were removed and prepared to obtain single-cell suspensions. Cells were immunostained at 4 °C in the dark for 30 min with antibodies against B220, CD3, CD4, CD8, CD11b, CD11c, CD19, CD27, CD44, CD49b, CD62L, CD69, CD127, CD152 (CTLA-4), CD197 (CCR7), CD279 (PD-1), F4/80, Ly6G, MHCII, NK1.1 and NKG2D (all reagents from BD Pharmingen, San Diego, CA, USA). Absolute cell numbers were obtained using CountBright absolute counting beads (Invitrogen), according to manufacturer's instructions. Flow cytometry data were acquired on a FACS Canto II cytometer and analysed using FACS Diva software (BD Biosciences, Oxford, UK). Flow cytometry gating strategies are illustrated in [Supplementary Fig. 1](#).

### 2.3.7. IFN- $\gamma$ measurement

In independent experiments, which did not include surgery, tumor-bearing mice were sacrificed at day 16 post LVR01-treatment (day 27 pti). Spleens were collected and splenocytes purified by erythrocyte lysis. Cells were counted and seeded in 96-well plates along with B16F1 cells at a ratio of 100:1 (re-exposure). After 18 h, supernatants were collected and stored at -80 °C until analysed. IFN- $\gamma$  determination was performed by ELISA using a commercial pair of antibodies (capture ab Cat. No. 551216; recombinant mouse IFN- $\gamma$  protein Cat. No. 554587 as the standard and detection ab Cat. No. 554410, all from BD), according to the manufacturer's instructions.

## 2.4. Statistical analysis

The statistical significances of differences between groups were analysed using Student's t-test, 2-way ANOVA or Tukey's multiple comparisons test. A value of  $p < 0.05$  was considered statistically significant. Differences in survival times were determined using Kaplan–Meier and log-rank test.



## 3. Results

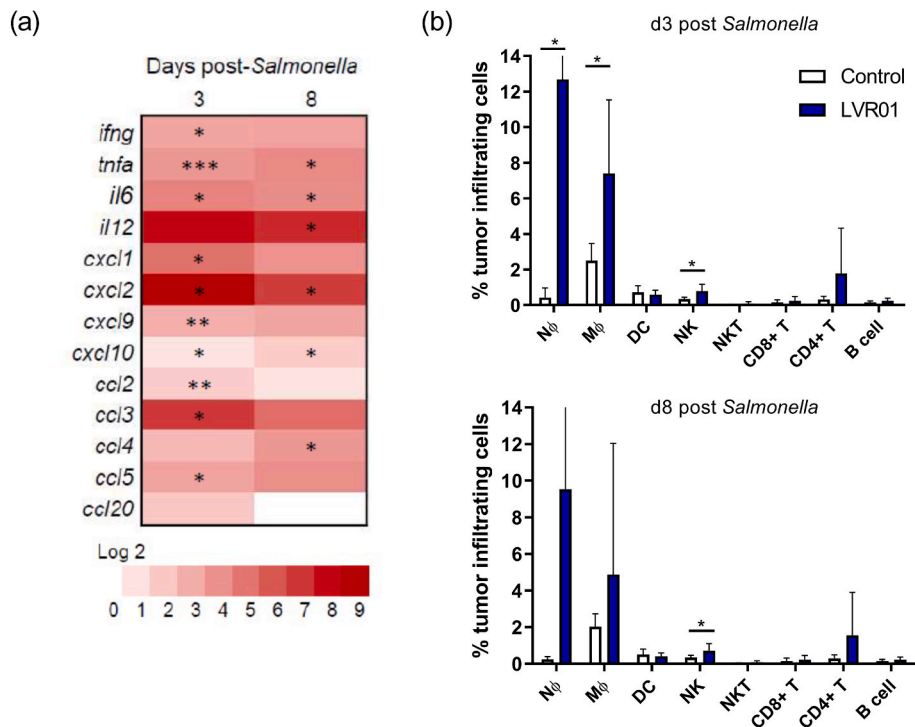
### 3.1. *Salmonella* neoadjuvant treatment improves surgery outcome

As we had previously described, intratumoral *Salmonella* LVR01 monotherapy resulted in tumor growth retardation and prolonged survival in a melanoma-bearing mice model (Vola et al., 2018; Chilibroste et al., 2021; Mónaco et al., 2022). In this work we studied the potential of *Salmonella* immunotherapy in a low burden tumor model, achieved by surgery to remove primary tumors. B16F1 melanoma-bearing mice were treated with one single dose of intratumoral *Salmonella* LVR01 a week before exeresis of primary tumor (Fig. 1a). Consistently with our previously reported results, a marked difference in tumor burden was found between *Salmonella*-treated and control (mock-treated) mice at the time of surgery ( $p = 0.0013$ , Student's T test) (Fig. 1b). Upon surgery, animals treated with LVR01 exhibited an improved outcome, determined by a prolonged disease-free survival ( $p < 0.0001$ , Log-rank test) (Fig. 1c) and overall survival ( $p < 0.0001$ , Log-rank test) (Fig. 1d). Bacteria-based neoadjuvant treatment helped to protect from disease recurrence or metastasis, and hence death, in approximately half of the animals (Fig. 1d). The observed effect could be due to tumor growth restriction and therefore impairment of metastasis occurrence, or to metastatic cell elimination by the immune response elicited by LVR01 treatment, which allows disease control. Of note, this effect is not only restricted to low metastatic potential cells, as similar results were observed using B16F10 highly metastatic cell line (Supplementary Fig. 2).

### 3.2. LVR01 treatment induces broad local and systemic immune responses

Thus, we analysed the immune response elicited by *Salmonella* immunotherapy which could be contributing to the elimination of residual cells. Firstly, we deepened into the kinetics of cytokines and chemokines expression over time upon treatment. *Salmonella* rapidly induced a strong proinflammatory tumor microenvironment, characterized by upregulated expression of many chemokine and cytokine mRNAs, including *Ifng*, *Tnfa*, *Il6*, *Il18*, *Cxcl1*, *Cxcl2*, *Cxcl9*, *Cxcl10*, *Ccl2*, *Ccl3* and *Ccl5*, which slowly dissipated by day 8 post-*Salmonella* administration (day 19 pti, day of surgery) (Fig. 2a). Besides, an increase in immune cell recruitment to the tumor, mainly neutrophils and macrophages but also natural killer (NK) soon after treatment ( $p = 0.0292$ ,  $p = 0.0277$  and  $p = 0.0151$ , respectively, Student T test) was found at an early time point (Fig. 2b, top). However, this rapid infiltration disappeared as soon as day 8 post-*Salmonella* administration, being NK the

**Fig. 1.** Tumor size, free of disease and overall survival of B16F1 melanoma bearing mice treated with LVR01 prior to surgery. (a) Schematic representation of treatment schedule. Mice were treated with  $1 \times 10^6$  cfu LVR01 i.t. as described in M&M. (b) Tumor size at day 18, prior to surgery. Volume was calculated as  $(L \times W \times D) \times \pi / 6$  and results are shown as median and quartiles. \*\* $p < 0.01$  Student's T test. (c) Reemergence of the disease is plotted as disease-free survival (DFS) curve. Then tumor size was measured every 2–3 days. (d) Animal survival is plotted as overall survival (OS) curve. Survival was followed up for 100 days, \*\*\* $p < 0.001$ , Log-rank Test (Control group  $n = 12$ , LVR01 group  $n = 30$ ).



**Fig. 2. Tumor setting at days 3 or 8 induced by LVR01 neoadjuvant intralesional administration in mice prior to surgery.** (a) Cytokine/chemokine profile in LVR01-treated mice, normalized against control untreated mice, expressed as mean Log<sub>2</sub> (n = 5–6) (b) Tumor immune cell phenotypification. Tumor homogenates were stained for detecting different tumor infiltrating populations of granulocytes and lymphocytes. Gating strategies are shown in Suppl. Fig. 1. All results are shown as mean ± SD (n = 5–11). Student's T test, \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001.

only analysed cell population which remained higher in LVR01-treated mice at the time of surgery ( $p = 0.0151$ , Student's T test) (Fig. 2b, bottom). Noteworthy, this low, yet significant, percentage of NK cells is in accordance with previous reports (Bascuas et al., 2018).

As adaptive immune response takes longer time to develop, we studied it in lymphoid tissues at a later time point (16 days post-LVR01 treatment). For that, mice did not undergo surgery. LVR01-treated tumor draining lymph nodes (TDLN) exhibited a broad activation profile, characterized by expansion of lymphocytic populations. Significantly higher absolute numbers of NK, NKT, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were counted ( $p < 0.0001$  in all cases, Student T test) (Fig. 3a). Furthermore, a marked reduction in CTLA-4 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $p = 0.0072$  and  $p = 0.0047$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, Student T test), as well as PD-1 expression reduction on CD8<sup>+</sup> T cells ( $p = 0.0228$ , Student T test) was observed (Fig. 3b), suggesting a reinvigoration in T cell function. Of note, this decrease is not due to higher naive or recently activated T cell recruitment, since the percentages of these cells remained unchanged (data not shown). In addition, LVR01 treatment expanded effector T cells ( $p < 0.001$  in all cases, Student's T test) (Fig. 3c) and effector memory cell subpopulations within both CD4<sup>+</sup> and CD8<sup>+</sup> T cell population ( $p < 0.001$  and  $p = 0.0046$ , respectively, Student's T test) (Fig. 3d). Moreover, splenocytes from LVR01-treated mice released higher levels of IFN- $\gamma$  upon re-exposure to tumor cells ( $p = 0.0305$ , Student T test) (Fig. 3e).

### 3.3. NK cells participate in the antitumor effect exerted by Salmonella

Due to broad immune activation, we sought to evaluate the individual contribution of different cell types, namely neutrophils and cytotoxic NK and CD8<sup>+</sup> T lymphocytes, in *Salmonella*-mediated anti-tumor effect. Total CD8<sup>+</sup> T cell depletion did not alter the outcome of *Salmonella* treatment (Fig. 4a). Likewise, transient neutrophil depletion did not impact on tumor growth retardation upon LVR01 administration (Fig. 4b). Nonetheless, partial NK cell depletion abrogated early tumor growth control induced by LVR01 ( $p = 0.0252$ , Tukey's multiple comparisons test) (Fig. 4c), suggesting a role in *Salmonella*-mediated anti-tumor response.

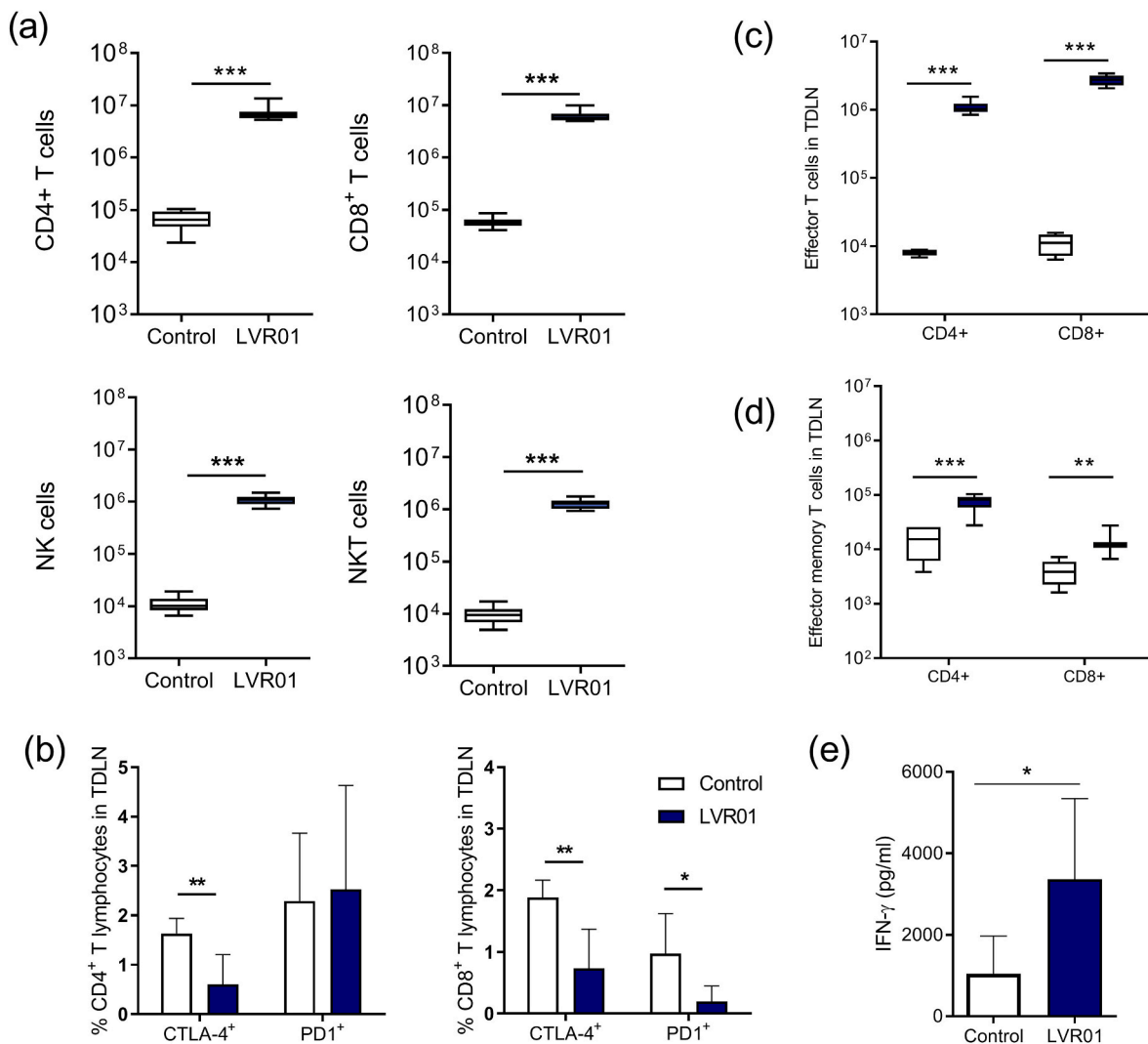
### 3.4. Salmonella treatment induces tumor-specific memory response that contributes to tumor control upon rechallenge

In order to evaluate the potential of *Salmonella*-induced immune response to eliminate tumor cells, we rechallenged mice that survived to primary melanoma after *Salmonella* treatment followed by surgery (tumor-free mice from Fig. 1d). For that, we injected the same melanoma cell line in the opposite flank at day 76 post-primary tumor resection and compared disease development to that in naïve mice (Fig. 5a). Tumor growth was slower in mice that were previously treated with LVR01 compared to naïve mice ( $p < 0.0001$ , two-way ANOVA) (Fig. 5b) and, as a consequence, overall survival was extended ( $p < 0.0001$ , Log-rank test) (Fig. 5c). Noteworthy, a few animals did not develop tumors, suggesting the presence of a tumor-specific memory immune response capable of eliminating melanoma cells (Fig. 5c).

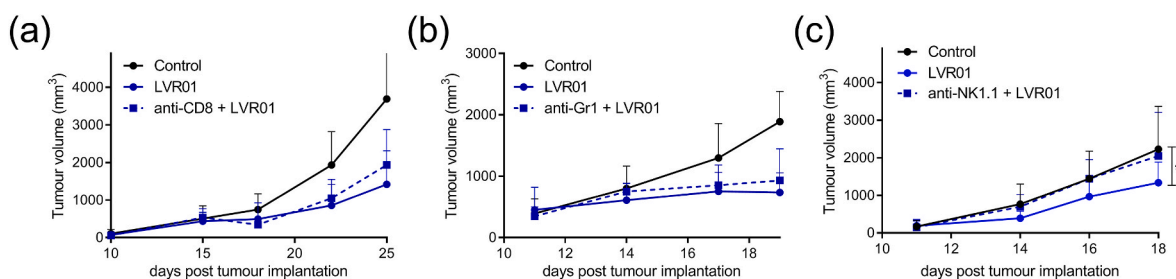
*Salmonella* LVR01 anti-tumor potential is not restricted to neoadjuvant application. We treated mice that underwent surgery for primary tumor removal with weekly LVR01 injection (Fig. 6a). Treatment resulted in tumor growth delay ( $p < 0.0001$ , Two-way ANOVA) (Fig. 6b), and subsequently prolonged overall survival ( $p < 0.0001$ , Log-rank test) (Fig. 6c). Yet, this approach did not provide complete protection, as all animals eventually developed disease.

## 4. Discussion

Microbe-based immunotherapies have been extensively studied for cancer treatment, and recently regained more attention (Forbes et al., 2018). However, to date only two of these therapies have been approved. Firstly, the use of BCG for superficial bladder cancer that is still in use and has many decades of significant results (reviewed in (Gontero et al., 2010)) and more recently, talimogene laherparepvec for advanced melanoma (Andtbacka et al., 2015). Due to *Salmonella* characteristics, i.e. tumor tropism, intrinsic antitumor activity and broad immune response induction, it is one of the most attractive bacteria to be used as a microbe-based cancer immunotherapy (reviewed in (Moreno et al., 2010)). To date, it has been evaluated in different tumor models, with optimistic results. Within TME, *Salmonella* elicits activation of



**Fig. 3. Immune profile induced by LVR01 in secondary lymphoid organs at day 16 after *Salmonella* treatment.** TDLN homogenates were stained for detecting different lymphocyte subpopulations. (a) Counts of lymphocytes are expressed as absolute numbers. (b) CTLA-4 and PD-1 expression on CD4<sup>+</sup> T and CD8<sup>+</sup> T cells. (c) Effector and (d) effector memory CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts. (e) Secreted IFN $\gamma$  levels by B16F1 re-stimulated splenocytes, measured by ELISA. All gating strategies are available in Suppl Fig. 1. Results in (a), (c) and (d) are shown as box and whiskers (min to max), while (b) and (e) are shown as mean  $\pm$  SD (n = 5–6). Student’s T test, \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001.

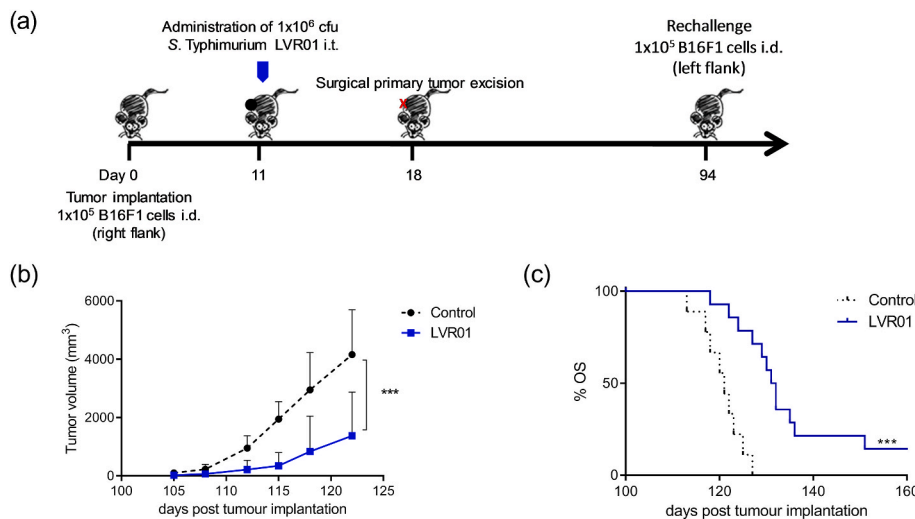


**Fig. 4. Relevance of different cell types in tumor control induced by LVR01.** Tumor growth after *Salmonella*-treated melanoma-bearing mice depleted of (a) CD8<sup>+</sup> T cells, (b) Neutrophils, and (c) NK cells. Gating strategies and depletion followup are shown in Suppl. Fig. 3. All results are shown as mean  $\pm$  SD (n = 11–12). Student’s T test, \**p* < 0.05.

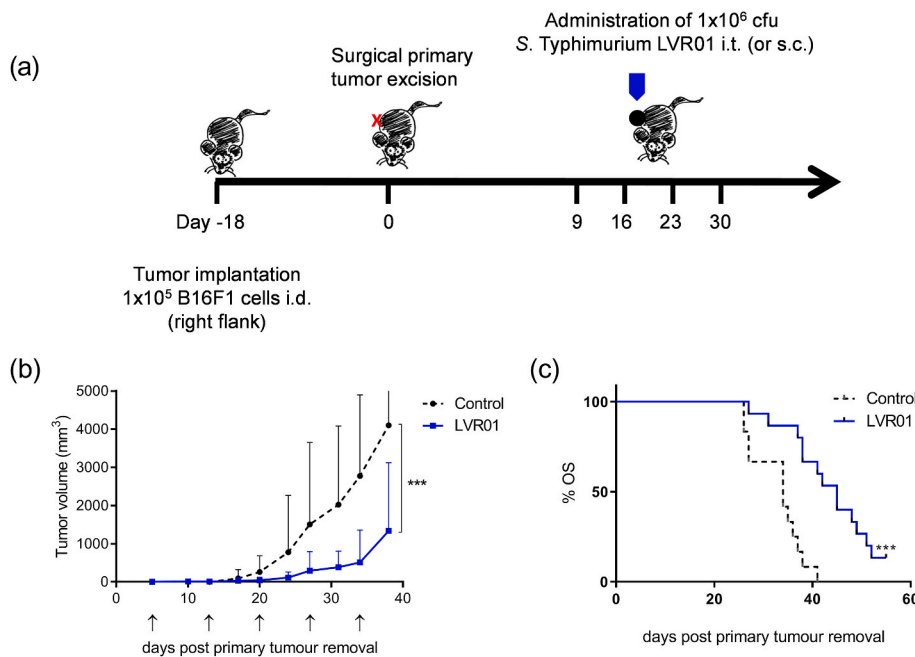
immune responses through different mechanisms: by activation of macrophages -evidenced by the enhanced expression of soluble mediators (e.g. inducible nitric oxide synthase and IFN- $\gamma$ )-, M2/M1 shift, inhibition of the expression of immunosuppressive factors (e.g. arginase-1, interleukin-4, transforming growth factor- $\beta$  and vascular endothelial growth factor), and activation of different pathways of cell death, such

as apoptosis, autophagy and pyroptosis (Mónaco et al., 2022; Kaimala et al., 2014; Ganai et al., 2011; Lee et al., 2014). Besides, released tumor antigens in this inflammatory setting are captured and presented by dendritic cells, triggering a specific antitumor response (Saccheri et al., 2010).

We have recently shown the potential of neoadjuvant *Salmonella*



**Fig. 5. Tumor size and overall survival of B16F1 melanoma bearing mice treated with neoadjuvant LVR01, after rechallenge.** (a) Schematic representation of treatment schedule. Mice were treated with  $1 \times 10^6$  cfu LVR01 i.t., tumors were excised a week later and 76 days later surviving mice were rechallenged subcutaneously in the opposite flank with  $1 \times 10^5$  B16F1 cells. (b) Tumor growth curve, expressed as days post primary tumor implantation. Tumor size was measured every 2–3 days and volume was calculated as  $(L \times W \times D) \times \pi / 6$ . Results are shown as mean  $\pm$  SD ( $n = 12$ – $16$ ) \*\*\* $p < 0.001$ , Student's T test. (c) Survival was followed up for 160 days \*\*\* $p < 0.001$ , Log-rank Test.



**Fig. 6. Tumor size and overall survival of B16F1 melanoma bearing mice treated with LVR01 post surgery.** (a) Schematic representation of treatment schedule. 18 days post tumor implantation, tumors were excised and starting 9 days later mice were treated weakly with  $1 \times 10^6$  cfu LVR01 i.t., or s.c. when tumors were not palpable. (b) Tumor growth curve, expressed as days post primary tumor removal. Tumor size was measured every 2–3 days and volume was calculated as  $(L \times W \times D) \times \pi / 6$ . Results are shown as mean  $\pm$  SD ( $n = 12$ ) \*\*\* $p < 0.001$ , two-way ANOVA. (c) Survival was followed up for 60 days after primary tumor removal \*\*\* $p < 0.001$ , Log-rank Test.

treatment in animals undergoing chemotherapy, highlighting the relevance of low tumor burden for *Salmonella* success (Bascuas et al., 2018; Chilibroste et al., 2021). In line with this, neoadjuvant LVR01 prolongs animal survival upon surgery to remove high tumor burden in a metastatic 4T1 breast cancer model (Kramer et al., 2015). In this work we assessed the potential of *Salmonella*-induced antitumor immune response in a minimal residual disease melanoma model to prevent tumor relapse and metastasis occurrence. This approach resulted in more than 50% complete recovery.

It is known that the immune system and cancer are intimately related, the immune system controls and shapes tumor cells immunogenicity, while tumor cells condition TME to impair anti-tumor immunity (Mittal et al., 2014). Immunotherapies can help to generate neoantigens, stimulate recruitment and activation of anti-tumor immune cells and override immunosuppression (Ward et al., 2016; Guerrouahen et al., 2020). Likewise, *Salmonella* induces local and systemic antitumor immunity as it mediates tumor cell death in a pro-inflammatory microenvironment, triggering a short-term immune

cell tumor infiltration and an long-term adaptive immune response (reviewed in (Pangilinan and Lee, 2021)).

In the early stage of the disease, tumor growth is controlled by innate effectors, such as NK cells and macrophages, recruited to the TME upon *Salmonella* administration. We have previously shown that macrophages recruited to the tumor site, along with tumor cells, exhibit inflammasome activation upon LVR01 treatment. Besides, both macrophages and caspase-1/11 axis proved to be central to *Salmonella* antitumor effect (Mónaco et al., 2022). In addition Kupz et al have pinpointed the NLRP4 inflammasome essentiality to the *in vivo* IFN- $\gamma$  secretion by NK cells in response to *Salmonella* (Kupz et al., 2014), providing a possible link between NK and macrophages in the context of *Salmonella*-based immunotherapy success.

We have shown that NK cells are important to control tumor growth at an early stage. In addition, we observed upregulation of *Ifng* gene expression in the TME, as well as IFN- $\gamma$  increased secretion by LVR01-treated mice' splenocytes re-exposed to tumor cells. Our results also showed that both the neoadjuvant or adjuvant treatment improved

tumor outcome to surgery, which could be indicating that the immunotherapy is successful at suppressing metastasis development. This is in line with what Lin and collaborators have recently shown, that NK cells are the main cell type involved in *Salmonella*-elicited control of metastatic cells through the secretion of IFN- $\gamma$ , which at the same time shapes NK cells to eliminate cancer cells, in a positive feedback loop (Lin et al., 2021).

We have also shown here by systemically depleting Gr1 cells that neutrophils are not essential for the antitumor effect exerted by immunotherapy. This is in accordance with our previous results in which besides inducing an increase of Th17-associated gene expression, *Salmonella* immunotherapy benefited both wild type and IL17a knock-out melanoma bearing mice (Chilibroste et al., 2021). It has also been shown that neutrophils are dispensable for preventing metastasis by *Salmonella* treatment in the 4T1 breast cancer model (Lin et al., 2021). However, a few works demonstrate different neutrophils-mediated pro-tumor mechanisms. Neutrophils represent a ring-barrier that contains *Salmonella* within the necrotic area, restraining its antitumor activity (Westphal et al., 2008). In addition, *Salmonella*-induced neutrophil extracellular trap release facilitates tumor cell dissemination (Coolis-Lartigue et al., 2013; Najmeh et al., 2017). Neutrophils are a very heterogeneous cell population (Fridlender et al., 2009) and actually some of them, namely N2 neutrophils, are indeed immunosuppressive and associated with cancer progression (Masucci et al., 2019). In addition, the depletion regime consisting of anti-Gr1 antibodies could also be depleting other Gr1+ cells, such as myeloid-derived suppressor cells (MDSC), which are also known for their pro-tumoral potential (Yang et al., 2010). Hence, Gr1+ cell-depletion of both N2 and MDSC could be masking the therapeutic efficacy of anti-tumor N1 neutrophils.

The role of CD8+ T cells has shown to be non-essential in early stages of disease in our model. Nevertheless, as adaptive immunity takes time to develop, CD8+ T cells could be relevant in latter stages. Upon LVR01 treatment, CD8+ T cells reinvigorate, as demonstrated by expansion of effector subpopulations, and downregulation of CTLA-4, as well as PD-1 expression. Besides, CD8+ T resident memory (Trm) cells, which are relevant in the skin scenario because they persist as alarm sensors long after initial threat, are not sensitive to depletion (Galvez-Cancino et al., 2018). In this way, Trm may remain to exert their antitumor potential. Melanoma-specific Trm cells may also act upon rechallenge, steadily killing tumor cells by the production of IFN- $\gamma$ , TNF- $\alpha$ , granzyme B and proinflammatory cytokines (Cheuk et al., 2017; Ariotti et al., 2014). However, in our hands LVR01 administration does not induce Trm (data not shown), but instead triggers T effector memory cell expansion in TDLN, which remains after surgery. This subpopulation could turn into T central memory cells (Martin and Badovinac, 2018) that may rapidly differentiate to Trm following tumor challenge (Enamorado et al., 2017).

It has been reported that *Salmonella*-mediated downregulation of tumor PD-L1 contributes to the reactivation of tumor-specific T cells (Chen et al., 2019). We have shown that LVR01 induces downregulation of PD-1 expression in CD8+ T lymphocytes. In this way, *Salmonella* controls both sides of the immune synapse, releasing the brakes that allow an increased cytotoxic response.

In addition to *Salmonella*-induced T cell reinvigoration, we observed the generation of memory cells upon LVR01 treatment. At this time, tumor escapes immune-mediated control, and tumor mass is no longer manageable by the immune system. Surgery provides for a window of time of action that allows the adapted immune system to eliminate minimal residual cells. Indeed, LVR01 treatment before surgery induces a tumor-specific immune response that controls tumor establishment and growth as evidenced by tumor rechallenge long after surgery.

To sum up, intratumoral *Salmonella* administration prior to surgery represents an interesting strategy for the treatment of melanoma. Primary lesion injection helps to control tumor growth that allows a better resection outcome and besides, the induction of systemic tumor-specific immunity constrains distant metastasis. Of note, intralesional approach

is already used in the clinic with Talimogene laherparepvec for advanced melanoma patients.

## 5. Conclusions

LVR01-induced immune response impacts tumor control in two levels. Firstly, LVR01 generates a proinflammatory tumor microenvironment characterized by immune cell recruitment, which restrains tumor growth and impairs the occurrence of metastasis. Secondly, LVR01 induces a tumor-specific immune response that helps to clear residual tumor cells, partially preventing relapse.

In conclusion, neoadjuvant LVR01 administration could be considered as a promising alternative treatment for advanced melanoma patients, by diminishing the occurrence of tumor metastasis upon primary surgery.

## Institutional review board statement

The study was conducted according to the guidelines approved by the Comisión Honoraria de Experimentación Animal (CHEA), the Ethics Committee of Universidad de la República (protocol approval file N° 070153-000446-18, approved on August 29, 2018).

## CRedit authorship contribution statement

**Amy Mónaco:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Preparation, Writing – review & editing. **María C. Plata:** Formal analysis, Investigation, Methodology, Writing – original draft, Preparation, Writing – review & editing. **Sofía Chilibroste:** Formal analysis, Investigation, Methodology. **Magdalena Vola:** Funding acquisition, Methodology. **Jose A. Chabalgoity:** Formal analysis, Funding acquisition, Writing – review & editing. **María Moreno:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Preparation, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crimmu.2022.08.001>.

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