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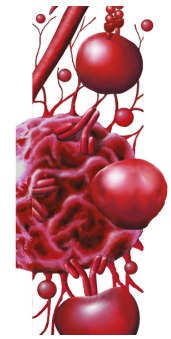
Immunotherapy



TLR7 agonist in combination with *Salmonella* as an effective antimelanoma immunotherapy

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Aim: We evaluated a novel approach combining the use of attenuated *Salmonella* immunotherapy with a Toll-like receptor agonist, imiquimod, in B16F1 melanoma-bearing mice. **Materials & methods:** B16F1 melanoma-bearing mice were daily treated with topical imiquimod in combination with one intratumoral injection of attenuated *Salmonella enterica* serovar Typhimurium LVR01. **Results:** The combined therapy resulted in retarded tumor growth and prolonged survival. Combination treatment led to an enhancement in the expression of pro-inflammatory cytokines and chemokines in the tumor microenvironment, with a Th1-skewed profile, resulting in a broad antitumor response. The induced immunity was effective in controlling the occurrence of metastasis. **Conclusion:** *Salmonella* LVR01 immunotherapy in combination with imiquimod is a novel approach that could be considered as an effective antimelanoma therapy.

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Keywords: experimental model • imiquimod • immunotherapy • melanoma • *Salmonella*

Melanoma is a severe form of skin cancer with increasing incidence [1]. Although it can be cured with surgery at early stages, the median survival rate when it becomes metastatic is 6.2 months with limited treatment options [2].

For many years, dacarbazine and IL-2 were the only approved drugs for treatment of metastasic melanoma. However, in the last few years, this scenario changed with the introduction of two types of new therapies: antibodies against immune checkpoints, such as CTLA-4 and PD-1, and molecular inhibitors of *BRAF* mutations [3]. Nevertheless, the increase in the median survival rate secondary to the use of these new therapies comes along with major side effects, highly elevated costs and induction of resistance to treatment [4–9].

Since the end of the 19th century, when William Coley tested the so-called 'Coley Toxin's' [10], bacteria have been proposed as an alternative for the treatment for cancer. The use of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has shown great potential as an antitumor immunotherapy thanks to its capacity to accumulate in tumors, intrinsic antitumor activity and ability to trigger an immune response in the tumor microenvironment [11]. Particularly, we have shown that *Salmonella*-based immunotherapies are suitable alternatives for the treatment of melanoma [12]. We have previously described the construction and evaluation of the attenuated strain *S.* Typhimurium LVR01, with excellent safety profile in mouse and others animals [13–15], and effective antitumor activity on B-cell lymphomas [16].

Imiquimod, an imidazoquinoline amine, is a TLR-7 agonist with potent antiviral and antitumor activity, mediated by plasmocytoid dendritic cells (pDC) [17]. It is approved by the US FDA for the use on actinic keratosis, viral warts and basocellular carcinomas, but its use in melanoma is still controversial [18–23]. It has been demonstrated that imiquimod-TLR7 signaling axis induces pDC with potential to kill tumor cells [24]. Hence, imiquimod could thereby provide an additional TLR stimulus with putative antitumor effect and potentiate *Salmonella*-mediated antitumor immunity.

Future Medicine

Due to the complexity and plasticity of tumor biology, the combination of two or more therapeutic approaches seems to be the best modality to treat melanoma. In the present study, we evaluate the antitumor effect of a combined therapy consisting of topical application of imiquimod together with intratumoral injection of LVR01 in murine melanoma models, demonstrating that the combined immunotherapy induces an antitumor immune response that prolongs the survival of melanoma-bearing mice and reduces occurrence of distant metastases.

Materials & methods

Bacterial strain & imiquimod formulation

S. Typhimurium LVR01, an attenuated strain constructed by introducing a null deletion into the *aroC* gene of parental *S.* Typhimurium isolate P228067, was used in this study [13]. Bacteria were routinely grown at 37°C with shaking in Luria Broth (LB) or on LB Agar (LBA) (Difco, MI, USA).

The imiquimod formulation used in this study was a commercially available cream of imiquimod 5% (Miquimod, Lazar, Montevideo, Uruguay). The control (placebo) ointment was requested to and obtained from the company.

Animals & tumor cell lines

Female C57BL/6 mice (Dilave, Uruguay), 6–8 weeks old, were used for *in vivo* experiments. All animal experimentation protocols were carried out in accordance with procedures authorized by the University's Ethical Committee for Animal Experimentation, Uruguay. Animals were kept with water and food *ad libitum* in the animal house facility at the Instituto de Higiene. All procedures in animals were performed under anesthesia with intraperitoneal injection of ketamine 80 to 100 mg/kg (PHS, Pharmaservice, Montevideo, Uruguay) and xylazine 10 mg/kg (Xylased, Vetcross, Montevideo, Uruguay).

B16F1 mouse melanoma cell line was obtained from American Type Culture Collection (VA, USA) and maintained in DMEM (PAA, Austria) supplemented with 10% fetal bovine serum (PAA) and 2 mM L-glutamine (Sigma-Aldrich, MO, USA) as recommended.

For B16F1 lysate, 5×10^7 B16F1 cells were collected and washed three-times with phosphate-buffered saline (PBS, pH 7.4). Cells were resuspended in 5 ml PBS and lysed by 15 freeze–thaw cycles. Cells were then centrifuged at 12,000 $\times g$ for 20 min. The soluble fraction was passed through a 0.22-µm membrane filter. Finally, the protein concentration in supernatant was measured by Bradford assay (Sigma-Aldrich).

In vitro invasion & intracellular replication assays

B16F1 melanoma cells were seeded in 24-well plates to a density of 1×10^6 cells per well. *S.* Typhimurium LVR01 was diluted in the appropriate prewarmed culture medium, and added to the tumor cells at a multiplicity of infection (MOI) 100:1. Cell culture plates were incubated at 37°C in 5% CO_2 atmosphere. After 1 h, the cell cultures were rinsed and incubated with medium containing gentamycin sulfate (50 µg/ml) to kill extracellular, but not intracellular bacteria. Viable intracellular bacteria were recovered by lysing the tumor cells in distilled water with 0.1% of Triton X-100 (Biopack, Argentina) for 10 min at room temperature. Quantification of bacteria was performed by plating serial dilutions on LBA plates. For intracellular replication assay, intracellular *Salmonella* were quantified at 3, 6, 24 and 48 h after infection.

Tumor cell viability (MTT assay)

Cytotoxicity against melanoma cells was determined by MTT assay. Briefly, 5×10^4 B16F1 cells were seeded in 96-well tissue culture plates and infected with *S.* Typhimurium LVR01 at a MOI 100:1 for 1 h. Cell cultures were rinsed and incubated with culture medium containing gentamycin sulfate (50 µg/ml) to kill extracellular, but not intracellular, bacteria for 48 h. Cell cultures were then rinsed and incubated for further 1 h with culture medium containing tetracycline (10 µg/ml) to kill intracellular bacteria. Finally, 50 µl/well of 5 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) were added and incubated for 4 h. Formazan crystals were dissolved with 20% SDS solution (Sigma-Aldrich) overnight at room temperature prior to measurement of absorbance at 570 nm.

Salmonella biodistribution assay

B16F1 cells were harvested in log phase, washed and resuspended in PBS to a final concentration of 2.5×10^6 cells/ml. Groups of mice were injected subcutaneously into the right flank with 2.5×10^5 cells and were then monitored for tumor growth. Once tumors became palpable, approximately 11 days post-tumor implantation (pti),

mice were intratumorally inoculated with 1×10^6 CFU of LVR01. At designated time points postintratumoral bacterial injection, animals were sacrificed and tumors, spleens and livers were removed and weighted. All tissues were homogenized in PBS and the number of viable S*almonella* was determined in each tissue by plating serial dilutions on LBA plates.

Tumor challenge & immunization schedule

For subcutaneous melanoma model, mice were shaved on the right flank and inoculated subcutaneously with 2.5×10^5 B16F1 cells as described above. Animals were divided in four groups: control, imiquimod, *Salmonella* and combined therapy (imiquimod plus *Salmonella*) groups. From days 11 to 18 pti, mice received a daily application of Imiquimod (0.2 mg) or control ointment on previously shaved skin. At day 13 pti, a suspension of 1×10^6 CFU of *S.* Typhimurium LVR01 or 100 µl PBS was intratumorally injected. Tumors were periodically measured with a caliper. Tumor volumes were calculated using the formula:

Tumor volume = (length × width × depth) × π/6.

Mice were sacrificed by cervical dislocation when tumor volume exceeded 4000 mm^3 or before if they showed any sign of distress.

For the immunological studies, animals were sacrificed at days 16 and 23 pti. Dissected tumors, tumor draining lymph nodes (TDLN) and spleens were disposed in saline solution and/or TRIzol reagent (Invitrogen, CA, USA) for further studies.

Spontaneous metastatic melanoma model was set up in our lab, as a modification of the previously described by Grinshtein *et al.* [25]. Briefly, B16F1 cells were harvested in log phase, washed and resuspended in PBS to a final concentration of 3×10^6 cells/ml. Mice were shaved on the right flank and intradermally inoculated with 1×10^5 B16F1 cells. Imiquimod and *S.* Typhimurium LVR01 treatments were applied as described above for classical subcutaneous melanoma model. Primary tumor resection was performed on day 17 pti, and relapse and/or occurrence of metastasis were followed up for 80 days pti. The number of lymph nodes (LN) affected (palpable) and their sizes were recorded every 3–4 days. Affected LN were measured with a caliper and sizes were calculated as for tumor using the above-mentioned formula.

Flow cytometric analyses

For obtaining single cell suspension of tumor-infiltrating cells, tumors were mechanically dissected using the plunger of a syringe. Released cells were filtered through a 70 µm strainer and washed twice with PBS before further use. Cells were immunostained with the appropriate antibodies against CD3 (clone 145–2C11), CD4 (clone GK1.5), CD8 (clone 53–6.7), CD11b (clone M1/70), CD11c (clone HL3), CD19 (clone 1D3), CD25 (clone 3C7), CD45 (clone 30-F11), CD49b (clone DX5), CD103 (clone M290), CD253 (TRAIL) (clone N2B2), B220 (clone RA3–6B2), Gr1 (clone RB6–8C5), MHC class II (I A^b, clone AF6–120.1) (all from BD Pharmingen, CA, USA) and F4/80 (clone BM8, Serotec, Oxford, UK). Optimal antibody concentrations were previously defined by titration. Flow cytometry data were collected on an FACS Canto II cytometer and analyzed using FACS Diva software (BD Biosciences, Oxford, UK). Cytometry analysis was performed gating on total living cells, followed by a gate on $CD45^+$ cells. For lymphocytes, a gate on lymphocyte population was performed on FSC versus SSC dot plot, followed by a final gate on cell-specific surface markers (B cells: $CD3^-$ $CD19^+$; $CD4^+$ T cells: $CD3^+$ $CD4^+$; $CD8^+$ T cells: $CD3^+$ $CD8^+$; NK cells: $CD3^-$ $CD49b^+$). For the remaining studied cell populations, the following strategy was assayed: macrophages were determined as $F4/80^+$ cells, whereas neutrophils were identified as $Gr1^+$ $CD11b^+$ cells and conventional DC (cDC) as $CD11b^+$ $CD11c^+$, both within NOT $F4/80^+$ gate. Plasmocytoid DCs were determined as $B220^+$ $CD19^-$ within $CD11c^+$ $CD11b^-$ cells. The percentage of tumor infiltrating cells was expressed in relation to total living cells, to normalize tumor sizes.

Cytokine & chemokine quantitative reverse transcription-PCR

Tumors and TDLNs were homogenized in TRIzol reagent with an Ultra Turrax Homogenizer (IKA, Königswinter, Germany) and were stored at -80°C. RNA was extracted according to the manufacturer's instructions. RNA quality and quantity were assessed by spectrophotometric measurement at 260/280 nm. Prior to cDNA synthesis, 1 µg total RNA was treated with DNase I (Invitrogen), and first-strand cDNA synthesis was carried out using random primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCR was performed using a QuantiTect SYBR green PCR kit (Qiagen, Hilden, Germany) in a Rotorgene 600 (Corbett, Qiagen). β-actin encoding gene (*Actb*) was used as housekeeping gene. The primers were used at a final

concentration of 0.9 μM (primer sequences are available under request). The relative mRNA amount in each sample was calculated using the $2^{-\Delta\Delta Ct}$ method [26] where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{actb}$, and expressed as relative mRNA levels in the test group compared with the control group.

Splenocyte proliferation assay

Spleens were removed from mice at day 23 pti and prepared as a single-cell suspension. Splenocytes were washed three-times with Roswell Park Memorial Institute (RPMI) 1640 medium (PAA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 100 μg/ml penicillin–streptomycin (Sigma-Aldrich) (supplemented RPMI) as recommended, counted and resuspended at a concentration of 1×10^6 cells/ml. Cells were labeled with 3 μM CFSE (Sigma-Aldrich) and then seeded in duplicates into 24-well plates (Nunc, IL, USA) at 2×10^6 cells per well in 2 ml of supplemented RPMI. Cells were incubated for 5 days with concanavalin A (Sigma-Aldrich), B16F1 cell lysate or left without stimuli. Cells were collected and stained as described above to assess lymphocyte proliferation by flow cytometry. Cytometry analysis was performed gating on total living cells. A gate on lymphocyte population was performed on FSC versus SSC dot plot, followed by a final gate on cell-specific surface markers as described above. Cell generation gates were finally defined within each cell population. The number of proliferative cells (events) in each gate was recorded, and the number of parental cells from which each generation of proliferative events derived was calculated.

Stimulation index was then calculated as: $\sum$ of proliferative cells / $\sum$ of parental cells, and normalized by the nonstimulated condition.

Flow cytometric assay of cytotoxicity

Cytotoxicity was assessed by flow cytometry as described before [27]. Unlabeled splenocytes were used as effectors (E) and B16F1 melanoma cells served as targets (T). Tumor cells were labeled with CFSE and seeded into 24-well plates (Nunc) at 1×10^4 cells per well in 0.5 ml of supplemented RPMI. Splenocytes were seeded in 200:1, 100:1 and 25:1 E:T ratios in 0.5 ml of supplemented RPMI. In parallel, target cells were incubated alone to measure spontaneous cell death. Cells were incubated for 18 h and then collected and stained with propidium iodide (Sigma-Aldrich) prior to analysis by flow cytometry. The percentage of tumor cell death (cytotoxicity) was calculated as:

$$\%\ \text{Cytotoxicity} = \frac{\%\ \text{target cell death} - \%\ \text{spontaneous target cell death}}{100 - \%\ \text{spontaneous cell death}} \times 100$$

Antibodies detection assay

Humoral immune response against B16F1 melanoma cells was assessed by ELISA using melanoma cell lysate to coat the plates. Briefly, plates were coated with whole B16F1 cell lysate (0.75 μg/well) in PBS and incubated overnight at 4°C. Plates were washed thrice with PBS-0.05% (v/v) Tween-20 (PBS-T) and then blocked using PBS-1% BSA at 37°C for 1 h. Serum samples diluted 1/200 in PBS-T-1% BSA (PBS-T-BSA) were added to the plates in duplicates and incubated overnight at room temperature. Plates were extensively washed with PBS-T. A secondary antibody goat anti-mouse IgG-biotinylated (Southern Biotech, AL, USA) diluted 1/4000 in PBS-T-BSA, followed by Streptavidin-PO (Sigma-Aldrich) diluted 1/2000 in PBS-T-BSA, were used to amplify the signal. Finally, color was developed using o-Phenylenediamine dihydrochloride (OPD) tablets (Sigma-Aldrich) according to manufacturer's instructions.

Statistical analysis

Differences in tumor volumes at all time points were assessed by ANOVA (*t*-test). Differences in survival rates were determined using Kaplan–Meier method, and groups were compared using the log-rank test. Synergistic effect was assessed by mixed-factorial ANOVA and multiple linear regression analysis. For *in vitro* and *in vivo* assays, statistical significance of differences for one variable between studied groups was analyzed using one-way ANOVA with Tukey's HSD test. Student's t-test was applied to evaluate the differences between a selected pair of groups (GraphPad Prism). A value of $p < 0.05$ was considered as statistically significant.

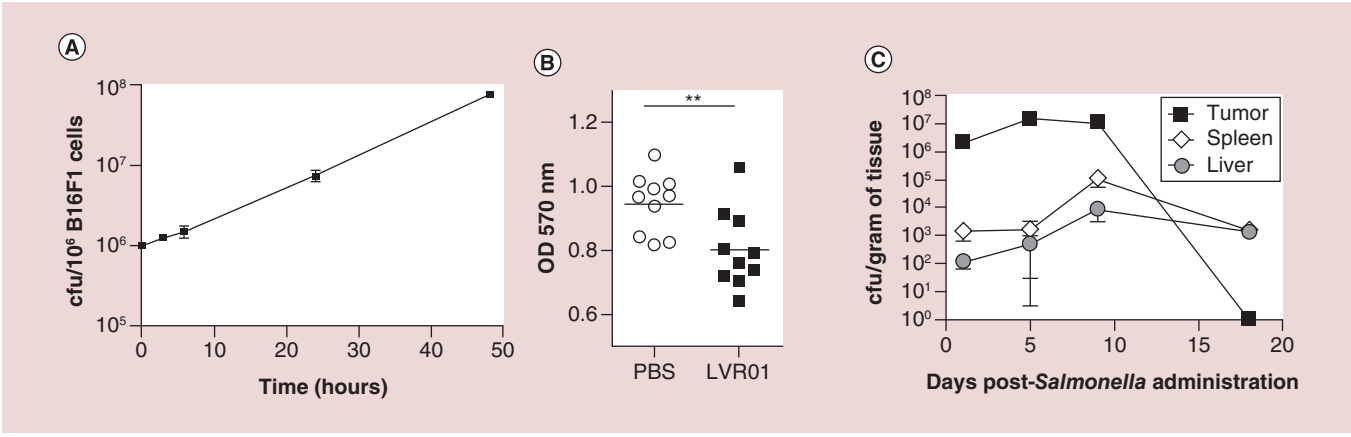

Figure 1. Evaluation of *Salmonella* Typhimurium LVR01 as monotherapy for B16F1 melanoma. (A) Capacity of *S.* Typhimurium LVR01 to invade and survive within melanoma B16F1 cells *in vitro.* Invasion was carried out at a MOI of 100:1 (bacteria:tumor cell ratio) for 1 h, and replication was determined in the continuous presence of gentamycin by counting number of viable bacteria at different time points for up to 48 h (n = 3/time point). One representative experiment out of three performed is shown. **(B)** *In vitro* cytotoxicity of *S.* typhimurium LVR01 in B16F1 melanoma cells. Infection assays were carried out as described above and cell viability was determined by MTT assay at 48 h post-infection. Results are shown as absorbance at 570 nm of the replicates (n = 10). One representative experiment out of three performed is shown. **p < 0.01, Student's *t*-test. **(C)** Biodistribution of *S.* Typhimurium LVR01 in B16F1 melanoma-bearing mice. B16F1 melanoma-bearing mice received 1×10^6 CFU of LVR01 intratumorally. At days 1, 5, 9 and 18 post-*Salmonella* administration, mice were sacrificed and the number of bacteria in tumor, spleen and liver was determined. Graphs show mean ± SD of CFU per gram of tissue (n = 3/time point). One representative experiment out of three performed is shown.
CFU: Colony-forming unit; MOI: Multiplicity of infection; PBS: Phosphate-buffered saline.

Results

S. Typhimurium LVR01 infects & replicates within B16F1 melanoma cells *in vitro*, inducing cell death

Invasion was determined by gentamicin protection assay at a MOI 100:1. We found that after 1 h, 1% of the bacterial inoculum can be recovered (Figure 1A), confirming that LVR01 can invade B16F1 melanoma cells. *Salmonella* counts were further determined after 3, 6, 24 and 48 h. We observed that bacterial counts consistently increased over time, recovering almost 10^8 CFU after 48 h (Figure 1A), implying that bacteria can replicate inside tumor cells. At that time point, B16F1 melanoma cell viability was determined by MTT assay. As shown in Figure 1B, there was a 15% reduction in cell viability after *Salmonella* infection (p = 0.0077, Student's *t*-test).

Salmonella LVR01 can be recovered from inside tumors for at least 9 days after intratumoral injection

We examined the biodistribution and persistence of *Salmonella* LVR01 *in vivo* when administrated intratumorally. The number of bacteria in tumor, as well as in liver and spleen, were evaluated at different time points (days 1, 5, 9 and 18) after inoculation. As it can be seen in Figure 1C, 24 h after *Salmonella* administration, bacteria were recovered from the tumor at numbers higher than the initial inoculum (p = 0.0342, Student's *t*-test). These numbers increased by day 5 (p = 0.0208, Student's *t*-test) and they could be recovered from tumor up to 9 days after intratumoral injection. These results confirmed that *Salmonella* replicates *in vivo* at tumor site. *Salmonella* was also found in spleen and liver, although in numbers markedly lower than those found in tumor (Figure 1C).

The combination of imiquimod & *Salmonella* treatment resulted in tumor growth delay & prolonged survival

B16F1 melanoma-bearing mice were treated with one of the two different monotherapies, topical application of 5% imiquimod cream or intratumoral injection of 1×10^6 CFU of *S.* Typhimurium LVR01 or with the combination of both as described in 'Materials & methods' (Figure 2A). By day 20 pti, each one of the monotherapies resulted in tumor growth delay (p = 0.014 and p = 0.044 for imiquimod and *Salmonella* groups, respectively when compared with control mice receiving no treatment), but the combined therapy resulted in a slower melanoma progression (p = 0.004, 0.025 and 0.014 compared with control, imiquimod- and *Salmonella*-treated groups, respectively) (Figure 2B). The differences in tumor size were maintained during all the duration of the experiment and they were maximal by day 22 pti, when tumor volume means were 4103, 2659, 2292 and 1187 mm^3 for control, imiquimod,

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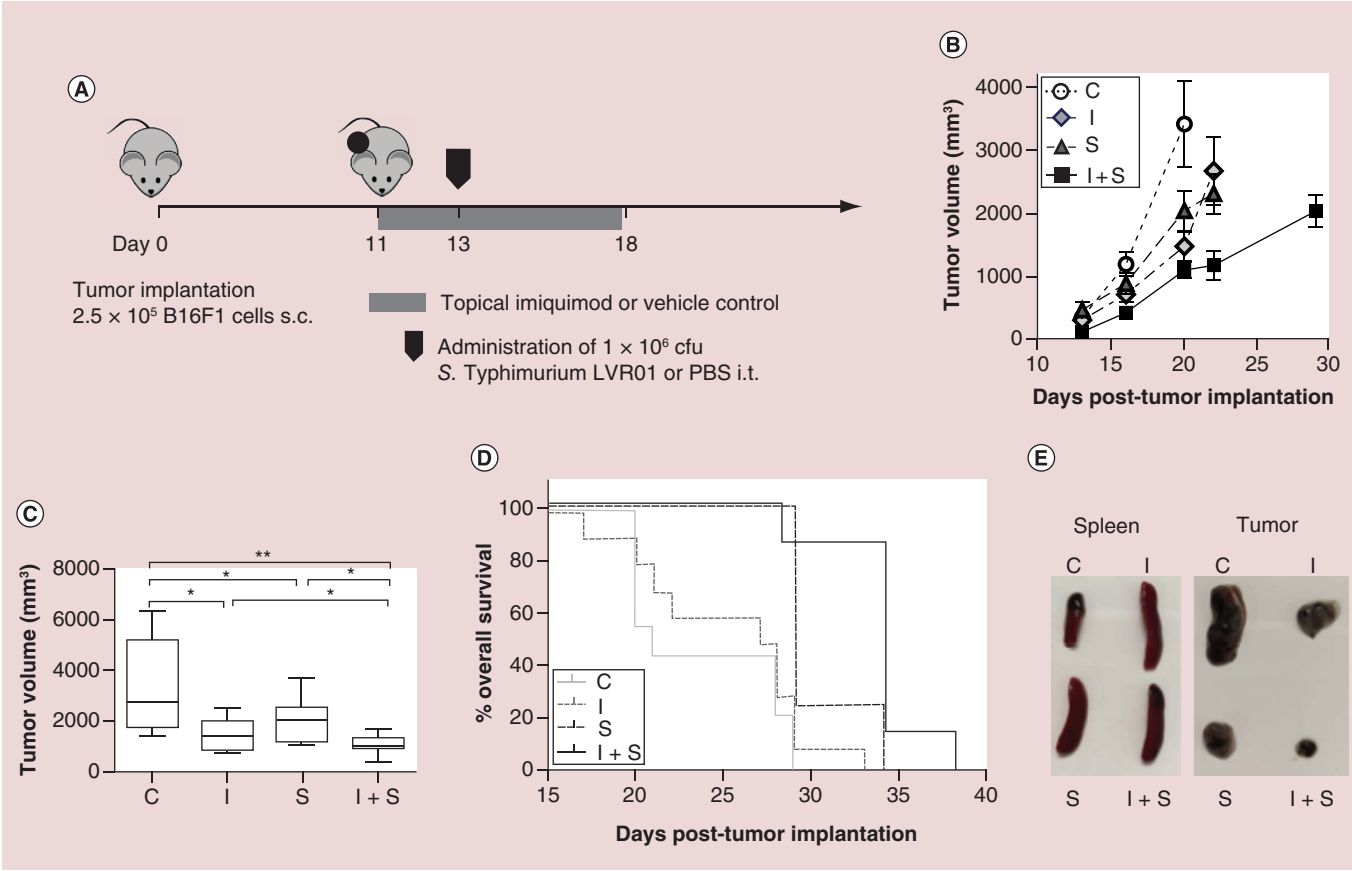

Figure 2. Combined treatment in subcutaneous melanoma model. (A) Schematic representation of treatment schedule. C57BL/6 mice were inoculated subcutaneously with 2.5 × 10^5 B16F1 melanoma cells. Animals were divided in four groups: control **(C)**, imiquimod **(I)**, *Salmonella* **(S)** and combined therapy (imiquimod plus *Salmonella*, **I + S**) groups. Imiquimod or vehicle ointment were applied daily from days 11 to 18 pti, and *S.* Typhimurium LVR01 (1 × 10^6 CFU) or PBS was injected intratumorally at day 13 pti. One representative experiment out of four performed is shown. **(B)** Tumor volume was measured every 2–3 days and calculated as (W×H×D) × π/6. Results are shown as mean ± SEM (n = 12–14/group). **(C)** Box plot of tumor volume at day 22 pti (n = 12–14/group). *p < 0.05 and **p < 0.01, one-way ANOVA. **(D)** Survival was followed up for 37 days. Kaplan–Meier plot. **(E)** Representative pictures of spleens and tumors were taken at day 16 pti. PBS: Phosphate-buffered saline; pti: Post-tumor implantation; SEM: Standard error of the mean.

Salmonella and combined therapy groups, respectively (Figure 2C & E). Consistently with that, administration of the combined therapy significantly extended animal survival as compared with control group (median survival 21 vs 34 days pti, $p < 0.001$), as well as compared with imiquimod- or *Salmonella*-treated groups ($p = 0.0013$ and $p = 0.0481$, respectively) (Figure 2D). Of note, we observed that daily application of imiquimod did not induce inflammatory changes on the skin, but we could observe higher incidence of tumor ulcers than in control group (18 and 5% for imiquimod and control groups, respectively) as well as a marked splenomegaly (Figure 2E). We also observed tumor ulceration after intratumoral administration of *Salmonella*, and hence the occurrence of tumor ulcers was even higher when we combined both treatments (13 and 23% for *Salmonella* and combined therapy groups, respectively).

The combined therapy elicited a pro-inflammatory response in the tumor microenviroment

We analyzed the local immune response 3 days post-*Salmonella* administration (i.e., day 16 pti) in tumors as well as in TDLN. There was a marked increase in the relative mRNA levels of several pro-inflammatory chemokines and cytokines, including *Cxcl1*, *Cxcl2*, *Cxcl9*, *Cxcl10*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ifna*, *Tnfa* and *Il6*, as well as Th1-skewed cytokines, such as *Ifng* and *Il12*, and *Il10* in tumors of animals treated with the combined therapy ($p < 0.05$, one-way ANOVA with Tukey's HSD test) (Figure 3A). Some cytokines were also upregulated in animals treated with a single monotherapy (imiquimod or *Salmonella*) but expression levels were always higher in animals receiving

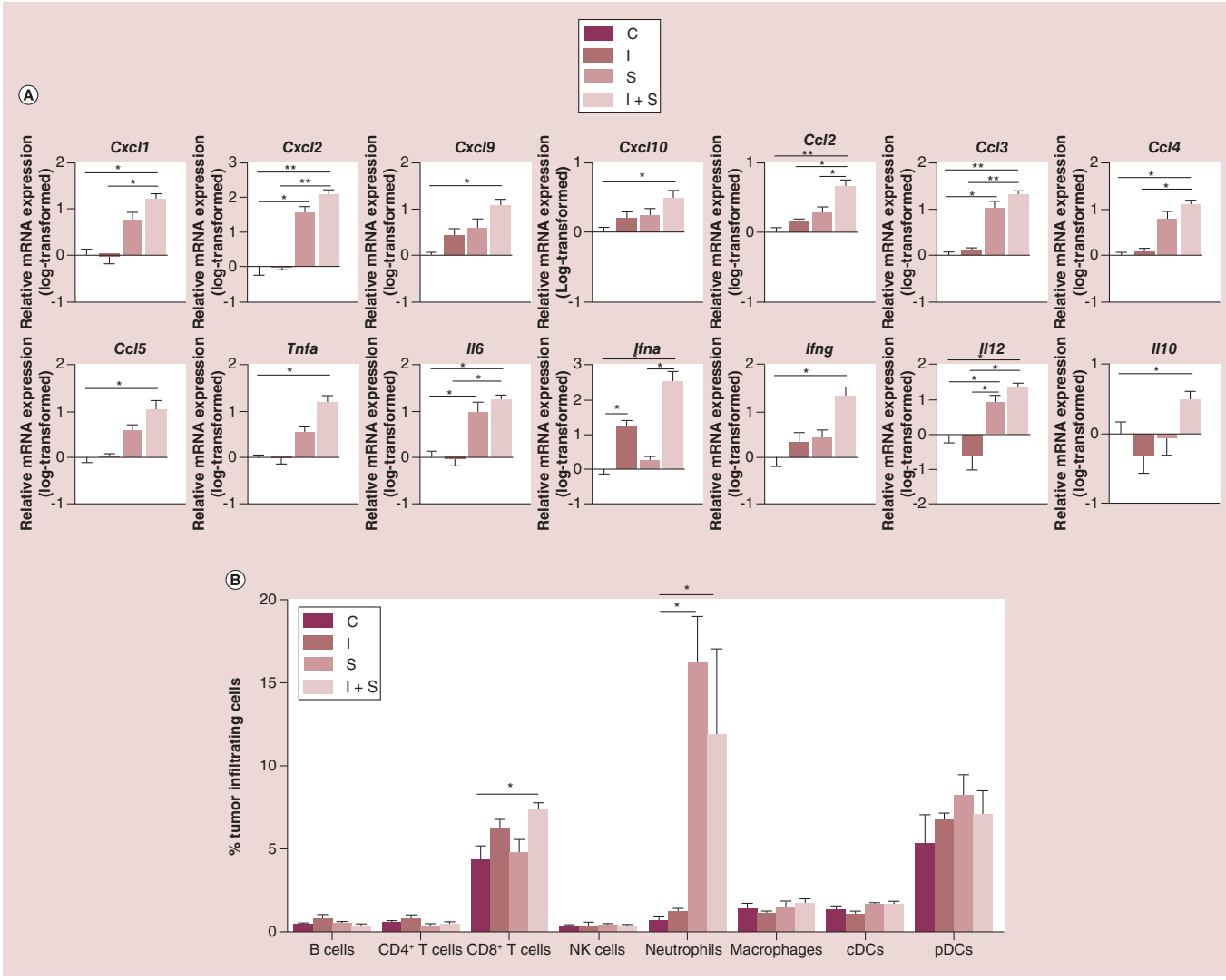

Figure 3. Local immune response elicited by combined treatment. Mice were treated with the different monotherapies or the combination of both: control **(C)**, imiquimod **(I)**, *Salmonella* **(S)** and combined therapy (imiquimod plus *Salmonella*, **I + S**) groups. Tumors were removed 3 days after *Salmonella* administration (day 16 pti). One representative experiment out of three performed is shown. **(A)** Cytokine/chemokine profile was assessed by quantitative RT-PCR and **(B)** Percentages of tumor-infiltrating cells: B cells ($CD3^-$ $CD19^+$ lymphocytes), $CD4^+$ T cells ($CD3^+$ $CD4^+$ lymphocytes), $CD8^+$ T cells ($CD3^+$ $CD8^+$ lymphocytes), NK cells ($CD3^-$ $CD49b^+$ lymphocytes), neutrophils ($Gr1^+$ $CD11b^+$ cells), macrophages ($F4/80^+$ cells), cDCs ($CD11b^+$ $CD11c^+$ $F4/80^-$ cells) and pDC ($CD11c^+$ $CD11b^-$ $B220^+$ $CD19^-$) within total living cells were determined by flow cytometry. Results are shown as mean ± SEM (n = 5–6/group). *$p < 0.05$ and **$p < 0.01$, one-way ANOVA with Tukey's HSD test.
cDC: Conventional dendritic cell; pDC: Plasmocytoid dendritic cell; pti: Post-tumor implantation; RT-PCR: Real-time PCR; SEM: Standard error of the mean.

the combined therapy. mRNA expression levels of *Il4*, *Il5* and *Il17* remained unchanged in all groups (data not shown).

We also observed increased numbers of tumor-infiltrating $CD8^+$ T cells in mice that received imiquimod in combination with *Salmonella* (p = 0.0469, one-way ANOVA with Tukey's HSD test) (Figure 3B). However, we could not find any differences in $CD8^+$ T-cell activation status determined by the expression of CD25 and TRAIL in their surface compared with control group (data not shown). A marked neutrophil accumulation was also observed in tumors of animals that received *Salmonella* either alone or in combination with imiquimod (p = 0.0236, one-way ANOVA with Tukey's HSD test) (Figure 3B). Instead percentages of macrophages, cDC, plasmocytoid DC, NK cells, B cells or T helper lymphocytes that infiltrate tumors remained unchanged between groups (Figure 3B).

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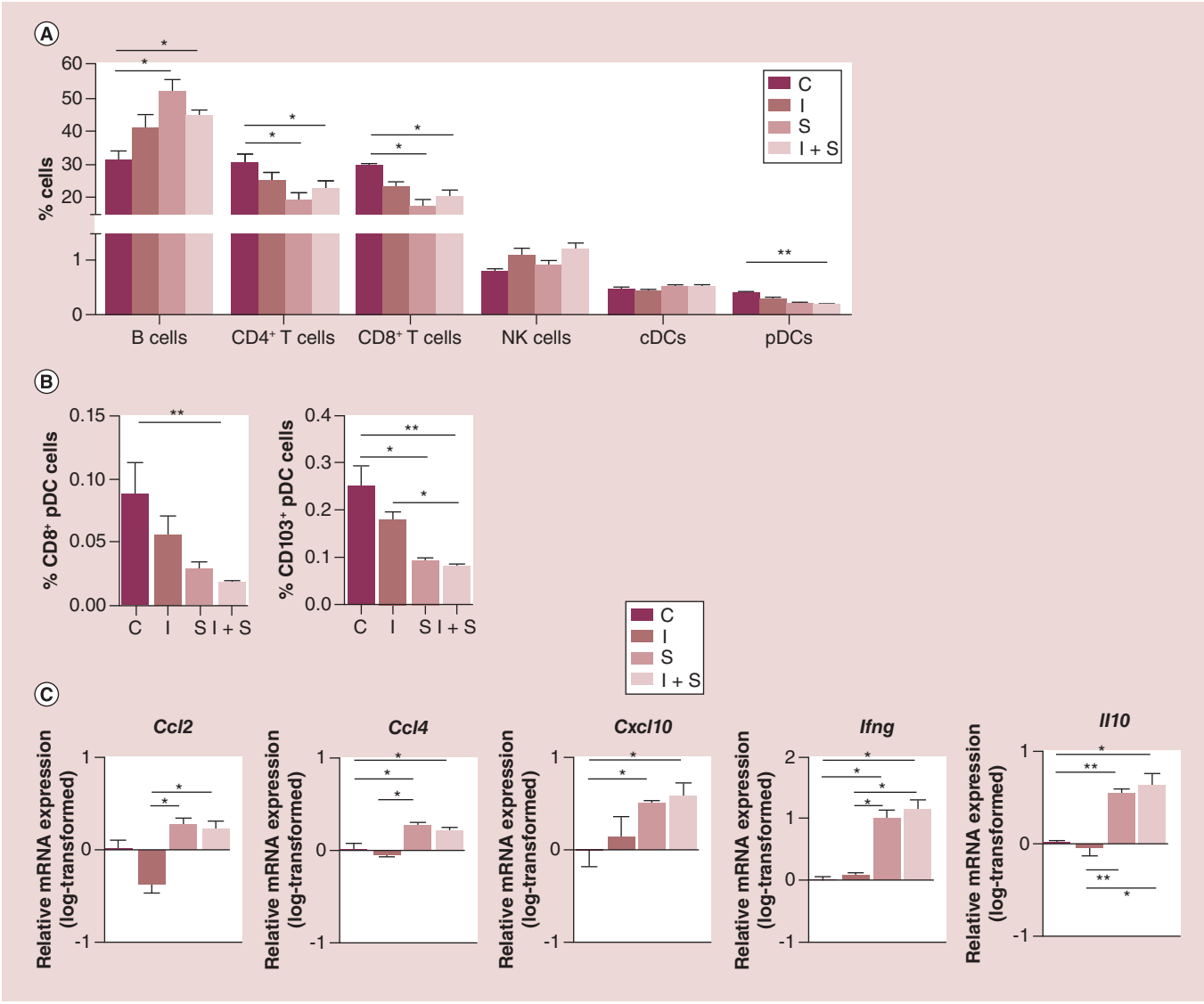

Figure 4. Immune modulation at tumor draining lymph nodes. Tumor draining lymph nodes (TDLN) were removed 3 and 10 days after *Salmonella* administration. One representative experiment out of three performed is shown. **(A)** Percentages of B cells (CD3$^-$ CD19$^+$ lymphocytes), CD4$^+$ T cells (CD3$^+$ CD4$^+$ lymphocytes), CD8$^+$ T cells (CD3$^+$ CD8$^+$ lymphocytes), NK cells (CD3$^-$ CD49b$^+$ lymphocytes), cDCs (CD11b$^+$ CD11c$^+$ cells) and pDC (CD11c$^+$ CD11b$^-$ B220$^+$) in TDLN total living cells determined by flow cytometry at day 16 dpi. **(B)** TDLN pDC characterization determined by flow cytometry at day 16 dpi. **(C)** Cytokine/chemokine mRNA profile assessed by quantitative RT-PCR at day 23 dpi. Results are shown as mean ± SEM (n = 5–6/group). *p < 0.05 and **p < 0.01, one-way ANOVA with Tukey's HSD test. Control **(C)**, imiquimod **(I)**, *Salmonella* **(S)** and combined therapy (imiquimod plus *Salmonella*, **I + S**) groups.
cDC: Conventional dendritic cell; pDC: Plasmocytoid dendritic cell; RT-PCR: Real-time PCR; SEM: Standard error of the mean.

In TDLN we found an increase in the percentage of B cells, concurrently with a decrease in the percentage of T cells, in animals that received *Salmonella* either alone or in combination with imiquimod ($p < 0.05$, one-way ANOVA with Tukey's HSD test) (Figure 4A). Besides, the combined therapy led to a reduction in the percentage of plasmocytoid DC in TDLN ($p = 0.0085$, one-way ANOVA with Tukey's HSD test) (Figure 4A). Flow cytometric analyses revealed that there was a decline in both CD8$^+$ and CD103$^+$ plasmocytoid DC populations in TDLN after treatment with imiquimod plus *Salmonella* (Figure 4B).

Analysis of the cytokine/chemokine profile in TDLNs revealed no changes in mRNA expression levels at that time point (data not shown), but we did observe an increased transcriptional expression of *Ccl2*, *Ccl4*, *Cxcl10*, *Ifng* and *Il10* in TDLN of mice treated with the combined therapy taken a week later ($p < 0.05$, one-way ANOVA

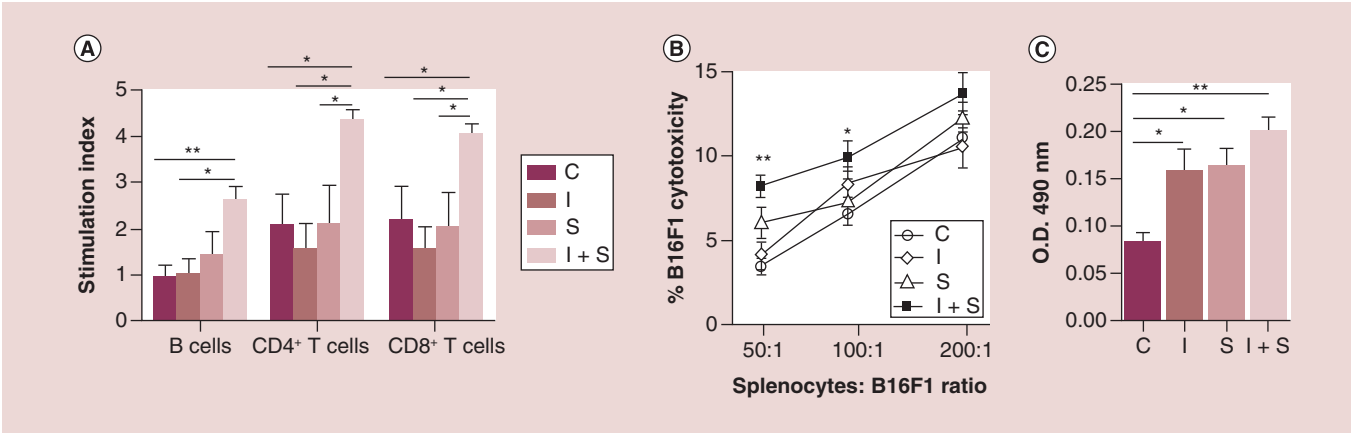

Figure 5. Cellular and humoral systemic antitumor immune response elicited by combined treatment. Mice were treated with the different monotherapies or the combination of both: Control **(C)**, imiquimod **(I)**, *Salmonella* **(S)** and combined therapy (imiquimod plus *Salmonella*, **I + S**) groups. Sera and spleens were obtained at day 10 after *Salmonella* administration (day 23 pti). One representative experiment out of three performed is shown. **(A)** Splenocytes proliferation upon restimulation with tumor antigens and **(B)** splenocyte-mediated cytotoxicity against B16F1 melanoma cells, both assessed by flow cytometry analysis. **(C)** IgG levels against melanoma antigens were determined by ELISA at a serum dilution 1/200. Results are shown as mean ± SEM (n = 5/group). *$p < 0.05$ and **$p < 0.01$, one-way ANOVA with Tukey's HSD test.
SEM: Standard error of the mean.

with Tukey's HSD test) (Figure 4C). Of note, *Tnfa*, *Ifna*, *Il4*, *Il6*, *Il12* and *Il17* mRNA expression levels remained unchanged in all groups (data not shown).

The combined therapy elicited a systemic antitumor immune response

We also analyzed the cellular and humoral systemic immune response elicited by the combined therapy 10 days after *Salmonella* administration. Splenocytes were obtained, labeled with CFSE and restimulated with whole B16F1 melanoma cell homogenate for 5 days. Remarkably, splenic $CD4^+$ and $CD8^+$ T cells as well as B lymphocytes derived from mice treated with the combined therapy proliferated upon restimulation with melanoma antigens ($p < 0.05$, one-way ANOVA with Tukey's HSD test) (Figure 5A).

In parallel, splenocytes cytotoxic capacity was determined using a flow cytometric assay as described in 'Materials and methods'. We found that splenocytes from mice treated with imiquimod plus *Salmonella* exhibited enhanced cytotoxic activity against B16F1 melanoma cells compared with untreated (control) mice ($p = 0.0010$ and 0.0348 for 50:1 and 100:1 E:T ratio, respectively) (Figure 5B).

Regarding humoral response determined by ELISA with whole B16F1 melanoma cell homogenate, we observed a modest but significant increase in IgG levels against melanoma antigens in serum of all treated animals, either with the monotherapies or with the combined therapy, compared with control group ($p < 0.05$, one-way ANOVA with Tukey's HSD test) (Figure 5C).

The combined therapy reduced the occurrence of metastatic disease

Finally, we evaluated the efficacy of the combined treatment as a neoadjuvant immunotherapy in the context of primary tumor resection. We first inoculated B16F1 cells intradermally, instead of subcutaneously, which promotes cell invasion and migration to LN. Mice were then treated with the combined therapy as described above for B16F1 subcutaneous melanoma model and at day 17 pti, when metastatic disease occurred in most of untreated animals, surgical excision of the primary tumor was performed (Figure 6A). Animals were followed up for the appearance of clinical evidence of disease, either recurrence or metastases at the LN. At the time of surgery, there is a tendency, still not significant, for tumor growth retardation in the combined therapy treated mice (tumor volume means: 665 and 488 mm^3 for control and combined therapy groups, respectively, $p = 0.1380$) (Figure 6B). Upon primary tumor resection, the combined therapy abrogated the occurrence of disseminated disease ($p = 0.0090$) (Figure 6C). Strikingly, four out of eleven treated-animals showed complete recovery. At day 46 pti, 73% of the untreated mice exhibited metastases in LN, whereas only 25% of the treated animals did. In addition, animals that received

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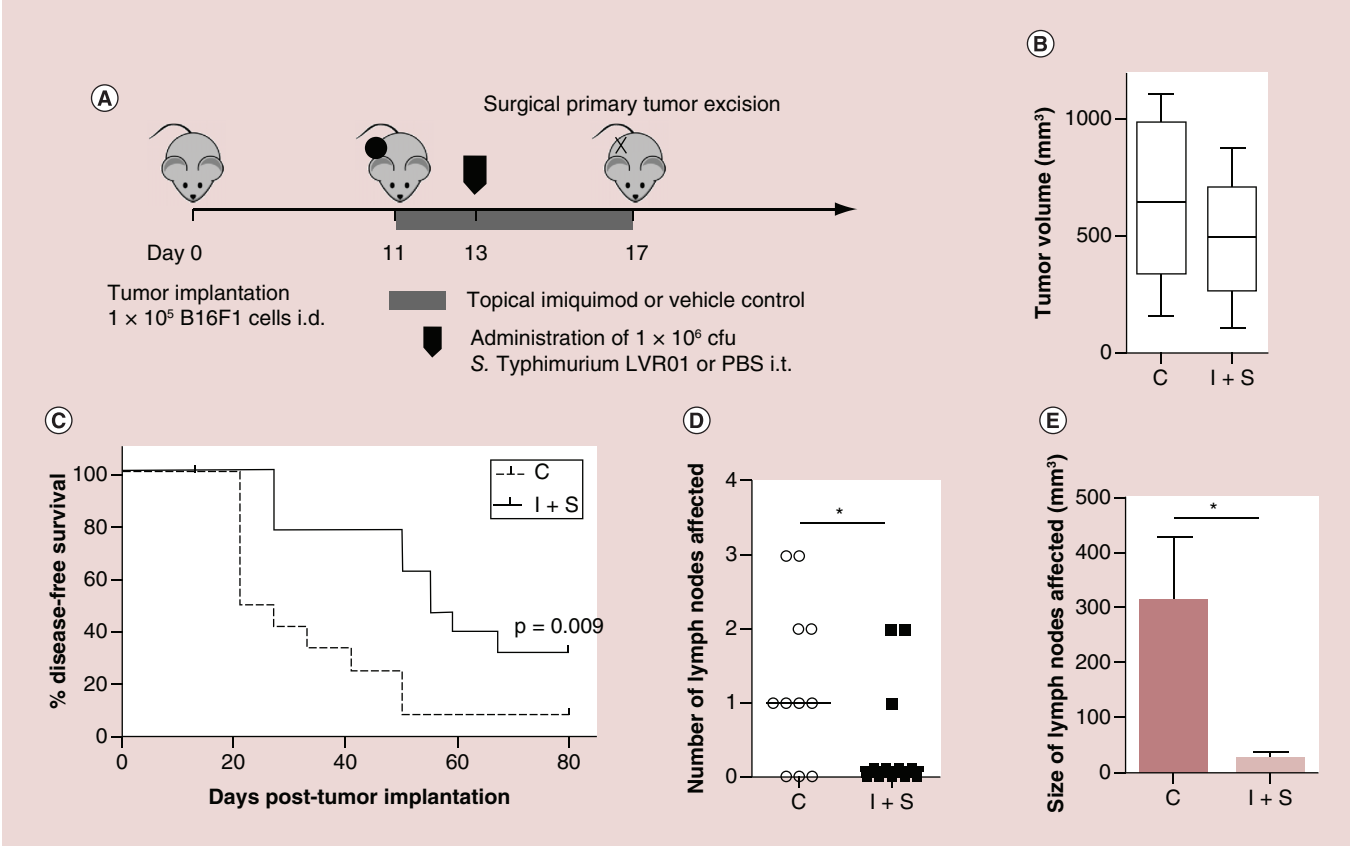

Figure 6. Combined treatment as neoadjuvant immunotherapy for melanoma. (A) Schematic representation of treatment schedule. C57BL/6 mice were intradermally inoculated with 1×10^5 B16F1 melanoma cells. Imiquimod or placebo ointment were applied daily from days 11 to 17 pti. *S.* Typhimurium LVR01 (1×10^6 CFU) or PBS were intratumorally injected at day 13 pti. Primary tumors were removed by surgery at day 17 pti. One representative experiment out of two performed is shown. **(B)** Box plot of tumor volume before surgery (n = 10–12/group). **(C)** Occurrence of lymph node (LN) metastasis was followed up for 80 days. Kaplan–Meier plot. **(D)** Frequency of compromised LN at day 46 pti. Results are shown as individual values and median (n = 10–12/group). *$p < 0.05$, Mann–Whitney test. **(E)** Size of compromised LN calculated as $(W \times H \times D) \times \pi/6$. Results are shown as mean ± SEM (n = 10–12/group). *$p < 0.05$, Student's *t*-test. Control **(C)** and combined therapy (imiquimod plus *Salmonella*, **I + S**) groups.
PBS: Phosphate-buffered saline; pti: Post-tumor implantation; SEM: Standard error of the mean.

the neoadjuvant treatment with imiquimod plus *Salmonella* had less number of compromised LN (p = 0.0378, Mann–Whitney test) (Figure 6D), which were less enlarged (p = 0.0124, Student's *t*-test) (Figure 6E).

Discussion

Immunotherapies can be regarded as the most advantageous approach for the treatment of cancer thanks to their response rate sustained over time. Combination of two or more therapies, mainly an immunotherapy combined with radiotherapy, chemotherapy or another immunotherapy, has been considered as a promissory modality to treat melanoma because of its potential to restrain tumor plasticity to acquire resistance. In this work, we assessed the potential use of attenuated *S.* Typhimurium combined with topical imiquimod in murine experimental melanoma model. The results presented show that imiquimod potentiates *Salmonella*-induced antitumor effect.

Topical imiquimod has been shown to induce remissions of basal cell carcinoma, lentigo maligna and breast cancer skin metastasis [28–30]. We observed that imiquimod application as monotherapy has a modest effect retarding B16F1-melanoma growth, with no consequences in animal overall survival. On the other hand, intratumoral administration of attenuated *S.* Typhimurium LVR01 as monotherapy significantly retards tumor growth and prolongs the survival of melanoma-bearing mice. However, when we combined both therapies we found a greater delay in tumor growth and extended animal survival. If we considered these two parameters as readout of our experimental trial, the combination of both monotherapies resulted in an additive effect. Nonetheless, we also observed a synergistic effect in many aspects of the immune response induced by the combined intervention. We

believe that the potentiating effect of the combined therapy on the tumor area enhances ulceration, which could be somehow beneficial as it denotes cytotoxicity but also deleterious by increasing morbidity and mortality of treated animals.

In a clinical setting, a patient lacks the primary melanoma lesion because it should be completely removed for diagnosis. Therefore, topical treatment could be applied only to lentigo maligna and cutaneous melanoma metastasis. Since the first case report of imiquimod-treated lentigo maligna in 2000 [31], numerous reports using topical imiquimod as a nonsurgical treatment for lentigo maligna have been published with dissimilar results concerning its efficacy. Recently Mora *et al.* reported a systematic review that shows good clinical and histological clearance rates, highlighting in the importance of cumulative dose and treatment intensity for a beneficial outcome [32]. Likewise, Sisti *et al.* reviewed published reports using topical imiquimod as monotherapy for melanoma skin metastases and concluded that it can be considered an effective treatment due to its potential in clearing cutaneous metastases spreading from melanoma primary tumor, albeit it does not stop disease progression [33]. In these two clinical scenarios, we believe that imiquimod plus *Salmonella* has a great potential as local neoadjuvant immunotherapy since it controls the occurrence of metastatic stage of disease. Whether the treatment of the primary tumor with the combined therapy prevents melanoma cell migration, invasion and metastasis or the antitumor immune response hereby induced eliminates and restrains the growth of metastases upon primary tumor resection is still not known. Recently, Liu *et al.* have demonstrated the superior efficacy of neoadjuvant compared with adjuvant immunotherapy to eradicate disseminated disease, owing its potential to the induction of IFN-γ and systemic tumor-specific $CD8^+$ T cells [34]. In line with their findings, the combination of imiquimod and *Salmonella* also primes both immune effectors.

The combined use of attenuated bacteria and imiquimod has been assessed with success in melanoma-bearing mice, as well as in patients with in-transit melanoma [33–35]. In the former mentioned work, imiquimod was used to increase the potential of *Listeria* as a vaccine vector expressing a recombinant murine melanoma antigen (TRP2) [35]. However, the high toxicity of *Listeria* limits its use only as a prophylactic strategy. On the contrary, attenuated *Salmonella* LVR01 has proven to be safe in mouse and other animals [13–15], and could be used in a therapeutic setting. Indeed, preliminary results obtained from two independent clinical trials performed with a small number of patients with in-transit melanoma treated with an attenuated bacteria with safety profile, such as BCG, in combination with imiquimod underpins the potential of the hereby evaluated combined immunotherapy [36,37] .

Evidences suggest that the mechanism responsible for the induction of antitumor immunity following the imiquimod plus *Salmonella* treatment could be related to DCs, which in turn would initiate an antitumor immune response. It has been reported that topical imiquimod treatment recruits and stimulates pDC resident in the skin to kill tumor cells in a ccl2-dependent manner. Imiquimod-stimulated pDC are then prone to produce IFN-α/β that led to the upregulation of death receptors on tumor cells, and to express death receptor ligands, such as TRAIL and granzyme B, resulting in tumor cells killing [24]. Strikingly, although we observed clinical evidences of imiquimod treatment as skin irritation and splenomegalia, we could not find any feature of immune activation induced by imiquimod monotherapy as described by other authors but for increased *Ifna* mRNA expression in tumor [36–39]. This difference could be attributed to the imiquimod formulation used. In this work, we applied Miquimod (Lazar, Uruguay) and not Aldara cream (Meda Pharma, Wangen-Brüttisellen, Switzerland) used in the above-mentioned works. In this regard, a short communication states that isostearic acid, the major component of Aldara vehicle (25% w/w), promotes inflammasome activation, keratinocyte death and Il-1α release in the absence of imiquimod formulation, and therefore contributes to the observed effects of Aldara [40]. It is worth mentioning that Miquimod does not contain isostearic acid. Furthermore, no pDC infiltration was observed in melanoma of combined therapy-treated mice despite upregulation of transcription of certain pDC chemoattractants, namely *Ccl2*, *Ccl5* and *Cxcl9* [24]. However, there was an upregulation of *Ifna* transcription in tumor microenvironment, in the absence of TRAIL expression on tumor infiltrating pDC. Nonetheless, there is a reduction in the percentage of pDC at the TDLN, particularly $CD8^+$ and $CD103^+$ pDC, suggesting that these cells may have exited the node, entered circulation and trafficked into the skin area where imiquimod was applied, as we could not find them in the tumor.

The combined therapy leads to an enhancement in the expression of *Cxcl1* and *Cxcl2* in tumors, which was accompanied by the recruitment of $Gr1^+$ $CD11b^+$ cells to the tumor site. The nature of these cells, which may represent neutrophils or myeloid-derived suppressor cells, cannot be determined since their phenotype and functions are still not clearly defined. However, since they correlate with a better outcome, we assume that they are not myeloid-derived suppressor cells, but a subset of neutrophils with antitumor functions. Neutrophil dichotomy

in tumor immunobiology has been described where chronic inflammation results in recruitment and activation of neutrophils with mainly protumor activity, whereas acute activation, for example, that described for BCG immunotherapy in bladder cancer, promotes their potent antitumor activity [41]. This latter phenomenon may be occurring in our model as we observed a pro-inflammatory cytokine profile (TNF-α and IL-6) together with a massive neutrophil infiltration in tumors, mainly related to *Salmonella* administration. This was previously described by us [16] and other group in different tumor models [42]. Bacteria-activated neutrophils can act directly as potent antitumor effector cells via TRAIL [43], and indirectly by activating local T-cell immunity [44]. Indeed, there is significant increase in tumor infiltrating cytotoxic T lymphocytes. It has been demonstrated that the presence of this cell population in melanoma metastases correlates with the local expression of *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Cxcl9* and *Cxcl10* [45], where the last three chemokines have been identified as immune signature in a clinical trial aiming at evaluating the use of topical imiquimod in combination with a multipeptide cancer vaccine in melanoma metastases [46]. It is known that Ccl2 and Ccl4 have a role in the recruitment of transferred T cells to the primary tumor site, preventing tumor recurrence [47], while IL-6 promotes the infiltration of effector T cell at distant tumor sites [48]. In concordance, we found increased mRNA expression of all these chemokines, but *Cxcl10*, in the tumor microenviroment of imiquimod plus *Salmonella*-treated mice, which is associated with the enhanced tumor immunity and reduced occurrence of metastasis. Moreover, Alatrash *et al.* showed that neutrophils can enhance adaptive immunity by modulating melanoma cell susceptibility to CTL lysis [49]. The relevance of this mechanism in our model needs to be addressed. We know hitherto that splenocytes from animals that received the combined therapy have enhanced cytotoxic activity against melanoma cells. It is worth mentioning that despite the great array of chemokines and cytokines with upregulated expression in melanoma, we did not observe increased infiltration of other cells such as macrophages, DC, B lymphocytes and NK cells, the latter with well-established antitumor activity. Other authors also found similar scenario with neutrophil infiltration in the absence of NK cell activity [50].

At local level, the combined therapy elicits a Th1-skewed response, characterized by an increased expression of *Ifng* in TDLN. IL10 mRNA expression is also upregulated. IL10 is considered as a key cytokine in immunoregulation, given it restrains immune response exacerbation. Interestingly, the combined therapy induces melanoma-specific humoral and cellular responses, suggesting that an optimal systemic antitumor immunity has been developed. Certainly, this induced immunity is effective in controlling the occurrence of disseminated stage of the disease.

Strikingly, all therapeutic approaches, either monotherapy or combined therapy, elicit humoral immunity against melanoma. Antibody-dependent cell-mediated cytotoxicity plays an important role in monoclonal antibody-based therapy against cancer. Besides classical NK cells, myeloid effector cells such as macrophages and neutrophils have been reported to mediate tumor cell killing through antibody-dependent cell-mediated cytotoxicity [51,52]. Given the fact that tumor-infiltrating neutrophils and antibodies against melanoma parallel with the observed antitumor effect, we inferred that antibody-dependent neutrophil-mediated killing might be an additional mechanism to eliminate melanoma cells besides tumor elimination by CD8 T cells.

Conclusion

The results show that topical application of imiquimod potentiates *Salmonella*-induced antitumor effect. The therapeutic approach overcomes the melanoma-mediated immunosuppressive status, allowing the development of local and systemic immunity. We demonstrate the potential of attenuated *Salmonella* and imiquimod as immunotherapy for melanoma, close to clinical application.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors adhere to licensing guidelines of the University's Ethical Committee for Animal Experimentation, Uruguay.

Summary points

- Melanoma-bearing mice treated with topical imiquimod in combination with an intratumoral injection of attenuated *Salmonella enterica* serovar Typhimurium LVR01 showed significant retard on tumor growth and prolonged survival as compared with nontreated animals or animals treated with only one of these therapies.
- The combined therapy led to an enhancement in the expression of pro-inflammatory cytokines and chemokines in tumor microenvironment, with a Th1-skewed profile.
- There was an infiltration of cytotoxic T lymphocytes and neutrophils in tumors of imiquimod plus *Salmonella*-treated mice.
- The combined therapy elicited a systemic specific antitumor response, characterized by splenic B- and T-cell proliferative responses.
- Splenocytes from mice treated with the combined immunotherapy had augmented cytotoxic activity against B16F1 melanoma cells.
- *Salmonella* LVR01-immunotherapy in combination with imiquimod induced specific anti-melanoma antibody response.
- The induced immunity was effective in controlling the occurrence of disseminated stage of the disease in a spontaneous metastatic B16F1-melanoma model.

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