Preclinical Evaluation of LVR01 Attenuated *Salmonella* as Neoadjuvant Intralesional Therapy in Combination with Chemotherapy for Melanoma Treatment

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Treatment of malignant melanoma has improved in the last few years owing to early detection and new therapeutic options. Still, management of advanced disease remains a challenge because it requires systemic treatment. In such cases, dacarbazine-based chemotherapy has been widely used, despite low efficacy. Neoadjuvant therapies emerge as alternative options that could help chemotherapy to achieve increased benefit. In this work, we evaluate LVR01, an attenuated *Salmonella enterica* serovar typhimurium, as neoadjuvant intralesional therapy in combination with dacarbazine in a preclinical melanoma model. B16F1 melanoma–bearing mice received intraperitoneal administration of dacarbazine for 3 consecutive days. LVR01 treatment, consisting of one single intratumoral injection, was applied 1 day before chemotherapy began. This therapeutic approach retarded tumor growth and prolonged overall survival, revealing a strong synergistic antitumor effect. Dacarbazine induced a drastic reduction of secondary lymphoid organ cellularity, which was partially restored by *Salmonella,* particularly potentiating activated cytotoxic cell compartments. Systemic immune reactivation could be a consequence of the intense inflammatory tumor microenvironment induced by LVR01. We propose that the use of LVR01 as neoadjuvant intralesional therapy could be considered as an interesting strategy with close clinical application to boost chemotherapy effect in patients with melanoma.

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INTRODUCTION

Melanoma is a severe form of skin cancer with a high incidence worldwide. To date, no treatment has been proven to be highly effective in the later stages of the disease, when melanoma becomes disseminated. In the last few years, two novel therapies were approved for the treatment of metastatic melanoma: antibodies against immune checkpoints, such as CTLA-4 and PD-1 (Hodi et al., 2010; Robert et al., 2014), and molecular inhibitors of BRAF (Chapman et al., 2011). In the case of BRAF inhibitors, its use is restricted to patients who carry *BRAF* mutation. In addition, these approaches present major side effects, are highly costly, and induce resistance to treatment, all of which contribute to narrowing its application (reviewed in Shah and Dronca, [2014]). Hence, efforts should be focused on pursuing novel strategies for the many patients not yet receiving optimal benefit from these approaches (Jenkins and Fisher, 2021).

Despite low response rates and overall survival benefits, dacarbazine (DTIC) has been considered the first-line standard treatment for metastatic melanoma in many countries (Serrone et al., 2000). DTIC can cause a direct cytotoxic effect on tumor cells, and more recently, it has been reported to have an immunostimulatory effect by modulating immune cells within the tumor microenvironment (Hervieu et al., 2013). DTIC remains a low-cost treatment but requires new alternative strategies to further boost its efficacy.

Immunotherapies are considered the most convenient approach for the treatment of cancer because they can elicit long-lasting immune responses. The use of bacteria as antitumor agents started a century ago when William B. Coley treated patients with cancer with bacteria or bacterial products. Later, intravesical Bacillus Calmette-Guérin for bladder cancer became the first bacteria-based immunotherapy approved, and it remains in the clinics after 40 years of use (Babjuk et al., 2020). Molecular engineering tools allowed the construction of mutants with precise attenuating deletions, making them safe to use. Nowadays, there is an increased interest in bacteria-based immunotherapies revealed by a recently published white paper (Forbes et al., 2018). Among bacteria, *Salmonella* has been shown to be highly effective as an antitumor agent in many solid cancer models (Hernández-Luna and Luria-Pérez, 2018) and safe, as tested in clinical trials I and II (Cunningham and Nemunaitis, 2001; Toso et al., 2002). It accumulates and replicates in the

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Abbreviations: DTIC, dacarbazine; p.t.i., post-tumor implantation; TDLN, tumor-draining lymph node

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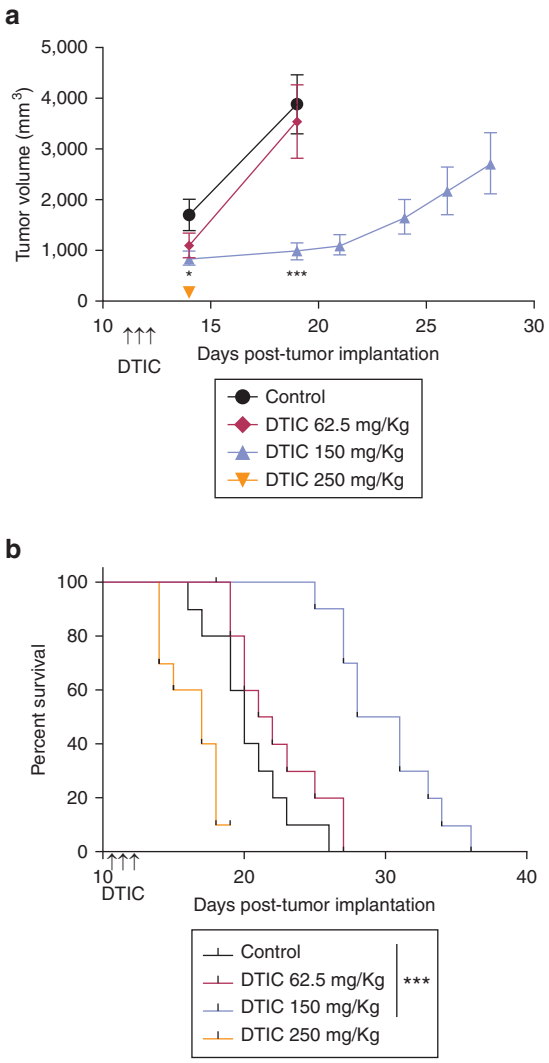

Figure 1. Standardization of DTIC treatment. Mice were treated with different doses of DTIC as described in Materials and Methods. (**a**) Tumor size was measured every 2−3 days (Student's *t*-test, $*P < 0.05$ and $***P < 0.001$). Results are shown as mean ± SEM (n = 10). (**b**) Survival was followed up for 36 days ($***P < 0.001$, log-rank test). DTIC, dacarbazine.

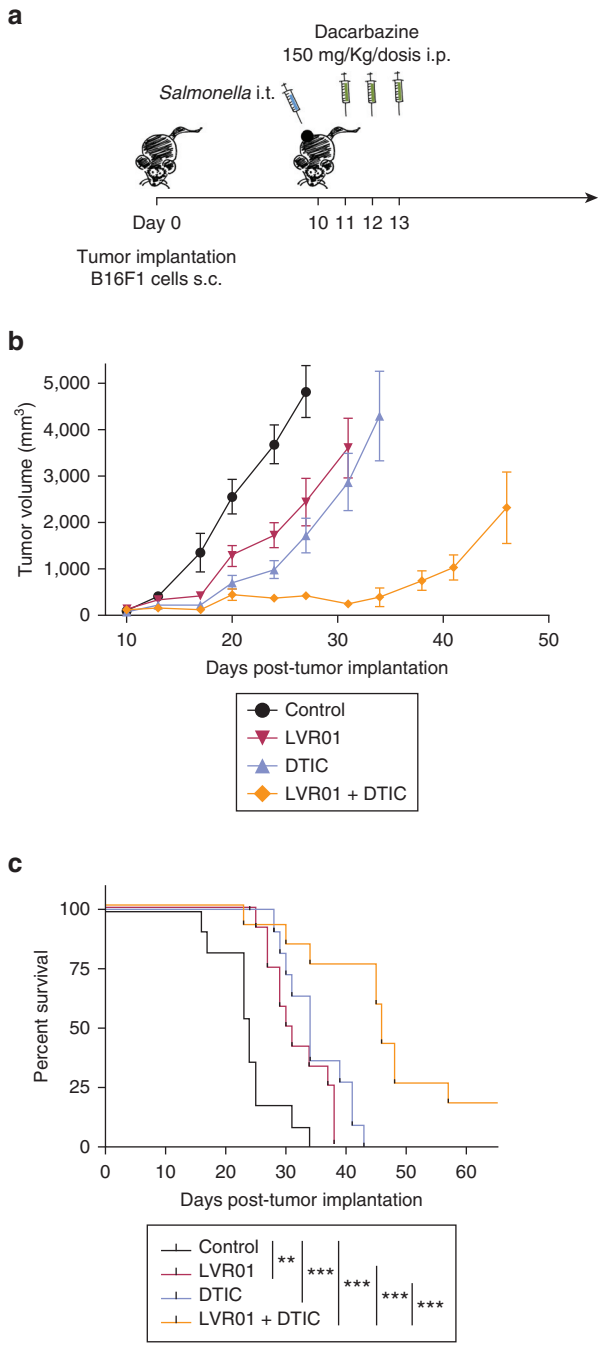

Figure 2. Tumor growth and survival in an s.c. B16F1 melanoma model. (**a**) Schematic representation of treatment schedule. Mice were treated with the different monotherapies (LVR01 or DTIC) or the combination of both (LVR01 + DTIC) as described in Materials and Methods. (**b**) Tumor size was measured every 2−3 days, and volume was calculated as (L × W × D) × π / 6. Results are shown as mean ± SEM (n = 12). **(c)** Survival was followed up for 100 days ($**P < 0.01$ and $***P < 0.001$, log-rank Test). One representative experiment of three was performed. D, depth; DTIC, dacarbazine; i.p., intraperitoneal; i.t., intratumoral; L, length; s.c., subcutaneous; W, width.

tumor microenvironment, showing tropism for necrotic and ischemic areas, where conventional therapies, such as chemotherapy and radiotherapy cannot reach. *Salmonella* can exert intrinsic antitumor effects and foster the immune system by inducing inflammation (review in Zhou et al. [2018]).

We have previously shown that intratumoral administration of LVR01, an attenuated *Salmonella enterica* serovar typhimurium, could be considered a potential treatment for melanoma, breast cancer, and B-cell non-Hodgkin lymphoma because it retards tumor growth and dissemination and thereby prolongs overall survival (Grille et al., 2014; Kramer et al., 2015; Vola et al., 2018). Moreover, LVR01 administration to B-cell non-Hodgkin lymphoma–bearing mice undergoing cyclophosphamide, doxorubicin, vincristine, and prednisone treatment resulted in increased therapeutic efficiency and enhanced overall health status of chemotherapy-treated mice (Bascuas et al., 2018).

Neoadjuvant therapy is emerging as a therapeutic option to manage melanoma in patients with advanced disease (Sun et al., 2019). In this study, we evaluate the potential of *Salmonella* LVR01 as neoadjuvant intralesional therapy in combination with DTIC chemotherapy in a preclinical melanoma model.

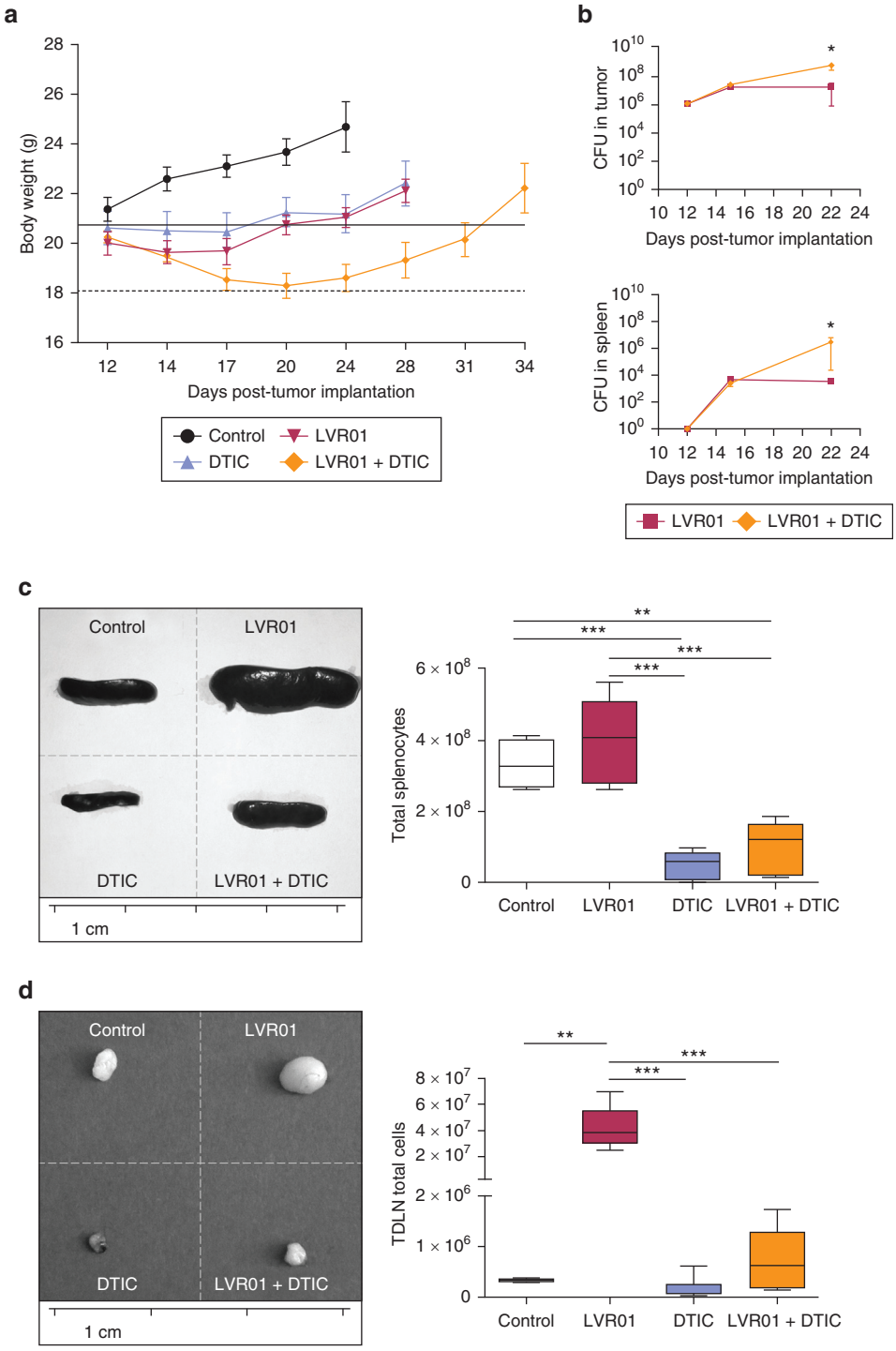

Figure 3. Treatment tolerability. Mice were treated with the different monotherapies or the combination of both as described in Materials and Methods. (**a**) Bodyweight was measured every 2–3 days. Results are shown as mean ± SEM (n = 12). The dotted line represents 10% of body weight loss related to the beginning of chemotherapy treatment (day 12 post-tumor implantation). (**b**) LVR01 biodistribution in tumor (top) and spleen (bottom). (**c**) Spleens and (**d**) TDLN were removed on day 27 post-tumor implantation, and absolute cell number in each organ was determined. Results are shown as mean ± SEM (n = 5–8) (ANOVA with Tukey's HSD test, Student's *t*-test, $^{**}P < 0.01$ and $^{***}P < 0.001$). One representative experiment of two was performed. CFU, colony-forming unit; DTIC, dacarbazine; HSD, honestly significant difference; TDLN, tumor-draining lymph node.

RESULTS

LVR01 neoadjuvant immunotherapy retards tumor growth and prolongs overall survival in melanoma-bearing mice undergoing chemotherapy

We first sought to set up a DTIC administration schedule on our melanoma model on the basis of previously published works (Hervieu et al., 2013; Jin et al., 2011; Wolf et al., 2006). Three different DTIC doses (250, 150, and 62.5 mg/kg) were assayed in a daily intraperitoneally administration schedule for 3 consecutive days: 11, 12, and 13 days post-tumor implantation (p.t.i.). The highest DTIC dose, 250 mg/kg, was found to be toxic, and all mice died a few days after the beginning of treatment (Figure 1a and b). The lowest dose, 62.5 mg/kg, showed no evidence of antitumor effect. However, the intermediate dose, 150 mg/kg, caused a delay in tumor growth ($P = 0.0002$ compared with that in the control group, Student *t*-test) in the absence of toxicity and hence prolonged overall survival ($P < 0.0001$ compared with that in the control group, log-rank test) (Figure 1a and b). This dosage was used for all subsequent experiments.

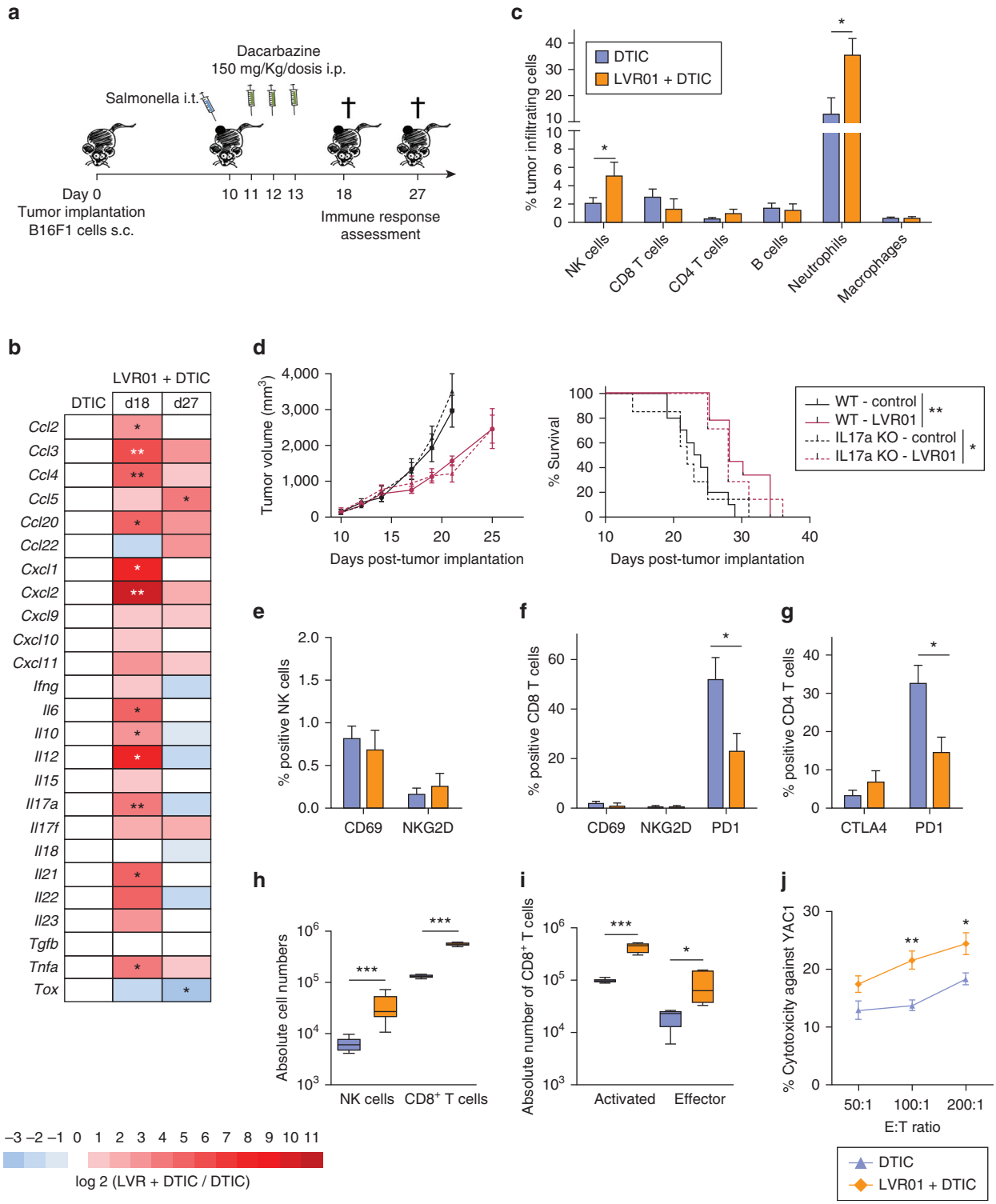

Figure 4. Immunomodulatory effects of LVR01 neoadjuvant intralesional administration in mice undergoing chemotherapy. (**a**) Schematic representation of treatment schedule. Tumors, TDLNs, and spleens were removed on days 18 and 27 post-tumor implantation. (**b**) Cytokine/chemokine profile in LVR01 + DTIC–treated mice, normalized against DTIC-treated mice. Mean $\log_2$(LVR01 + DTIC/DTIC) (n = 5–6). (**c**) Tumor immune cell phenotypification. (**d**) Tumor size and survival of *Il17a*-KO or WT mice treated with LVR01. Results are shown as mean ± SEM (n = 10 per group). Survival was followed up for 65 days (*$P < 0.05$ and **$P < 0.01$, log-rank test). (**e**) CD69 and NKG2d expression within tumor-infiltrating NK cells. (**f**) CD69, NKG2D, and PD-1 expression within tumor-infiltrating CD8$^+$ T cells. (**g**) CTLA4 and PD-1 expression within tumor-infiltrating CD4$^+$ T cells. (**h**) Absolute numbers of cytotoxic lymphocytes in TDLN. (**i**) Activated (CD44$^-$ CD62L$^{+/-}$ CD127$^{+/-}$) and effector (CD44^{+++} CD62L$^-$ CD127$^{+/-}$) CD8$^+$ T cells in TDLN. (**j**) Cytotoxic assay performed using splenocytes as E and NK-sensitive YAC1 cells as T by CFSE method. All results are shown as mean ± SEM (n = 5–11). Student's *t*-test, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. One representative experiment of four was performed. CFSE, carboxyfluorescein succinimidyl ester; DTIC, dacarbazine; E, effector; i.p., intraperitoneal; i.t., intratumoral; KO, knockout; s.c., subcutaneous; T, target; TDLN, tumor-draining lymph node; WT, wild type.

Table 1. Biodistribution of *Salmonella* in B16F1 Tumor–Bearing Mice on Day 5 Post-Bacteria Administration

Strain	CFU in Tumor	CFU in Spleen	Tumor-to-Spleen Ratio
LVR01	2×10^8	7×10^3	30,000:1
SL3261	1×10^8	1×10^5	1,000:1

Abbreviation: CFU, colony-forming unit.
The mean of CFU per gram of tissue is presented; n = 3.

Then, we evaluated the potential of *Salmonella* LVR01 as neoadjuvant therapy for melanoma. Bacteria (1×10^6 colony-forming units) were intratumorally administered on day 10 p.t.i. when melanoma became palpable, only 1 day before DTIC treatment began (Figure 2a). As previously described, DTIC (mentioned earlier) and *Salmonella* (Vola et al., 2018) monotherapies both resulted in tumor growth retard (Figure 2b). The combination of *Salmonella* LVR01 plus DTIC resulted in a synergistic effect on tumor growth delay, with a coefficient of drug interaction <1 (Bliss Independence model) (Figure 2b). The area under the curve$_{10-27dpi}$ were 35,336, 16,893, 10,168, and 4,635 for control, LVR01, DTIC, and LVR01 plus DTIC groups, respectively (Figure 2b). Consequently, *Salmonella* LVR01 neoadjuvant treatment prolonged overall survival compared with that in the control group and with that in the LVR01 and DTIC monotherapy groups ($P < 0.0001$, $P = 0.0005$, and $P = 0.0008$, respectively; log-rank test) (Figure 2c). The median survivals were 24, 32, 34, and 42 days p.t.i. for control, LVR01-, DTIC-, and LVR01 plus DTIC–treated mice, respectively.

The same strong antitumor effect was observed during treatment with the combined therapy in highly metastatic B16F10 melanoma–bearing mice (Supplementary Figure S1).

The neoadjuvant therapy was well-tolerated with <10% of transient weight loss (Figure 3a). Ten days after *Salmonella* administration, a higher bacterial load was found in the tumor as well as in the spleen in mice undergoing chemotherapy (Figure 3b). DTIC treatment induced a marked reduction in the spleen and tumor-draining lymph nodes (TDLNs) sizes and cellularity, whereas *Salmonella* treatment induced splenomegaly and TDLN enlargement. Hence, spleens and TDLNs from combined therapy–treated mice regained their normal appearance (Figure 3c and d), even though the absolute splenocyte numbers were not fully recovered (Figure 3d).

LVR01 induces inflammation and activation of cytotoxic lymphocytes in DTIC-treated mice

Salmonella-mediated antitumor effect is mostly attributed to its capacity to elicit a broad immune activation. We then investigated the potential of this nonspecific active treatment

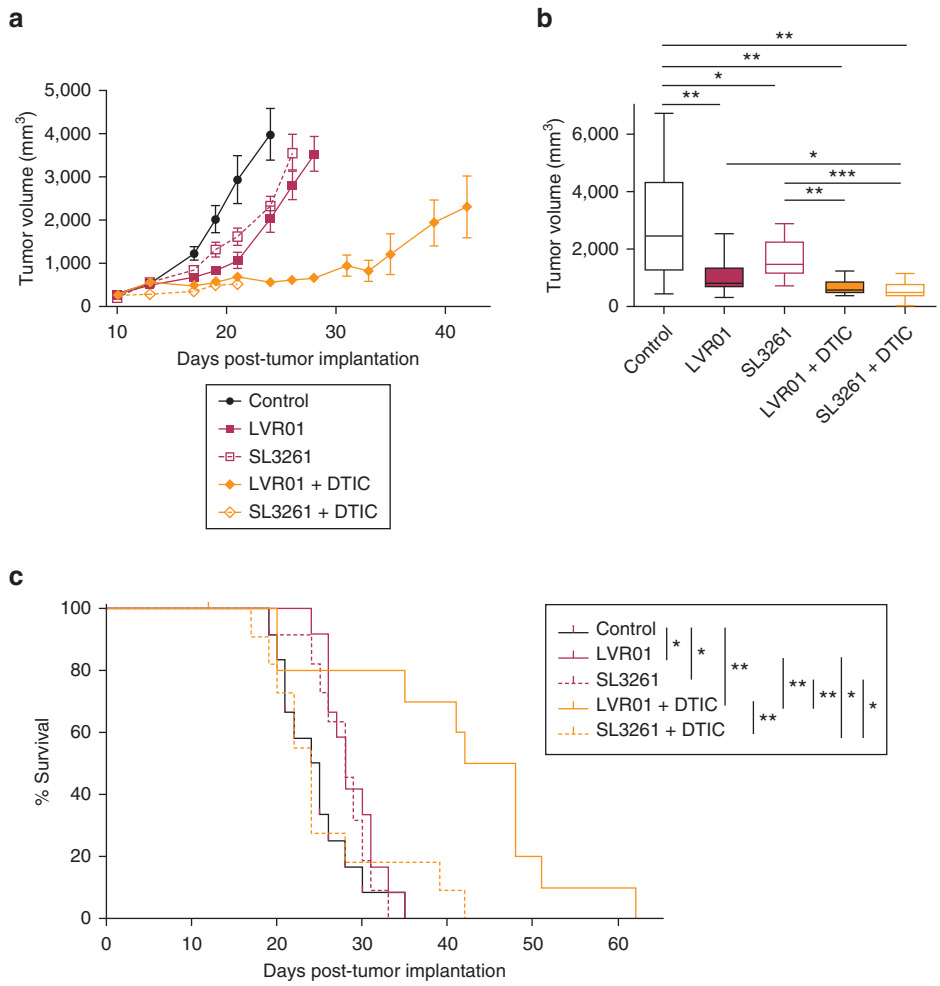

Figure 5. Tumor growth and survival with *Salmonella* SL3261 neoadjuvant treatment. B16F1 melanoma cells were implanted as previously described. Mice were then treated with the different *Salmonella*-based monotherapies (LVR01 or SL3261) or as neoadjuvant therapies with dacarbazine (LVR01 + DTIC or SL3261 + DTIC) as described in Materials and Methods. (**a**) Tumor size was measured every 2–3 days, and volume was calculated as (L × W × D) × π / 6. (**b**) Box-plot of tumor volume on day 21 post-tumor implantation (ANOVA with Tukey's honest significance difference test, Student's *t*-test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). Results are shown as mean ± SEM (n = 11–12). (**c**) Survival was followed up for 65 days ($*P < 0.05$ and $**P < 0.01$, log-rank test). One representative experiment of two was performed. D, depth; DTIC, dacarbazine; L, length; W, width.

in mice undergoing DTIC because chemotherapy is known to be cytotoxic for immune cells, in addition to tumor cells. Chemokine/cytokine transcriptional profile in the tumor microenvironment was determined 8 and 17 days after *Salmonella* LVR01 therapy in DTIC-treated mice (on days 18 and 27 p.t.i.; Figure 4a and b). Albeit DTIC treatment, *Salmonella* generated a proinflammatory tumor microenvironment characterized by increased expression of many chemokine and cytokine mRNAs. We observed increased expression of *Ccl2*, *Ccl3*, *Ccl4*, *Ccl20*, *Cxcl1*, *Cxcl2*, *Il6*, *Il10*, *Il12*, *Il17a*, and *Il21* mRNA levels on day 18 p.t.i., which slowly dissipated by day 27 p.t.i. (Figure 4b).

An increase in the frequency of NK cells and neutrophils could be detected as a consequence of *Salmonella* treatment by day 27 p.t.i. ($P = 0.0490$ and $P = 0.0167$, respectively; Student *t*-test) (Figure 4c). Despite increased IL-17–related gene expression and neutrophil infiltration on *Salmonella* treatment, this axis is not involved in *Salmonella*-mediated antitumor response as seen in experiments performed in *Il17a*-knockout mice (Figure 4d).

Salmonella administration also resulted in a marked reduction in PD-1 expression on tumor-infiltrating T cells compared with DTIC-alone treatment ($P = 0.0108$ and 0.0244 for $CD4^+$ and $CD8^+$ T cells, respectively; Student *t*-test) (Figure 4f and g), without affecting CD69, NKG2D, nor CTLA4 expression in lymphocytes (Figure 4e–g). In addition, a decrease in Tox transcription factor, which plays a crucial role in controlling the differentiation of exhausted T cells, was observed by day 27 p.t.i. (Figure 4b).

The effect of *Salmonella* treatment was more evident in lymphoid tissues, such as the spleen and TDLN. In these tissues, LVR01 treatment induced the expansion of cytotoxic lymphocyte populations. Higher numbers of NK and $CD8^+$ T cells were found in the TDLNs of mice receiving LVR01 plus DTIC compared with that in DTIC-treated mice ($P = 0.0006$ and $P < 0.0001$, respectively; Student *t*-test) (Figure 4h). Particularly, activated ($CD44^-$ $CD62L^{+/-}$ $CD127^{+/-}$) and effector ($CD44^{+++}$ $CD62L^-$ $CD127^{+/-}$) $CD8^+$ T-cell populations were expanded by *Salmonella* neoadjuvant treatment ($P < 0.0001$ and $P = 0.0226$, respectively; Student *t*-test) (Figure 4i). In addition, splenic NK cells from LVR01 plus DTIC-treated mice exhibited higher cytotoxic activity against NK-sensitive YAC1 cells (Figure 4j).

Neoadjuvant administration of *Salmonella* typhimurium LVR01 but not SL3261 prolongs the survival of melanoma-bearing mice undergoing chemotherapy

Finally, we evaluated the potential of another *Salmonella* typhimurium strain, SL3261, an *aroA* mutant that has been well-characterized, as neoadjuvant intralesional treatment in melanoma-bearing mice undergoing chemotherapy. In the B16F1 model, both strains accumulated in the tumor, but SL3261 showed higher systemic dissemination (Table 1). Both strains displayed comparable antitumor activity when used as monotherapy, showing similar tumor growth and overall survival curves (Figure 5). *Salmonella* SL3261 in combination with DTIC showed a potent early antitumor effect, evidenced by a retard in tumor growth (Figure 5a). By day 21 p.t.i., tumor volumes were 2,944 ± 550, 680 ± 101, and 529 ± 128 mm^3 for control, LVR01 plus DTIC, and SL3261 plus DTIC groups (Figure 5b). However, despite constraining tumor progression, SL3261 administration in combination with DTIC boosted mice susceptibility to therapy-related side effects, increasing animal mortality (Figure 5c). Thus, the potential of *Salmonella* neoadjuvant treatment is strain dependent, being SL3261 deleterious and LVR01 beneficial for mice undergoing DTIC treatment.

DISCUSSION

Chemotherapy, particularly DTIC, was routinely used at the bedside to treat inoperable metastatic melanoma before the recently approved therapies. For many years, the use of DTIC has been only focused on its cytotoxic activity against tumor cells, with doubtful clinical results. However, there is accumulating evidence to reconsider DTIC antitumor potential owing to its immunomodulating properties (Ugurel et al., 2013). Alkylating agents, such as DTIC, can attenuate immunosuppressive tumor microenvironment and increase the potential of effector immune cells against transformed cells (Fritzell et al., 2013; Hervieu et al., 2013; Tan et al., 2015).

DTIC does not act directly on immune cells. Instead, it triggers the upregulation of the expression of NKG2D ligands on tumor cells, leading to NK cell activation. Consequently, NK cells produce IFN-γ, which subsequently induces the upregulation of major histocompatibility complex class I molecules on tumor cells, rendering them sensitive to cytotoxic cells (Hervieu et al., 2013). DTIC also decreases the expression of PD-L1 on tumor cells and the production of CCL22, which suggests that DTIC might suppress the recruitment of regulatory T cells in the tumor site (Fujimura et al., 2018). Altogether, these mechanisms lead to an abrogation of the suppressive function of T-cell proliferation.

Nevertheless, DTIC monotherapy has shown low response rates and overall survival benefits. Large amounts of studies combining different chemotherapies failed to improve the clinical responses obtained with single agents (reviewed in Wilson and Schuchter [2016]). However, combining chemotherapy with another therapy, such as radiotherapy or immunotherapy, has been recently considered as an alternative approach because it helps to restrain tumor plasticity to acquire resistance. Furthermore, the use of therapies in neoadjuvant settings is attaining interest, particularly for metastatic melanoma, because it can reduce the size of locally advanced tumors and induce long-lasting strong immune responses (reviewed in Sun et al. [2019]). In some situations, intralesional approach has been contemplated as an alternative use of already developed therapies among others. The increasing interest in this type of treatment modality resides in the potential to generate favorable local responses that can be durable as well as potentiate systemic immune responses, with minimal toxicities. Besides, this approach induces a bystander effect, where noninjected lesions also respond to the treatment. It has been shown to be promising in patients with locoregional disease. In 2015, the Food and Drug Administration approved the first intralesional therapy, talimogene laherparepvec (modified oncolytic virus encoding *GM-CSF* gene), for the treatment of metastatic melanoma lesions in the skin and lymph nodes (Andtbacka et al., 2015).

Intralesional therapy was first reported in 1893 by Coley, who treated patients with a bacterial agent. Microbial-based anticancer therapy is being reconsidered (Forbes et al., 2018). Its potential relies on the capacity of the bacteria to induce, reactivate, or amplify a pre-existing antitumor immune response. We have previously shown that *Salmonella* typhimurium LVR01 is a potent antitumor agent for melanoma and B-cell non-Hodgkin lymphoma. LVR01 induces a broad immune response, characterized by a proinflammatory tumor microenvironment and systemic tumor-specific humoral and cellular immune response (Bascuas et al., 2018; Grille et al., 2014; Vola et al., 2018). This phenomenon could be explained by the fact that tumor antigens released at the site of LVR01 injection may serve as an autologous vaccine that in the presence of a proinflammatory microenvironment produced also by the bacteria stimulated a potent systemic immunity.

In view of the relevance of this setting, we evaluated the potential of LVR01 intralesional treatment in primary tumors in combination with DTIC. Although the B16F1 model has its limitations, such as low metastatic power and absence of relevant mutations among others, it is still relevant for primary assessment of immune-related aspects. DTIC treatment dampens immune cell compartments, which were restored on *Salmonella* LVR01 treatment. *Salmonella* LVR01 triggered a broad antitumor immune response that helped to partially counteract DTIC-mediated immunosuppression, facilitating the resetting of immunity by inducing a proinflammatory tumor microenvironment and a restock of immune cells in lymphoid organs. Particularly, neoadjuvant *Salmonella* LVR01 treatment favored the activation of cytotoxic lymphocytes, such as $CD8^+$ T cells and NK cells, known for their potent antitumor activity, resulting in longer overall survival. Similarly, the combination of CpG with DTIC showed a strong antitumor effect mediated by tumor-specific cytotoxic lymphocytes. In this setting, the antitumor response was dependent on both $CD4^+$ and $CD8^+$ T cells but not on NK cells (Najar and Dutz, 2008). Engagement of NKG2D with its ligand MICA, a stress-induced molecule, activated cytolytic responses of T cells and NK cells against tumor cells. *Salmonella* does not potentiate DTIC-mediated immune effect because no differences in CD69 and NKG2D expression were found within cytotoxic lymphocyte populations on bacteria administration in mice undergoing DTIC treatment (Figure 4d and e) nor CTLA-4 expression (Figure 4f). Instead, a decrease in PD-1 expression in T cells and *Tox* transcription factor on LVR01 treatment in DTIC-treated mice was found, suggesting a mechanism of reverting T-cell reinvigoration, by reverting either dysfunction and exhaustion, respectively.

LVR01 induces an upregulation of the expression of chemokines and cytokines, with a marked T helper type 1/T helper type 17 profile in the tumor microenvironment, which could recruit cytotoxic lymphocytes as well as repolarize immune cells, such as tumor-associated neutrophils and macrophages, into antitumoral phenotypes, as already reported (Fujimura et al., 2018; Yang et al., 2018). Thus, *Salmonella* helps to transform tumor microenvironment from an immune-suppressive to an immune-permissive one, restoring anticancer immunity. In contrast, DTIC constrains tumor growth by inducing cell death, assisting the immune system with time to sculpt an antitumor response and less tumor burden to cope with.

The use of attenuated *Salmonella* as nonspecific active immunotherapy combined with standard chemotherapy in melanoma would be an interesting alternative therapeutic strategy, which could be easily moved into clinical trials. Nevertheless, selecting the appropriate *Salmonella* strain is of particular concern. Our results show that the efficacy of *Salmonella* neoadjuvant therapy in combination with chemotherapy is strain dependent, being that LVR01 is of great value for melanoma as we also previously showed for B-cell non-Hodgkin lymphoma (Bascuas et al., 2018). Another attenuated *Salmonella* typhimurium, SL3261, which was already successfully used by us as an oral vector vaccine for melanoma (Agorio et al., 2007), fails to potentiate the DTIC-mediated antimelanoma effect. Although both bacteria strains showed comparable tumor accumulation and antitumor activity as monotherapy (Table 1 and Figure 5), only LVR01 enhanced the response to chemotherapy. The combination of SL3261 with DTIC boosted mice susceptibility to therapy-related toxic effects, increasing mouse mortality, probably because DTIC treatment augments vulnerability to *Salmonella* systemic infection (Figure 3b), and SL3261 is more prone to systemic dissemination than LVR01 (Table 1). Because the LVR01 parental strain was isolated from dogs (Chabalgoity et al., 2000), it could be less virulent for mice. Certainly, LVR01 treatment to B-cell non-Hodgkin lymphoma–bearing mice under cyclophosphamide, doxorubicin, vincristine, and prednisone treatment helps to enhance the overall health status of mice undergoing chemotherapy (Bascuas et al., 2018), a benefit not obtained to such extent for melanoma. Safety evaluation should be considered for LVR01 as well as for other attenuated *Salmonella* strains for its use in humans undergoing chemotherapy regimens. Still, even in the worst scenario, that is, bacteria dissemination, the situation can be easily controlled with antibiotic treatment because most *Salmonella* strains are sensitive to a wide range of antibiotics.

In conclusion, LVR01 could be considered as neoadjuvant therapy for the treatment of melanoma because the combination with DTIC induced the activation of both innate and adaptive cytotoxic lymphocytes, resulting in longer survival. We believe that the use of attenuated *Salmonella* LVR01 as non-specific active immunotherapy combined with standard chemotherapy in melanoma would be an interesting alternative therapeutic strategy, which could be rapidly moved into clinical trials.

MATERIALS AND METHODS

Tumor cell lines

B16F1 and B16F10 melanoma cells were purchased from ATCC (Manassas, VA) (CRL-6323 and CRL-6475, respectively) and maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% carbon dioxide atmosphere.

Bacterial strains

S. enterica serovar typhimurium LVR01, an attenuated strain constructed by introducing a null deletion into the *aroC* gene of the parental canine *Salmonella* typhimurium isolate P228067

(Chabalgoity et al., 2000), and *S. enterica* serovar typhimurium SL3261, an attenuated strain constructed by introducing a null deletion into the *aroA* gene of the parental *Salmonella* typhimurium SL1344 (Hoiseth and Stocker, 1981), were used in this study. Bacteria were grown at 37 °C in Luria–Bertani media shaking at 200 r.p.m. overnight and stored at −80 °C in 15% glycerol stocks until they were used.

Chemotherapy

DTIC was purchased in the form of a white powder from Fármaco Uruguayo (Montevideo, Uruguay), reconstituted by the addition of water for injections according to the manufacturer's instructions, and used immediately.

Mice

Female C57BL/6 mice (Dilave, Montevideo, Uruguay) and *Il17a*-knockout mice (IPMont, Montevideo, Uruguay), aged 6−8 weeks, were used for in vivo experiments. Mice were housed on 12:12 hours of light/dark cycles with controlled temperature (22 ± 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) (experiment number 071140-002259-12).

DTIC treatment setup

C57BL/6 mice were subcutaneously inoculated with 2.5×10^5 B16F1 melanoma cells. Chemotherapy treatment consisting of the daily intraperitoneal application of DTIC at 62.5, 150, or 250 mg/kg per dose was started on day 11 pti and continued for 3 days. Tumor size was measured every 2−3 days, and tumor volume was calculated as (length × width × depth) × π / 6. Mice were killed by cervical dislocation when tumor volume exceeded 4,000 mm^3 or before they showed any sign of distress.

In vivo melanoma tumor model and treatment

C57BL/6 or *Il17a*-knockout mice (when specified) were subcutaneously inoculated with 2.5×10^5 B16F1 or B16F10 melanoma cells. Mice were divided into four groups: control, *Salmonella* (LVR01), DTIC, and combined therapy (Salmonella plus DTIC) groups. When tumors were palpable (day 10 pti), *Salmonella* typhimurium LVR01 (1×10^6 colony-forming units) were intratumorally injected. On the following day (11 days pti), chemotherapy treatment consisting of daily intraperitoneal application of DTIC 150 mg/kg was started and continued for 3 days. Mice were followed up every 2−3 days, as described earlier. When needed, bacteria counts were determined by removing organs and plating dilutions in Luria–Bertani agar.

Absolute numbers

On day 18p.t.i. and 27 p.t.i., mice were killed, and tumors, TDLNs, and spleens were removed and prepared to obtain a single-cell suspension. Cell numbers were determined using Cellometer K2 automatic cell counter (Nexcelom Bioscience LLC, Lawrence, MA).

Flow cytometry analysis

On day 18 p.t.i. and 27 p.t.i., mice were killed, and tumors and TDLN were removed and prepared to obtain a single-cell suspension. Cells were immunostained at 4 °C in the dark for 30 minutes with antibodies against CD3, CD4, CD8, CD11b, CD11c, CD19, CD44, CD49b, CD62L, CD69, CD127, CD152 (CTLA-4), CD274 (PD-1), F4/80, Gr1, major histocompatibility complex II, and NKG2D (all reagents from BD Pharmingen, San Diego, CA). Absolute cell numbers were obtained using CountBright absolute counting beads (Invitrogen, Waltham, MA), according to the manufacturer's instructions. Flow cytometry data were acquired on a FACS Canto II cytometer and analyzed using FACS Diva software (BD Biosciences, Oxford, United Kingdom). Flow cytometry gating strategies are illustrated in Supplementary Figure S2.

Gene expression analysis

On days 18 and 27 p.t.i., mice were killed, and tumors were removed and collected in TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −80 °C until they were processed as previously described (Vola et al., 2018). The sequences of the primers used in this study are available on request.

NK cell cytotoxicity assay

Spleens were removed from mice on day 27 p.t.i. and prepared to obtain a single-cell suspension. NK cytotoxicity assay was performed using YAC-1 cells (TIB-160, ATCC) as target cells, as previously described (Vola et al., 2018).

Statistical analysis

Differences in survival times were determined using Kaplan−Meier and log-rank tests. Tumor growth analysis was performed by calculating the area under the curve, and the drug combination effect was evaluated using the Bliss Independence model (Foucquier and Guedj, 2015). For ex vivo assays, the statistical significances of differences between groups were analyzed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: CIA, MV, MM; Formal Analysis: SC, MM; Funding Acquisition: MV, MM; Investigation: SC, AM, MCP, MM; Methodology: SC, MM; Supervision: MM; Visualization: MM; Writing - Original Draft Preparation: SC, AM, MM; Writing - Review and Editing: AM, JAC, MM

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.08.442.

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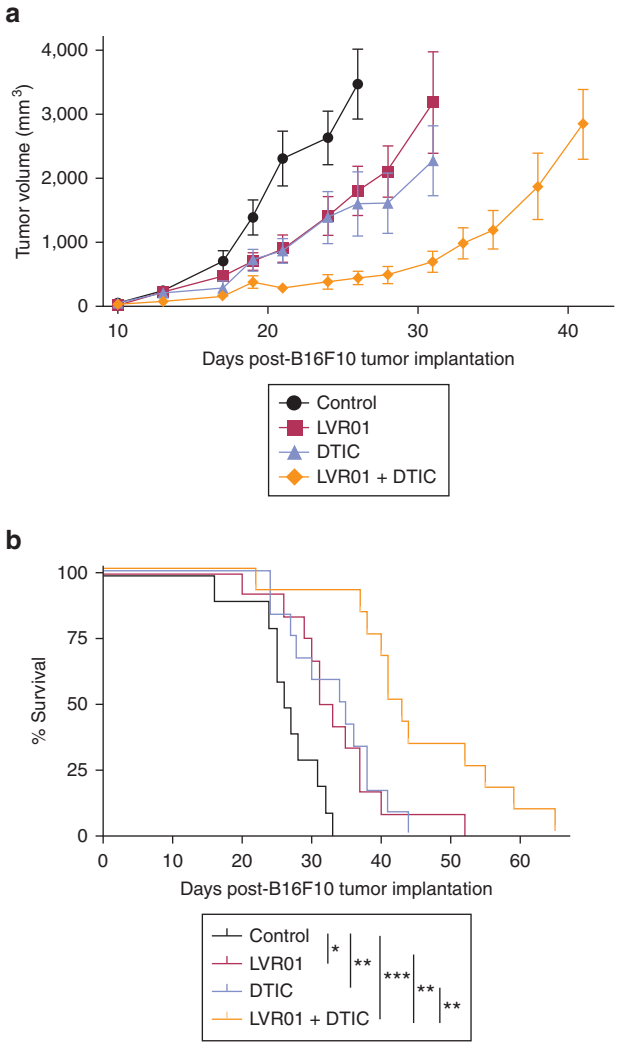
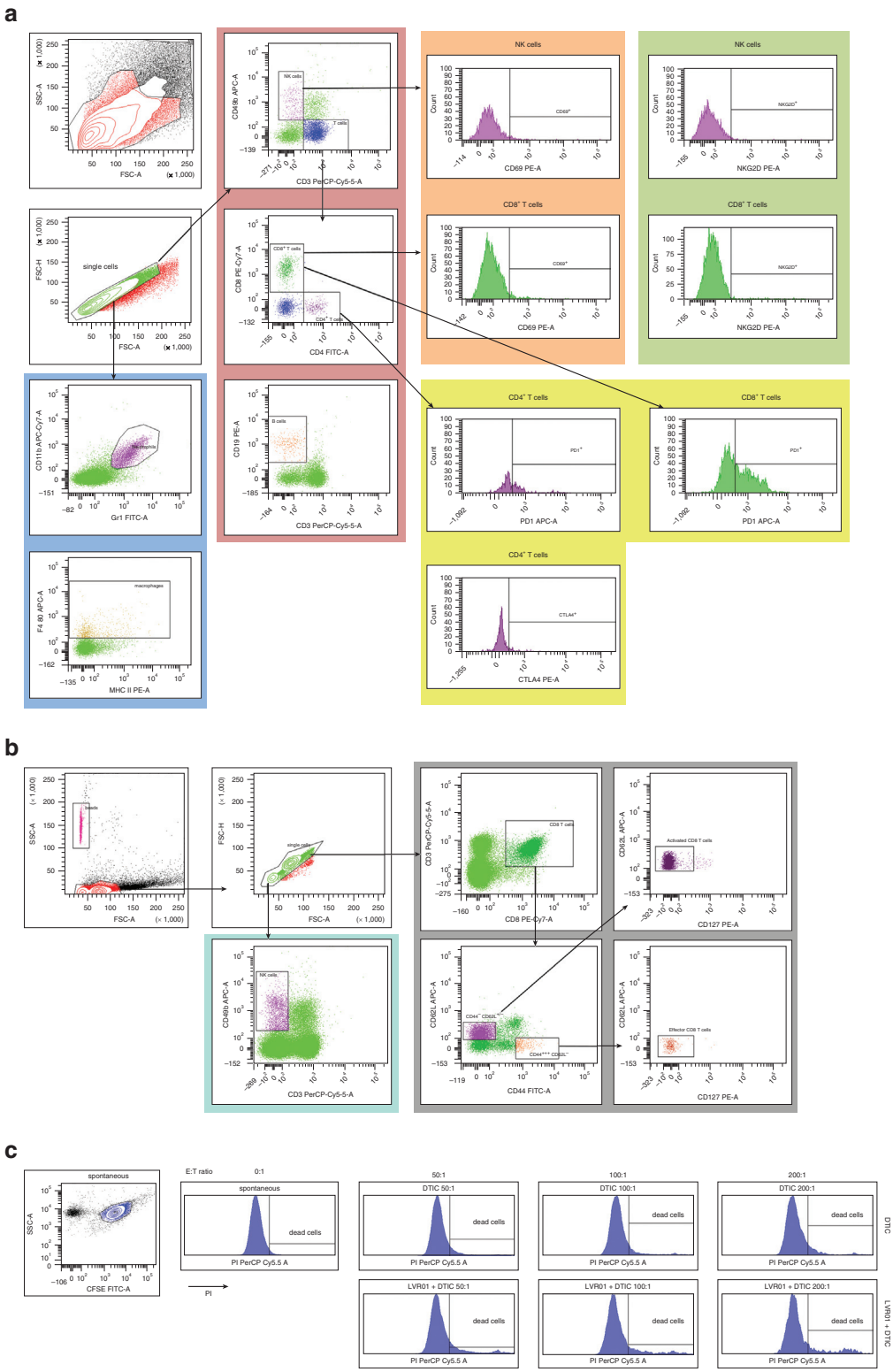
SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Tumor growth and survival in a subcutaneous highly metastatic B16F10 melanoma model. B16F10 melanoma tumors were subcutaneously implanted. Mice were treated with the different monotherapies (LVR01 or DTIC) or the combination of both (LVR01 + DTIC) as described for the B16F1 melanoma model. (**a**) Tumor size was measured every 2−3 days. Results are shown as mean ± SEM (n = 12). (**b**) Survival was followed up for 65 days (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, log-rank test). DTIC, dacarbazine.



Supplementary Figure S2. Flow cytometry analysis gating strategy. (**a**) Tumor homogenates were stained for CD4-FITC, CD19-PE, CD3-PerCP Cy5.5, CD8-PE Cy7, and CD49b-APC to define B cells ($CD19^+$ $CD3^-$), NK cells ($CD49b^+$ $CD3^-$), $CD8^+$ T cells ($CD8^+$ $CD4^-$ within $CD3^+$ cells), and $CD4^+$ T cells ($CD4^+$ $CD8^-$ within $CD3^+$ cells) (purple background) or with Gr1-FITC, MHC II-PE, F4/80-APC, and CD11b-APC Cy7 to define neutrophils ($CD11b^+$ $Gr1^+$) or macrophages ($F4/80^+$) (blue background) to determine tumor-infiltrating cell populations (gating strategy for Figure 4c). Tumor homogenates were also stained for CD4-FITC, NKG2D-PE or CD69-PE, CD3-PerCP Cy5.5, CD8-PE Cy7, and CD49b-APC to evaluate CD69 (orange background) and NKG2D (green background) expression on NK and CD8 T cells and with CD4-FITC, CTLA-4-PE, CD3-PerCP Cy5.5, CD8-PE Cy7, and PD-1-APC to evaluate CTLA-4 expression on CD4 T cells and PD-1 expression on $CD4^+$ and $CD8^+$ T cells (yellow background) (gating strategy for Figure 4e–g). (**a**) TDLN homogenates were stained for CD4-FITC, CD19-PE, CD3-PerCP Cy5.5, CD8-PE Cy7, and CD49b-APC to define NK cells ($CD49b^+$ $CD3^-$) and $CD8^+$ T cells ($CD8^+$ $CD4^-$ within $CD3^+$) (gating strategy for Figure 4h). TDLN homogenates were also stained for CD44-FITC, CD127-PE, CD3-PerCP Cy5.5, CD8-PE Cy7, and CD62L-APC to define activated ($CD44^-$ $CD62L^{+/-}$ $CD127^{+/-}$) and effector ($CD44^{+++}$ $CD62L^-$ $CD127^{+/-}$) within $CD8^+$ T cells (gray background) (gating strategy for Figure 4i). CountBright beads were included. (**c**) Cell death (PI^+) was determined within YAC1 cells ($CFSE^+$), and percentage of cell death was calculated as described in Materials and Methods (gating strategy for Figure 4j). APC, allophycocyanin; CFSE, carboxyfluorescein succinimidyl ester; E, effector; FSC-A, forward scatter area; FSC-H, forward scatter height; MHC, major histocompatibility complex; PE, phycoerythrin; PI, propidium iodide; SSC-A, side scatter area; T, target; TDLN, tumor-draining lymph node.