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Engineered Salmonella expressing IL-18 as a therapeutic strategy for colorectal cancer

Talita Motta Quiarim

tmquiarim@estudante.ufscar.br

Federal University of São Carlos (UFSCar) https://orcid.org/0000-0003-3740-9021

Bruna Fragelli

Universidade Federal de São Carlos

Adilson Silva

Universidade Federal de São Carlos

Maria Novo-Mansur

Universidade Federal de São Carlos

Rebecca Kesselring

Deutschen Konsortium für Translationale Krebsforschung

Fernanda Anibal

Universidade Federal de São Carlos

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Abstract

Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality worldwide, largely due to therapeutic resistance and metastatic progression. Novel strategies capable of targeting the tumor microenvironment and modulating key oncogenic pathways are urgently needed. In this study, we investigated the antitumor effect of attenuated *Salmonella* Typhimurium SL3261 genetically engineered to express interleukin-18 (IL-18) in both precursor (pro-IL-18) and bioactive (IL-18) forms. Treatment with bacterial supernatants significantly reduced cell viability in colorectal cancer cell lines (CT-26 and HCT-8), mouse organoids and patient-derived organoids. Notably, organoids exhibited pronounced sensitivity, with near-complete loss of viability after prolonged exposure, particularly in response to bioactive IL-18. Co-culture experiments further demonstrated enhanced cytotoxicity, with IL-18–expressing bacteria inducing a rapid and sustained reduction in organoid viability. IL-18 delivery was linked with modulation of tumor-associated inflammatory responses. These findings suggest a shift towards a pro-inflammatory and antitumor microenvironment. The effects observed with recombinant IL-18 alone partially recapitulated the bacterial-mediated responses, indicating a synergistic contribution of bacterial factors and cytokine signalling. Overall, our results demonstrate that *Salmonella*-mediated delivery of IL-18 exerts potent antitumor effects in CRC models, highlighting its potential as a novel immunotherapy.

1. INTRODUCTION

Colorectal cancer (CRC) remains a major global health burden, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality worldwide, with approximately 1.9 million new cases and over 900,000 deaths annually [1]. A major contributor to this high mortality is the development of metastatic disease, which occurs in a substantial proportion of patients and represents the primary determinant of poor clinical outcome [1–3]. Despite advances in early detection and treatment, the prognosis for metastatic CRC remains poor, highlighting the need for therapeutic strategies capable of targeting both primary tumours and metastatic progression [4].

CRC development is a complex and multifactorial process involving genetic alterations, environmental influences, and lifestyle factors [2]. Most cases are sporadic and evolve over a prolonged period through the adenoma–carcinoma–metastasis sequence, characterized by progressive dysregulation of key molecular pathways that control cell proliferation, survival, inflammation, and immune responses [5, 6]. Among these, aberrant activation of the Wingless-related integration site (Wnt)/β-catenin signalling pathway drives uncontrolled proliferation, while glycogen synthase kinase 3 beta (GSK-3β) regulates β-catenin stability and transcriptional activity [7, 8]. In parallel, pro-inflammatory signalling pathways such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) (including p38 MAPK) contribute to tumour progression and metastatic dissemination [8, 9]. Additionally, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway play a dual role, with signal transducer and activator of transcription 3 (STAT3) promoting tumour progression and immune evasion, whereas STAT1 is associated with antitumor immunity [7–11].

Although conventional treatments, including surgery, chemotherapy, and radiotherapy, have improved patient outcomes, they remain insufficient to control advanced and metastatic disease. Therapeutic resistance, limited drug penetration into hypoxic tumour regions, and the persistence of immunosuppressive tumour microenvironments contribute to treatment failure [6, 12]. Immunotherapy has emerged as a promising alternative by aiming to restore effective antitumor immune responses; however, its success in CRC has been limited, particularly in metastatic and microsatellite-stable tumours [13, 14].

Bacteria-based therapies have gained attention as innovative tools capable of overcoming some of these limitations [15]. Certain bacterial species, including *Clostridium*, *Listeria*, and *Salmonella*, exhibit a natural ability to preferentially colonize hypoxic and necrotic tumour regions and can be engineered to deliver therapeutic molecules directly into the tumour microenvironment [12, 15]. In addition to their delivery capacity, these microorganisms stimulate innate and adaptive immune responses and modulate key oncogenic and inflammatory pathways, including NF-κB, MAPK, and JAK/STAT signalling [9, 16–18].

Among these, *Salmonella enterica* serovar Typhimurium has been extensively studied due to its capacity to survive in diverse microenvironments and selectively accumulate in tumours and metastatic sites [15, 19]. Through activation of Toll-like receptor signalling, *Salmonella* induces robust immune responses and promotes the production of pro-inflammatory mediators, reshaping the tumour microenvironment toward an antitumor state [15, 19]. Additionally, *Salmonella* can directly impair tumour cell viability through apoptosis induction, metabolic competition, and increased tumour immunogenicity [20].

Previous studies from our research group demonstrated that recombinant *Salmonella* Typhimurium SL3261 engineered to express IL-2 and TRAIL exerts significant antitumor effects. In bladder cancer models, this system induced tumour cell death [21, 22], and similar cytotoxic effects were observed in colorectal cancer cell line (HCT-8) treated with bacterial supernatants containing these cytokines (data not published). These findings highlight the potential of this bacterial platform as a versatile immunotherapeutic strategy.

A central mechanism underlying *Salmonella*-mediated antitumor activity is the activation of inflammasomes within the tumour microenvironment, leading to caspase-1–mediated cleavage of pro-inflammatory cytokines such as interleukin-18 (IL-18) into their active forms [15]. IL-18 is a key immunoregulatory cytokine involved in gastrointestinal homeostasis and immune activation, produced as an inactive precursor that requires enzymatic processing to become biologically active [23, 24].

Once activated, IL-18 promotes interferon-γ (IFN-γ) production, enhances the cytotoxic activity of natural killer (NK) cells and CD8 + T lymphocytes, and drives Th1-type immune responses [25]. Importantly, IL-18 signalling is linked to modulation of pathways central to CRC progression, including activation of STAT1 and regulation of NF-κB and MAPK signalling, potentially counteracting pro-tumorigenic STAT3-driven responses and indirectly influencing β-catenin–mediated proliferation [25–28].

Based on this framework, we hypothesize that targeted delivery of IL-18 using engineered *Salmonella* Typhimurium SL3261 can modulate key signalling pathways involved in CRC progression, reducing tumour proliferation and enhancing antitumor immunity. Therefore, this study aims to investigate the therapeutic potential of *Salmonella* Typhimurium SL3261 expressing IL-18 in colorectal cancer models. Specifically, we evaluate the effects of precursor and bioactive IL-18 in tumour and metastatic organoids and cell lines, focusing on the modulation of JAK/STAT (STAT1/STAT3), NF-κB, Wnt/β-catenin, GSK-3β, and p38 MAPK pathways, to establish a mechanistic link between bacterial immunotherapy and pathway reprogramming in CRC.

2. MATERIAL AND METHODS

2.1. Plasmids construction

The mouse IL-18 nucleotide sequence (GeneID 16173), which is ortholog to human IL-18, has 579 base pairs (bp) that translates to 192 amino acids forming the precursor form of IL-18 (pro-IL-18) and was used in this work. The coding sequence of the Emerald Green Fluorescent Protein (EmGFP) was fused to the 5' end of the pro-IL-18 sequence and used as a fluorescent reporter to verify protein expression in the bacterial host. The fused EmGFP-pro-IL-18 coding sequence was synthesized by FastBio (Ribeirão Preto, SP, Brazil) and inserted between NedI and HindIII restriction sites of pET28a plasmid (Novagen), which was used to transform *E. coli* DH5α cells by heat shock.

For *in vivo* expression, the EmGFP-pro-IL-18 coding sequence was subcloned between the NedI and HindIII restriction sites of the pAE plasmid [29] under the control of the nirB promoter, which is activated under anaerobiosis, and has been shown to be functional in *Salmonella* Typhimurium SL3261 [21, 22]. The resulting plasmid was used as a template to obtain a similar construct but lacking the 105 initial bp from the pro-IL-18 sequence to obtain the bioactive form of IL-18 (IL-18*) [28]. This step was performed by "around-the-horn" cloning technique [30, 31] using the reverse primer PO_4^{3-}AACTTTGGTCGTCTGCATTGTACC and the forward primer CTTGTACAGTTCGTCCATACCCAGG. The PCR reaction was prepared with 50 ng of the template plasmid pAE+EmGFP-pro-IL-18, 25 μM of each primer, and 25 μL of Platinum SuperFi II PCR master mix (ThermoFisher). The reaction was run with an initial denaturation step at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 10 s, and extension at 72°C for 3 min, with a final extension step at 72°C for 5 min. The DNA template was digested by DpnI treatment at 37°C for 1 h. After that, the PCR product was gel purified and 50 ng were circularized in a reaction with 1 U T4 DNA ligase and 1 μL 10x T4 ligase buffer at room temperature, overnight. The reaction product was used to transform *E. coli* DH5α cells by heat shock, and the resulting colonies were screened for positive clones. All the constructs were confirmed by colony PCR, restriction assays, and Sanger sequencing.

2.2. *Salmonella* transformation and culture

Salmonella Typhimurium SL3261 is an attenuated strain carrying the deletion of the *aroA* gene and was kindly donated by Dr. Elisabeth Martins (Butantan Institute, Brazil). This strain was used in this work for the bacterial expression and delivery of IL-18.

The plasmids pAE+EmGFP-pro-IL-18 and pAE+EmGFP-IL-18* isolated from *E. coli* DH5α were used to transform the *Salmonella* Typhimurium SL3261 strain by electroporation using a Gene Pulser (Bio-Rad) set to 25 μF capacitance, 2.5 kV voltage, and 200 Ω resistance, and the recombinant strains were named pro_SL3261 and bio_SL3261, respectively. The transformation was confirmed by colony PCR, and the transformed cells were stored at -80°C in 10% glycerol stocks.

The recombinant strains were activated in LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar) added with 100 μg/mL ampicillin. For liquid cultures, isolated colonies were inoculated in LB medium with ampicillin and incubated for 16 h at 37°C and 220 rpm.

2.3. *In vitro* IL-18 expression by genetically modified SL3261

For *in vitro* tests, recombinant protein expression by the strains pro_SL3261 and bio_SL3261 was carried out in 50 mL conical tubes under anaerobic conditions. For each strain, a single colony was grown in liquid medium for 16 h at 37°C under agitation. Next, bacterial cells were transferred to a tube containing 15 mL of fresh medium with an adjusted Optical Density (OD) = 0.1 and were incubated at 37°C under agitation until OD = 1.0. The culture was then centrifuged at 4,000 g for 5 min, the supernatant was discarded, and fresh medium was added to the tube filling its maximum capacity to induce protein expression by the absence of oxygen (anaerobic condition). The tube was sealed with Parafilm® and maintained at 37°C for 72 h under agitation. At the end, the bacterial culture was centrifuged at 4,000 g for 5 min, and the supernatant was filtered sterilized (0.22 μm filter), and used for the *in vitro* assays. As a negative control, the SL3261 strain was submitted to the same conditions as described. Fresh cultures were prepared for every *in vitro* assay.

2.4. Fluorescence emission from IL-18 expression

One hundred microliters of bacterial supernatant were added to a black 96-well plate to measure the fluorescence intensity emitted by pro-IL-18 (pro_SL3261) and bioactive IL-18 (bio_SL3261) fused with EmGFP. The fluorescence emission was read for the excitation wavelength of 487 nm and emission wavelength of 509 nm on the SpectraMAX i3TM plate spectrophotometer (Molecular Devices, San Jose, CA, USA).

2.5. ELISA for IL-18

For the detection of IL-18 in the bacteria supernatants, enzyme-linked immunosorbent assay (ELISA) was used. The assay was conducted using pre-coated 96 well plate from the Mouse IL-18 ELISA Kit (ThermoFisher), the assay was conducted according to the manufacturer's manual instructions.

2.6. Colorectal cancer cell lines and organoids

Mouse cell lines, CT26-WT colon cancer cells and human colon adenocarcinoma HCT-8 cells, were obtained from Rio de Janeiro's cell bank (Rio de Janeiro, BR). The cell lines were grown in Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, LGC Biotechnology) and 5 mL penicillin/streptomycin maintained at 37°C in 5% CO_2 incubator.

APTAK organoids, an aggressive tumour organoid from mouse intestinal epithelial cells engineered to be devoid of adenomatous polyposis coli (Apc), tumor protein p53 (TP53), transforming growth factor beta receptor 2 (Tgfbr2) that express constitutively active Kirsten rat sarcoma viral oncogene homolog ($Kras^{G12D}$) and an activated/myristoilated isoform of AKT serine/threonine kinase 1 (Akt1), were also used. APTAK organoids were cultured in basal medium Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Advanced DMEM-F12 supplemented with 5 mL penicillin/streptomycin (Thermo Fisher), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mmol/L, Invitrogen), Glutamax 1X (Invitrogen), N2 1X (Gibco), B27 1X (Gibco), and N-Acetylcysteine (1mmol/L, Sigma-Aldrich). Hygromycin (200 µg/mL), puromycin (2 µg/mL) and normomycin (200 µg/mL) were added to maintain the mutated genes. The cells were maintained in 50% of Matrigel Cultrex® Basement Membrane Extract, R&D systems, Bio-Techne, USA) and grown at 37°C in 5% CO_2 incubator.

Mouse organoids (primary tumor, liver metastasis and peritoneal carcinomatosis) derived from orthotopic transplantation of APTAK organoids in mouse cecum were maintained in the same conditions as described for APTAK organoids.

Human organoids (primary tumor and liver metastasis) derived from biopsy of patients with CRC, donated by the Hospital University of Freiburg with the patient consent, were cultured in Advanced DMEM-F12 supplemented with 5 mL penicillin/streptomycin (Thermo Fisher), HEPES (10 mmol/L, Invitrogen), Glutamax 1X (Invitrogen), Wnt surrogate (80 ng/mL, Protein Express), R-spondin (100 ng/mL, Preprotech), Noggin (1,000 ng/mL, preprotech), B-27 1X (Thermo Fisher), N-Acetylcysteine (1mmol/L, Sigma-Aldrich), h-Epidermal growth factor (25 µg/mL, Peprotech), ALK5 inhibitor A83-01 (Tocris), p38 inhibitor SB202190 (Sigma-Aldrich), Primocin 1X (InvivoGen) and Y-27632 (Sigma-Aldrich). The cells were maintained in 100% of Matrigel Cultrex® Basement Membrane Extract, R&D systems, Bio-Techne, USA) and grown at 37°C in 5% CO_2 incubator.

2.7. Cell Viability assays

2.7.1. MTT assay

The tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylbromide (MTT) assay (Sigma-Aldrich) analyse the mitochondrial activity from the cells by the formation of formazan crystals and its directly proportional to the concentration of alive cells [32]. The tumor cells analysed by this method were CT-26 and HCT-8. The treatment groups were divided as it follows: negative control (cells only), LB control (LB medium), SL3261 (bacterial supernatant), pro_SL3261 (bacterial supernatant containing pro-IL-18), and bio_SL3261 (bacterial supernatant containing bioactive IL-18). $1x10^4$ cells/well were plated in a 96 well-plate and incubated for 24h. The medium was removed from the cells and 100 µL of the treatment was

added to each group, as described, and then 100 µL of DMEM medium was added to each well. For the negative control group, 200 µL DMEM medium was added to the wells. Three plates were prepared with eight replicates for each group and incubated in three different times (24 h, 48 h, 72 h). After each incubation time, the wells were washed two times with PBS 1X and 200 µL of the MTT solution (0,5 mg/mL) were added to each well and incubated for three hours at 37°C and 5% CO_2. After that, the reagent was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well followed by spectrophotometry reading at 570 nm using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Life Technologies Holdings Pte. Ltd, Singapore).

2.7.2. Luminescent assay

The luminescent assay using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega) is capable of determining the number of viable cells based on the quantification of Adenosine Triphosphate (ATP) present in culture, indicating the presence of metabolically active cells which is directly proportional to luminescence readout [33, 34]. For this analysis, mouse and human organoids were used. The treatment groups were divided as it follows: negative control (cells only), SL3261 (bacterial supernatant), pro_SL3261 (bacterial supernatant containing pro-IL-18), and bio_SL3261 (bacterial supernatant containing bioactive IL-18). $8x10^3$ cells/well in 50% Matrigel, in triplicates, were plated in a white 96 well-plate and incubated for 24 h. Medium was removed from the cells and 100 µL of the treatment was added to each group, as described, and then 50 µL of APTAK medium was added to every well. For the negative control group, 150 µL APTAK medium was added to the wells. Four plates were prepared and incubated in four different times (24 h, 48 h, 72 h, 96 h). After each incubation times, 150 µL of CellTiter-Glo® Reagent was added and homogenised to induce cell lysis. The plate was incubated at room temperature for 10 min and luminescence was read using Tecan Spark® Multimode microplate reader (Männedorf, Switzerland).

Moreover, recombinant IL-18 (BioLegend, Revvity) was used for the treatment of APTAK organoids to assess the role of the cytokine itself on the cells. Following the same protocol as described above, four different concentrations from the protein were tested: 0.1 µg/mL, 1.0 µg/mL, 7.0 µg/mL, 50.0 µg/mL.

2.8. Co-culture assay

Live bacteria were co-cultured with APTAK organoids to mimic the *in vivo* treatment in the tumor microenvironment. $8x10^3$ cells/well in 50% Matrigel (Cultrex® Basement Membrane Extract, R&D systems, Bio-Techne, USA), in triplicates, were plated in a white 96 well-plate and incubated for 24 h. The treatment groups were divided as it follows: negative control (cells only), SL3261 (bacteria), pro_SL3261 (bacteria containing pro-IL-18), and bio_SL3261 (bacteria containing bioactive IL-18). 5 mL inoculum of each bacterial strain (SL3261; pro_SL3261; bio_SL3261) were prepared in APTAK medium (without antibiotics) and incubated for 16 h at 37°C under agitation. OD was read and adjusted to OD = 0.5. The cells medium was removed, and 150 µL of fresh APTAK medium was added to the negative control wells, and 148 µL of fresh APTAK medium (without antibiotics) was added to the other wells. 2 µL of the bacterial cultures were added to each well in their corresponding group. Four plates were prepared for

different incubation times (24 h, 48 h, 72 h, 96 h). The plates were incubated for 2 h at 37°C and 5% CO_2. The wells were washed three times with 200 µL of PBS 1X and 150 µL of APTAK medium (without antibiotics) was added to the wells. The plates were sealed with Parafilm®, to create an anaerobic environment, and incubated at 37°C and 5% CO_2. After each incubation times, 150 µL of CellTiter-Glo® Reagent (Promega) was added and homogenised to induce cell lysis. The plate was incubated at room temperature for 10 min and luminescence was read using Tecan Spark® Multimode microplate reader (Männedorf, Switzerland).

2.9. ELISA for chemokines CXCL1 and CXCL2

The presence of pro-tumorigenic chemokines CXCL1 and CXCL2 was assessed from the organoids culture treated with the bacteria supernatants and analysed by ELISA. The assays were conducted using Mouse CXCL1/KC DuoSet ELISA kit and Mouse CXCL2/MIP-2 DuoSet ELISA kit (R&D Systems, Bio-Techne, Minneapolis, USA), according to the manufacturer manual instructions.

2.10. Griess Assay for Nitric Oxide (NO)

Production of nitic oxide (NO) was measured by Griess reaction, which is a colorimetric method used to detect nitrite (NO_2^-), a stable breakdown product of NO [35, 36]. Cells supernatants, after the treatment periods (as described in cell viability assay), were collected and 50 µL were transferred to a 96-well plate. 50 µL of the Griess solution: 1:1 mixture of solution A (1% sulphanilamide in 5% phosphoric acid) and solution B (0,1% N-1-naphthylethylenediamine dihydrochloride) were added to the samples and incubated at room temperature for 15 minutes. A standard curve was prepared to be used in the calculations for nitrate concentrations in the samples afterwards. The spectrophotometry reading was at 540 nm using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Life Technologies Holdings Pte. Ltd, Singapore). For the organoids supernatant samples, Nitric Oxide assay kit (Sigma-Aldrich, Merk) was used according to the manufacturer manual instructions.

2.11. Western blot

Western blot was performed to assess key markers (STAT1, STAT3, β-catenin, GSK-3β, p38 MAPK and NF-κB), non-phosphorylated and phosphorylated forms, responsible for CRC development and progression when the organoids are treated with bacterial supernatants. $8x10^3$ cells/well in 50% Matrigel, in triplicates, were plated in a 96 well-plate and incubated for 72 h. The organoids used for this assay was: APTAK and organoids extracted from primary tumor, liver metastasis and peritoneal carcinomatosis. The treatment groups were divided as it follows: negative control (cells only), SL3261 (bacterial supernatant), pro_SL3261 (bacterial supernatant containing pro-IL-18), and bio_SL3261 (bacterial supernatant containing bioactive IL-18). Medium was removed from the cells and 100 µL of the treatment was added to each group, as described, and then 50 µL of APTAK medium was added to every well. For the negative control group, 150 µL APTAK medium was added to the wells. The plates were incubated at 37°C and 5% CO_2 for 24 h. The cells were harvested and pelleted for the protein extraction step. Using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher),

nuclear and cytoplasmatic proteins were extracted from the cell samples, according to manufacturer manual guidelines. The proteins were measured, in triplicates, by Pierce™ BCA Protein Assay Kit (Thermo Fisher) and the concentration calculated, all according to manufacturer manual guidelines. Next, the proteins were mixed with Laemmli buffer (3:1) and incubated at 95°C for 10 minutes. Mini-PROTEAN® TGX™ Precast Gels 12% (Bio-Rad) were used to load the samples and electrophoresis were performed with 120 V and 45 minutes run. The gel was placed in the Trans-Blot Turbo Transfer Pack (Bio-Rad), which includes buffer, membrane, and filter paper combination, and then placed in the Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad, Hercules, CA, USA) for membrane transfer for 7 minutes, 2.5 A, 20 V. The membrane was blocked using albumin 5% (0.5 g albumin, 10 mL TBST – Bio-Rad), incubated at room temperature, for 1h, under agitation. Primary antibodies for the markers were prepared using 10 mL of albumin 5% (1:1,000) and the membrane placed in that solution. The tube was incubated overnight, at a cold room, under agitation. Next, the membrane was washed three times with TBST 1X (Bio-Rad), every 10 minutes. Secondary antibody was prepared with 10 mL of albumin 5% (1:1,000) and placed to the membrane. It was incubated at room temperature, for 1h, under agitation. Another washing step was performed, and the membrane was mixed with HRP substrate and chemiluminescence read using ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA).

2.12. Statistical analysis

The data obtained in this study were analysed using GraphPad Prism software, version 9.0 (San Diego, CA, USA). Outlier identification was performed using Grubbs' test, followed by the Shapiro–Wilk test to assess data normality. For parametric data, analysis of variance (ANOVA) was applied, followed by Tukey's multiple comparisons test, with results expressed as mean ± standard deviation. For nonparametric data, the Kruskal–Wallis test was used, followed by Dunn's multiple comparisons test, with results expressed as median and lower and upper quartiles [Me (Q1; Q3)]. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Generation of IL-18–expressing *Salmonella* SL3261 and protein detection

Construction of plasmids encoding pro-IL-18 or IL-18* was successfully achieved, as confirmed by DNA sequencing (**Supplementary Data**). Expression of IL-18 by transformed SL3261 strains was verified through detection of EmGFP fluorescence fused to the expressed proteins. As shown in Fig. 1A, supernatants from SL3261 exhibited only basal fluorescence, whereas supernatants from SL3261 expressing pro-IL-18 (pro_SL3261) or bioactive IL-18 (bio_Sl3261) showed markedly higher fluorescence intensity, indicating successful protein expression and secretion. This signal was particularly pronounced in the bioactive IL-18 group.

Consistent with these findings, ELISA analysis detected IL-18 exclusively in the supernatants derived from the modified SL3261 strains, with no detectable signal in the SL3261 control group (Fig. 1B). Notably, bio_SL3261 supernatants displayed higher IL-18 concentrations than those containing pro-IL-18.

3.2. Effects of bacterial supernatants on CRC cell line viability

Treatment with bacterial supernatants reduced mitochondrial activity in both CT-26 and HCT-8 colorectal cancer cell lines at 24, 48, and 72 hours (Fig. 2). In CT-26 cells, exposure to bacterial supernatants resulted in a reduction of cell viability within the first 48 hours. After 72 hours, the bio_SL3261 group exhibited the strongest cytotoxic effect, with significant cell viability reduction. HCT-8 cells displayed a distinct response pattern. During the first 48 hours, cells treated with bacterial supernatants exhibited increased proliferation compared to the control group. However, after 72 hours, a marked reduction in cell viability was observed, with the bio_SL3261 group showing a slightly stronger inhibitory effect compared to the other treatments.

3.3. Bacterial supernatants suppress viability of murine and human CRC organoids

Treatment with bacterial supernatants significantly reduced viability in both murine and human CRC organoids (Fig. 3). APTAK organoids showed an approximately 70% reduction in viability following exposure to IL-18–containing supernatants. Similar effects were observed in murine organoids derived from primary tumors, liver metastases, and peritoneal carcinomatosis, with sustained suppression of viability throughout the incubation period.

Human CRC organoids exhibited even greater sensitivity. Both precursor and bioactive IL-18 treatments significantly reduced viability within 24 hours, leading to near-complete loss of viable cells after 96 hours. In most models, bioactive IL-18 produced the strongest inhibitory effects.

3.4. Recombinant IL-18 partially reproduces the cytotoxic effects in APTAK organoids

Treatment of APTAK organoids with recombinant IL-18 resulted in a dose-dependent reduction in cell viability (Fig. 4). Concentrations of 0.1 and 1.0 µg/mL were ineffective. The 7.0 µg/mL concentration consistently suppressed organoid growth throughout the incubation period, whereas the 50.0 µg/mL concentration induced a transient reduction in viability at 72 hours, followed by partial recovery at 96 hours.

3.5. Co-culture with IL-18–expressing bacteria enhances cytotoxicity in organoids

Direct co-culture of APTAK organoids with modified SL3261 under anaerobic conditions resulted in a rapid reduction of organoid viability (Fig. 5). Within 24 hours, viability decreased to below 50% in all bacterial treatment groups. The bio_Sl3261 strain induced the strongest effect, reducing viability to approximately 20% at 72 hours and below 15% at 96 hours, and the pro_SL3261 strain also suppressed organoid viability, although to a slightly lesser extent.

3.6. Modulation of CXCL1 and CXCL2 secretion by bacterial supernatants

ELISA analysis revealed that CXCL1 was produced by APTAK and murine CRC organoids following treatment with bacterial supernatants (Fig. 6A-D). In all organoid models, SL3261 treatment induced the highest CXCL1 levels. In contrast, supernatants containing pro-IL-18 and bioactive IL-18 reduced CXCL1 secretion, with bioactive IL-18 levels approaching those observed in the negative control groups. A similar pattern was observed for CXCL2 secretion (Fig. 6E-H). SL3261 treatment consistently resulted in the highest CXCL2 concentrations across all organoid types, whereas pro-IL-18 and bioactive IL-18 treatments markedly attenuated chemokine production, yielding levels comparable to controls.

3.7. IL-18–expressing bacterial supernatants enhance nitric oxide production

Nitric oxide (NO) production was detected in CT-26 cell cultures following treatment with bacterial supernatants (Fig. 6I). Bioactive IL-18 consistently induced the highest NO levels at all time points. Pro-IL-18 induced a delayed increase in NO production, becoming more evident after 48 hours of incubation.

In murine organoids, NO production varied according to tumor origin (Fig. 6J-M). APTAK organoids exhibited a time-dependent increase in NO, particularly following exposure to IL-18–containing supernatants. Primary tumor organoids showed a heterogeneous but sustained NO response in the pro-IL-18 and bioactive IL-18 groups. Liver metastasis organoids displayed lower and more variable NO levels, whereas peritoneal carcinomatosis organoids exhibited a pronounced late-stage increase in NO, especially in response to pro-IL-18.

3.8. IL-18 alters key signalling pathways in CRC organoids

Western blot analysis was performed to assess signalling pathway modulation after 24 hours of treatment (Fig. 7). β-actin and histone H3 confirmed successful extraction of cytoplasmic and nuclear proteins. STAT-1 activation was limited across organoid types but was strongly induced in peritoneal carcinomatosis organoids treated with IL-18–expressing supernatants. Both pro-IL-18 and bioactive IL-18 suppressed phosphorylated STAT-3 in all tested organoids. Non-phosphorylated and phosphorylated β-catenin levels were markedly reduced in IL-18–treated groups. GSK-3β activation was enhanced by bacterial supernatants, particularly those containing bioactive IL-18. p38 MAPK activation was limited overall but detectable in APTAK organoids following bacterial treatment. NF-κB was largely

undetectable; however, in APTAK organoids, pro-IL-18 and IL-18* treatments led to marked suppression of cytoplasmic NF-κB, with complete inhibition observed in the bioactive IL-18 group.

5. DISCUSSION

Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide and is characterized by complex, multifactorial pathogenesis involving dysregulated signalling pathways, immune evasion, and a permissive tumour microenvironment [4–6]. These features limit the efficacy of single therapies and highlight the need for innovative and diverse treatment strategies [15]. In this context, our study demonstrates that attenuated *Salmonella* Typhimurium SL3261 engineered to express IL-18, in its precursor and bioactive form, elicits robust and sustained anti-tumour effects across multiple CRC models *in vitro*, including advanced murine and human organoids representing primary and metastatic disease.

The tumour-targeting capacity of *Salmonella* Typhimurium, a facultative anaerobic bacterium, has been extensively documented, with preferential accumulation in hypoxic and necrotic tumour regions driven by chemotaxis and metabolic adaptability [34]. The SL3261 strain, which is auxotrophic for aromatic amino acids due to *aroA* gene deletion, provides a safe and attenuated vector with limited systemic dissemination [35, 36]. These tumour-targeting bacteria can be genetically engineered to deliver immunomodulatory molecules directly into the tumour microenvironment, thereby enhancing therapeutic efficacy while minimizing systemic toxicity [17, 37]. Our findings reinforce the therapeutic potential of this vector by demonstrating that bacterial-mediated cytokine delivery enables sustained and localized exposure within the tumour microenvironment, overcoming key limitations associated with systemic cytokine administration, such as rapid degradation, poor bioavailability, and dose-limiting toxicity [16, 38].

Cytokines play a central role in anti-tumour immunity by promoting immune cell activation, proliferation, and migration [16]. Among cytokines, IL-18 has emerged as a pleiotropic regulator of immune responses with context-dependent roles in cancer biology [23, 27, 39]. Reduced IL-18 expression has been associated with CRC progression, whereas its restoration exerts anti-proliferative and immunostimulatory effects [39]. Mechanistically, IL-18 is processed via inflammasome activation into its bioactive form, which promotes IFN-γ production, enhances cytotoxic lymphocyte activity, and modulates inflammatory signalling [40, 41]. Importantly, our study provides evidence that the biochemical form of IL-18 is a critical determinant of therapeutic efficacy, as bioactive IL-18 consistently outperformed its precursor form across all experimental systems. This observation underscores the importance of cytokine maturation and suggests that effective therapeutic strategies must ensure not only expression but also functional activation of IL-18.

A central finding of this work is that bacterial delivery of IL-18 confers superior anti-tumour activity compared to recombinant cytokine administration. While recombinant IL-18 required higher concentrations and exhibited transient effects, bacterial supernatants and co-culture systems induced sustained tumour suppression at lower effective doses. This likely reflects continuous local production,

enhanced stability, and improved bioavailability within the tumour microenvironment. These results align with previous reports demonstrating that bacterial vectors can function as “microfactories” for therapeutic molecules, enabling controlled and prolonged exposure at the tumour site [27, 42]. Furthermore, the comparison between bacterial supernatants and co-culture conditions suggests that both secreted factors and bacteria–cell interactions contribute to the observed anti-tumour effects, highlighting the multifaceted nature of this therapeutic approach.

Consistent with previous reports, such as the work by de Lima Fragelli et al. (2021) [21], which demonstrated strong anti-tumour effects of SL3261 expressing IL-2 and TRAIL, our results further validate the use of engineered *Salmonella* as a versatile vector for cytokine-based cancer therapy. The ability of SL3261 to deliver IL-18 directly to the tumour microenvironment enables sustained and localized immune modulation, which is likely critical for achieving effective tumour control [38].

The use of organoid models in this study provides important translational insights. While conventional CRC cell lines demonstrated susceptibility to IL-18–based treatments, organoids derived from primary tumours, liver metastases, and peritoneal carcinomatosis exhibited more consistent and pronounced responses. These models preserve tumour architecture, genetic heterogeneity, and key molecular features, making them highly relevant for preclinical evaluation [43]. Notably, metastatic organoids retained sensitivity to IL-18–mediated cytotoxicity, supporting the potential applicability of this strategy in advanced CRC, where therapeutic options remain limited [3]. Despite these advantages, it is important to acknowledge that organoid systems lack immune and stromal components, which may influence cytokine responses and bacterial behaviour *in vivo* [43].

In tumour microenvironment, IL-18–expressing bacterial treatments induced significant modulation of tumour-associated inflammatory mediators. Specifically, the observed downregulation of CXCL1 and CXCL2 contrasts with the pro-inflammatory effect elicited by non-engineered SL3261, likely driven by lipopolysaccharide (LPS)-mediated signalling [44, 45]. Given that CXCL1 and CXCL2 are associated with tumour-promoting inflammation, angiogenesis, and metastatic niche formation [46–48], their suppression suggests that IL-18 reprograms the tumour microenvironment toward a less permissive state for tumour progression. This dual effect, pro-inflammatory stimulation by the bacterial backbone and selective modulation by IL-18, highlights the complexity and therapeutic potential of engineered bacterial systems.

In addition, bioactive IL-18 induced sustained nitric oxide (NO) production across CRC models, with more pronounced responses in aggressive organoids. NO is a well-established mediator of cytotoxicity, capable of inducing oxidative stress, mitochondrial dysfunction, and DNA damage in tumour cells [49–51]. The magnitude and persistence of NO production observed in this study suggest that it represents a key downstream effector of IL-18–mediated anti-tumour activity. Moreover, IL-18–driven NO generation may synergize with other inflammatory pathways to amplify tumour cell death.

Western blot analyses further revealed that IL-18–expressing treatments disrupt multiple oncogenic signalling pathways central to CRC progression. Dysregulation of pathways such as Wnt/β-catenin,

JAK/STAT, PI3K/AKT/mTOR, NF-κB, and MAPK is a hallmark of CRC and is driven by key genetic alterations, including mutations in APC, KRAS, and TP53 [8].

In our study, treatment with IL-18–expressing bacterial supernatants resulted in decreased phosphorylation of STAT3 and β-catenin, alongside increased activation of GSK-3β. Notably, reduced phosphorylation of STAT3 indicates inhibition of pro-survival and inflammation-associated signalling, while decreased β-catenin levels, coupled with activation of GSK-3β, suggest suppression of Wnt/β-catenin signalling, a critical driver of CRC tumorigenesis and metastasis [8, 52]. Additionally, modulation of NF-κB and p38 MAPK pathways points to context-dependent regulation of stress and inflammatory responses [8, 9]. Importantly, these effects were observed in highly aggressive APTAK organoids harbouring key oncogenic alterations (e.g., APC loss, KRAS activation), indicating that IL-18–based bacterial therapy can overcome intrinsic resistance mechanisms by targeting multiple signalling nodes simultaneously.

Another notable aspect of our findings is the time-dependent nature of tumour responses. In some models [53–55], delayed cytotoxic responses were observed following prolonged exposure, suggesting an initial stress-adaptive response followed by irreversible growth inhibition. This dynamic highlights the importance of sustained cytokine exposure and may reflect delayed activation of cytotoxic pathways or accumulation of toxic mediators such as NO.

In conclusion, this study demonstrates that *Salmonella* Typhimurium–mediated delivery of bioactive IL-18 represents a potent and multifaceted anti-tumour strategy for CRC. By combining tumour-targeting bacterial vectors with sustained, localized cytokine production, this approach effectively modulates inflammatory mediators, induces cytotoxic effector mechanisms, and disrupts key oncogenic signalling pathways. These findings provide a strong rationale for further investigation of IL-18–based bacterial therapies, particularly in combination with immune checkpoint inhibitors or other immunomodulatory strategies, to enhance therapeutic efficacy in advanced colorectal cancer.

Declarations

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AUTHOR'S CONTRIBUTIONS

TMQ: Conceptualization, Methodology, Investigation, Data curation, Analysis, Validation, Resources, Funding, Writing - original draft, Writing - review & editing. **BDLF:** Methodology, Investigation, Data curation, Analysis, Validation, Writing - original draft, Writing - review & editing. **AJS and RK:** Methodology,

Investigation, Data curation, Analysis, Validation, Writing - review & editing. **MTMNM.:** Methodology, Writing - review & editing. **FFA:** Supervision, Funding acquisition, Writing - review & editing.

COMPETING INTERESTS

None to report. 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.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figures

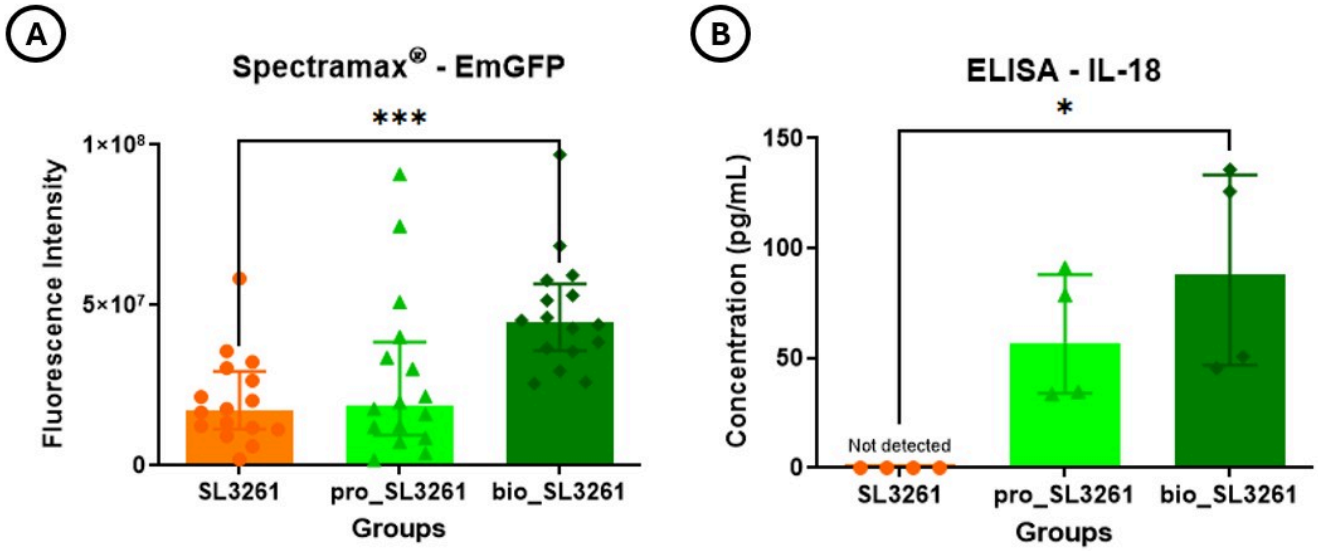


Figure 1

Fluorescence intensity of EmGFP fused to pro-IL18 and bioactive IL-18 and ELISA for IL-18 detected in bacterial supernatants from modified SL3261. [A] Supernatant from non-transformed SL3261 was used as a negative control and exhibited only basal fluorescence. Increased fluorescence emission was observed in supernatants containing pro-IL-18 and IL-18*, indicating successful protein expression and secretion, with higher intensity observed in the IL-18* group. (*) vsSL3261: *** p < 0,001. [B] Supernatants from non-transformed SL3261 were used as a negative control and showed no detectable IL-18. Supernatants from SL3261 expressing pro-IL-18 or bioactive IL-18 displayed measurable IL-18 levels, with higher concentrations detected in the bio_SL3261 group. (*) vsSL3261: * p < 0, 05; *** p < 0,001.

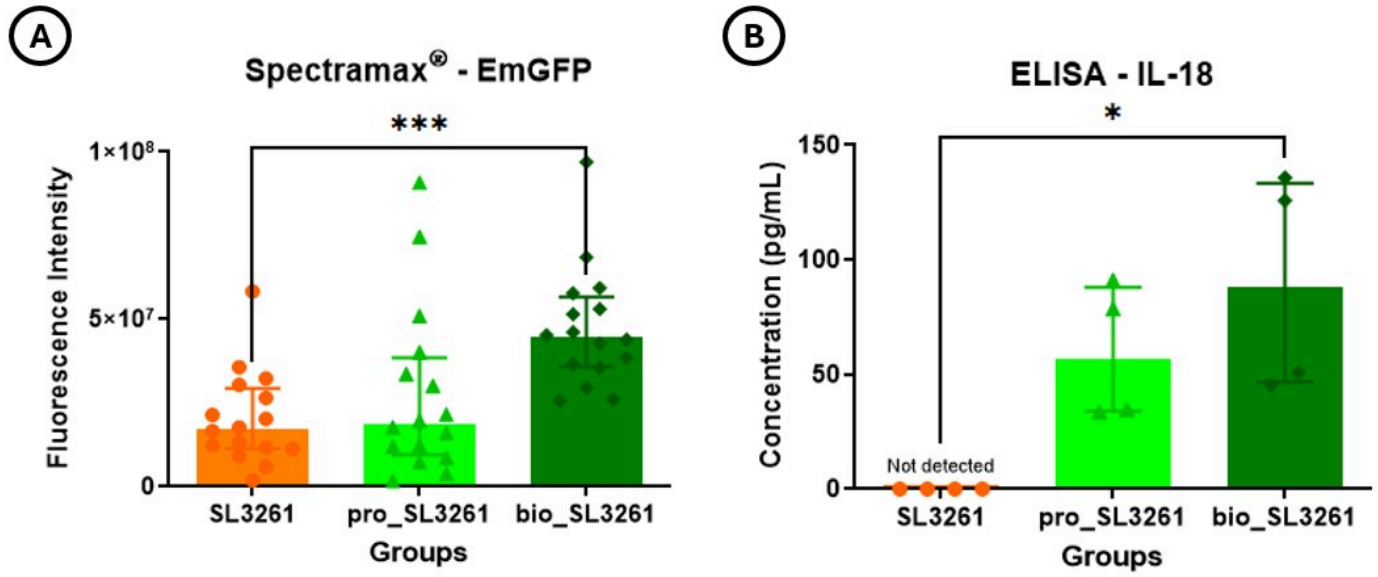


Figure 2

Mitochondrial activity analysis by MTT assay following treatment with bacterial supernatants. [A] CT-26 and [B] HCT-8 colorectal cancer cell lines were treated with supernatants from SL3261, SL3261 expressing pro-IL-18 (pro_SL3261), or SL3261 expressing bioactive IL-18 (bio_Sl3261). Negative control received no treatment, and LB medium was used as a bacterial culture control. [A] CT-26 cells exhibited a marked reduction in viability within 48h, with the strongest effect observed in the bio_SL3261 group at 72h. (*) vs negative control: * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

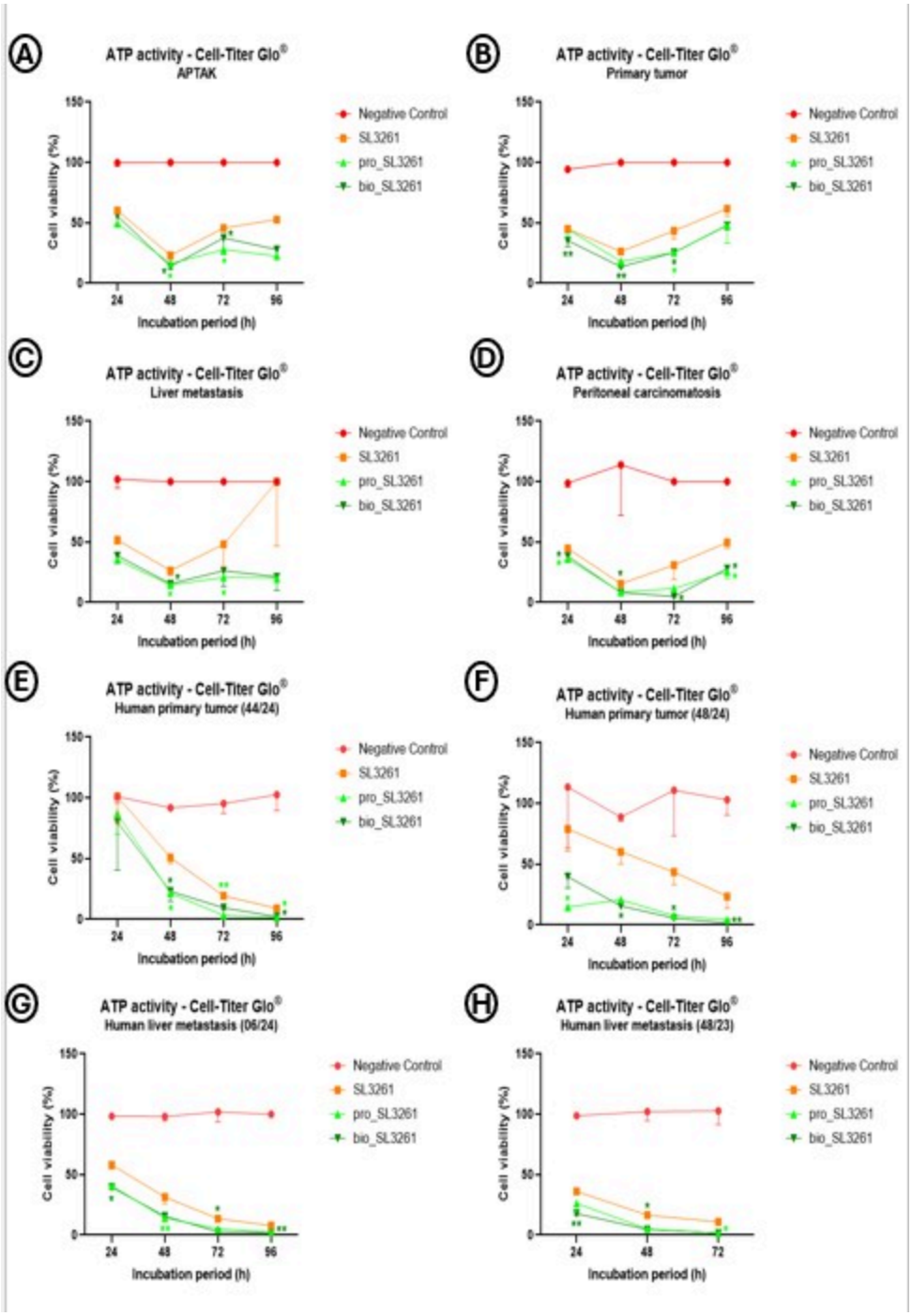


Figure 3

Cell viability of APTAK, murine and human organoids assessed by CellTiter-Glo® Luminescent Cell Viability Assay following treatment with bacterial supernatants. Untreated organoids served as negative control. Treatment with bacterial supernatants reduced organoid viability, with sustained suppression observed in groups receiving pro-IL-18 (pro_SL3261) or bioactive IL-18 (bio_SL3261) over the 96h incubation period. [A] APTAK. [B] Primary tumor. [C] Liver metastasis. [D] Peritoneal carcinomatosis. [E] Human primary tumor (48/24). [F] Human primary tumor (44/24). [G] Human liver metastasis (06/24). [H] Human liver metastasis (48/23). (*) vs negative control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ e **** $p < 0.0001$.

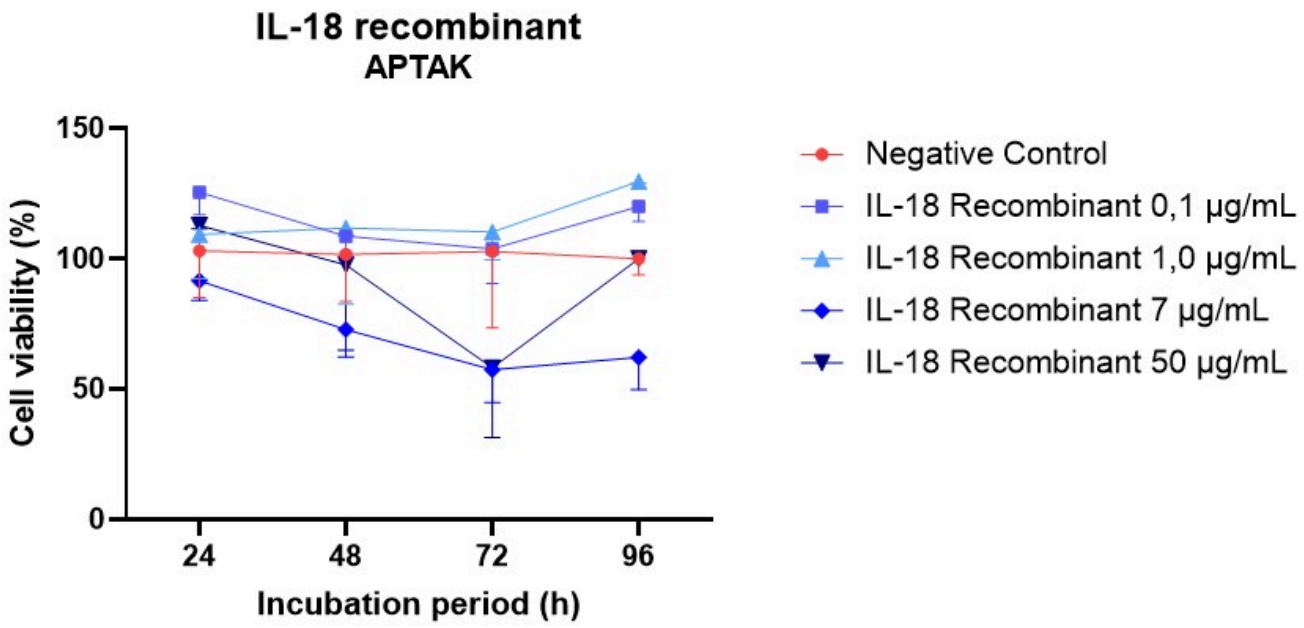


Figure 4

Cell viability of APTAK organoids treated with different concentrations of recombinant IL-18, measured by CellTiter-Glo® assay. Organoids were treated with increasing concentrations of recombinant IL-18 (0.1, 1.0, 7.0, and 50.0 µg/mL). Untreated organoids served as negative control (·). The 7.0 µg/mL concentration consistently reduced organoid viability throughout the incubation period, whereas lower concentrations were ineffective.

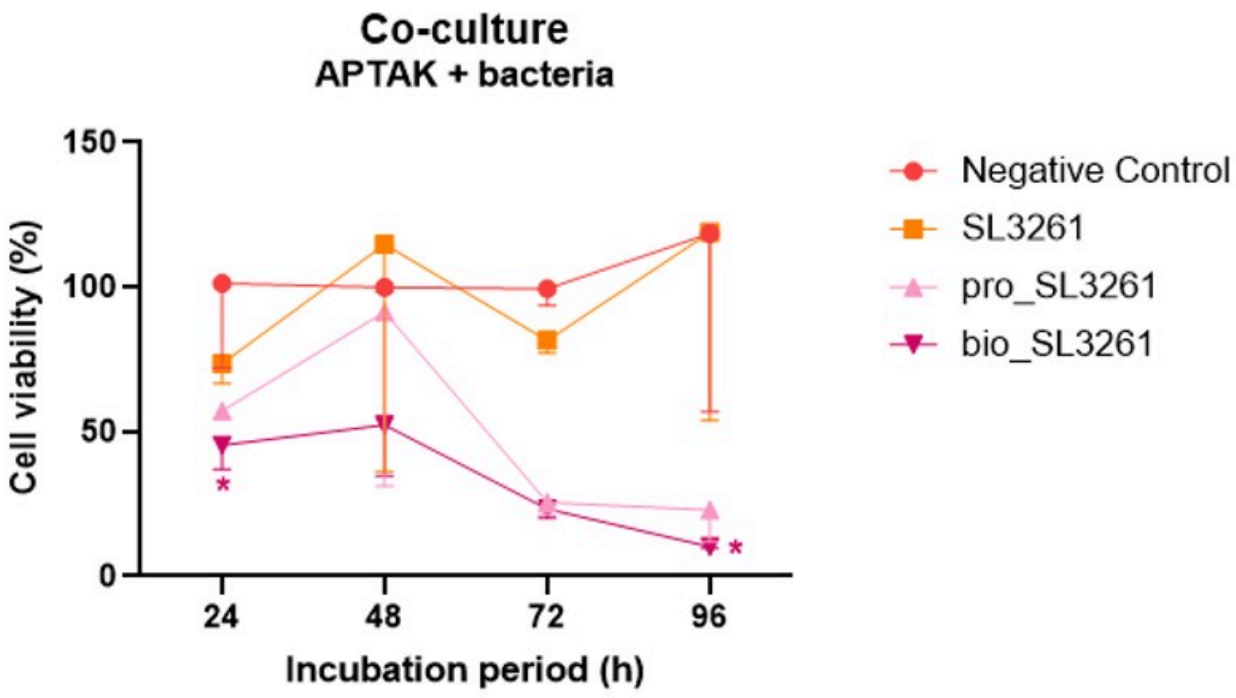


Figure 5

Cell viability of APTAK organoids co-cultured with SL3261 strains under anaerobic conditions, measured by CellTiter-Glo® assay. Organoids were co-cultured with non-transformed SL3261 or SL3261 expressing pro-IL-18 (pro_SL3261) or bioactive IL-18 (bio_SL3261). Untreated organoids served as negative control. Recombinant bacteria expressing IL-18 significantly reduced organoid viability over 96h, with the strongest effect observed in the bio_SL3261 group.

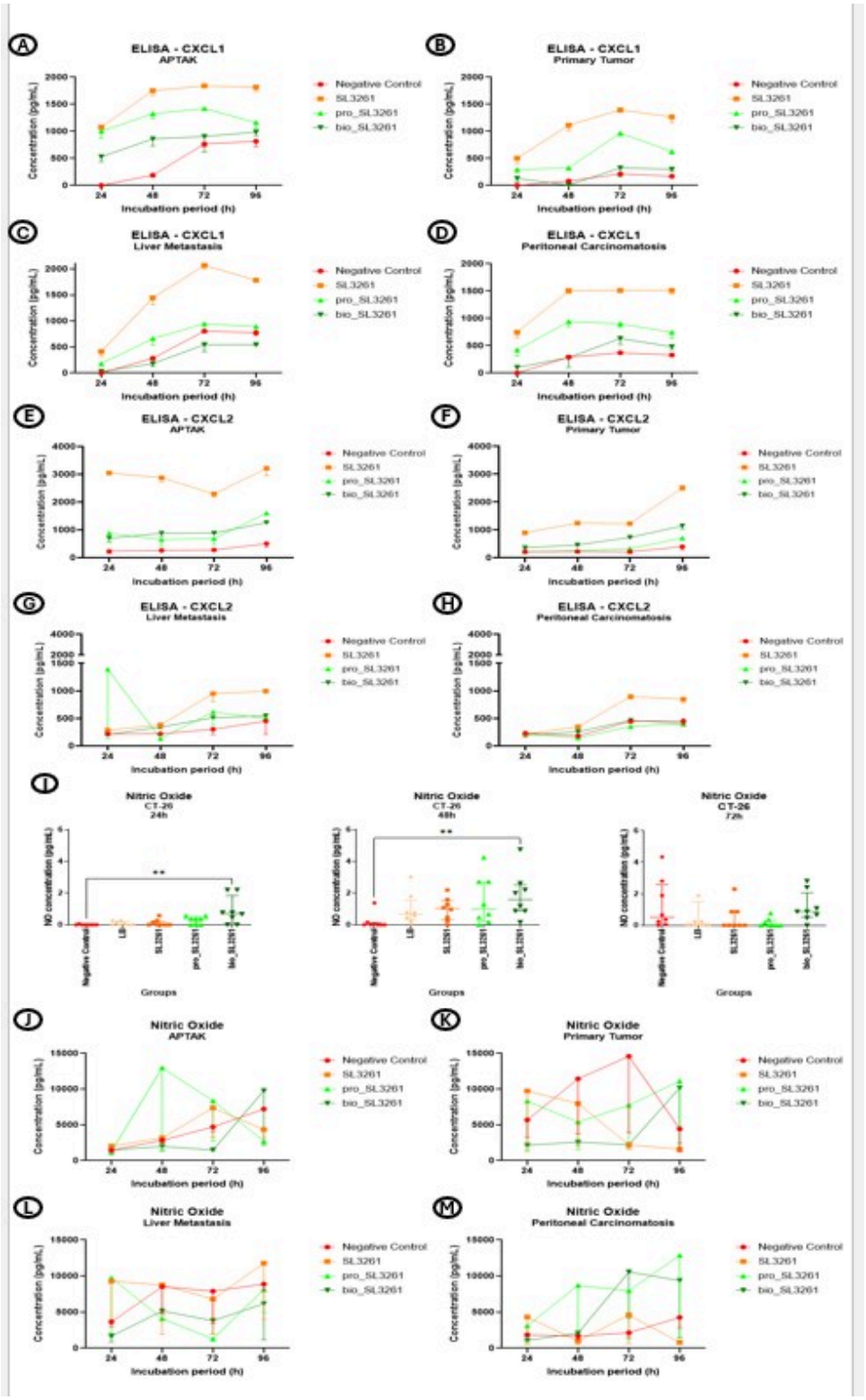


Figure 6

CXCL1 and CXCL2 levels measured by ELISA and Nitric oxide (NO) production measured by Griess assay from cell culture supernatants treated with bacterial supernatants. Organoids included [A,E,J] APTAK, [B,F,K] primary tumor, [C,G,L] liver metastasis, and [D,H,M] peritoneal carcinomatosis, and cell line CT-26 [I]. [A, B, C, D] SL3261 treatment induced higher CXCL1 production, whereas pro-IL-18 and bioactive IL-18 treatments reduced CXCL1 levels to values comparable to negative controls. [E, F, G, H]

SL3261 treatment resulted in the highest CXCL2 concentrations, while pro-IL-18 and bioactive IL-18 treatments significantly attenuated CXCL2 production. [I] bio_SL3261 treatment induced the highest NO production at all time points, whereas pro_SL3261 induced a delayed increase. [J, K, L, M] NO production varied by organoid origin and incubation time, with increased levels observed particularly in organoids treated with pro-IL-18 and bioactive IL-18.

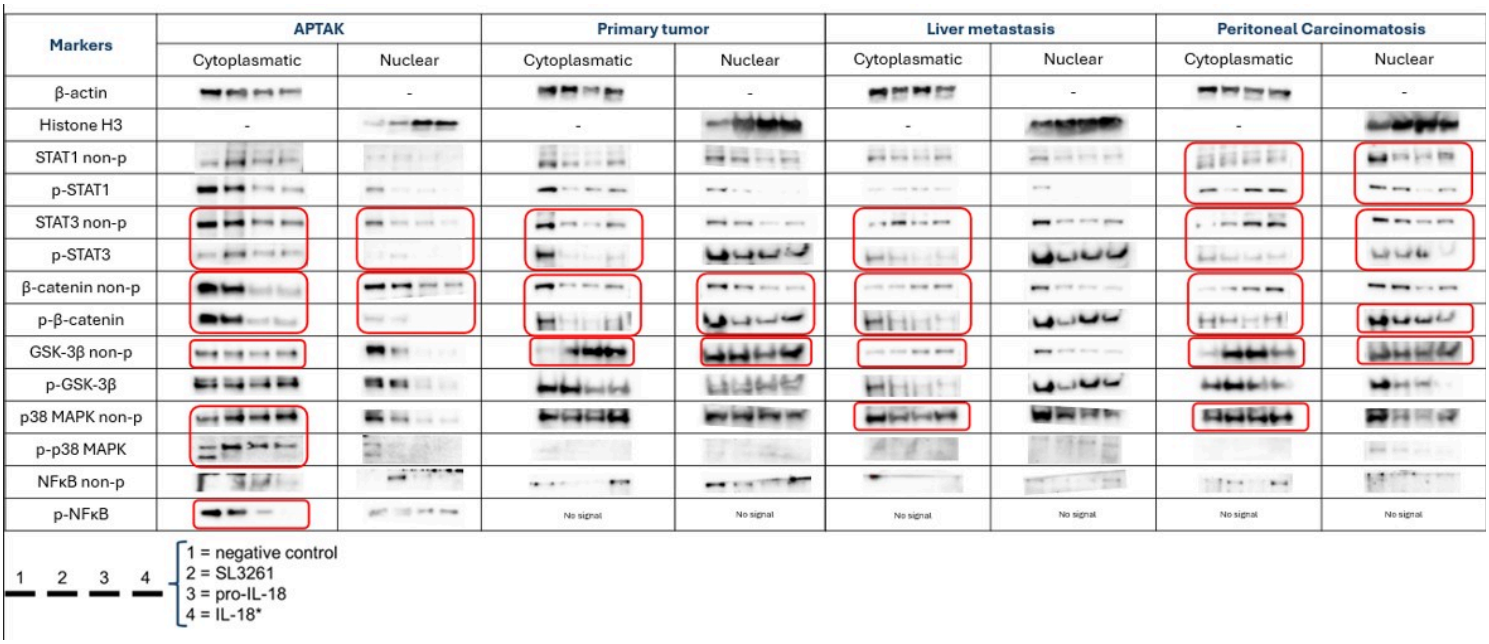


Figure 7

Western blot analysis of key signalling proteins involved in colorectal cancer cell regulation after 24 h of treatment with bacterial supernatants. Organoids included APTAK, primary tumor, liver metastasis, and peritoneal carcinomatosis. β-actin (cytoplasmic) and histone H3 (nuclear) were used as loading controls. Non-phosphorylated (non-p) and phosphorylated (p) forms of STAT-1, STAT-3, β-catenin, GSK-3β, p38 MAPK, and NF-κB were evaluated. Highlighted regions indicate relevant changes in protein activation or suppression induced by the treatments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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