Archivum Immunologiae et Therapiae Experimentalis
72, 2 (2024), DOI: 10.2478/aite-2024-0002

ORIGINAL

Human Soluble TRAIL Secreted by Modified *Lactococcus lactis* Bacteria Promotes Tumor Growth in the Orthotopic Mouse Model of Colorectal Cancer

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Abstract
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis of sensitive cancer cells, including colorectal cancer (CRC). Due to its short biological half-life after intravenous administration and related clinical ineffectiveness, novel formulations of TRAIL need to be developed. Here we propose *Lactococcus lactis* bacteria as a vehicle for local delivery of human soluble TRAIL (hsTRAIL) in CRC. The use of common probiotics targeting guts as carriers for TRAIL could ensure its sustained release at the tumor site and extend the duration of its activity. We have already engineered hsTRAIL-secreting *L.lactis* bacteria and showed their effectiveness in elimination of human CRC cells in vitro and in vivo in a mouse subcutaneous model. Here, *L.lactis*(hsTRAIL+) were administered by gastric gavage to SCID mice with orthotopically developed HCT116 tumor in cecum, in monotherapy or in combination with metformin (MetF), already shown to enhance the hsTRAIL anti-tumor activity in subcutaneous CRC model. Oral administration of *L.lactis*(hsTRAIL+) resulted in significant progression of HCT116 tumors and shortening of the colon crypts. Secretion of hsTRAIL in the colon was accompanied by infiltration of the primary tumor with M2-macrophages, while MetF promoted transient colonization of the gut by *L.lactis*. Our study indicates that *L.lactis* bacteria after oral administration enable delivery of biologically active hsTRAIL to colon, however its potential therapeutic effect in CRC treatment is abolished by its pro-tumorigenic signalling, leading to the recruitment of M2-macrophages and tumor growth promotion.

Keywords
Colorectal cancer • TRAIL • Metformin • *Lactococcus lactis* • Orthotopic mice model of CRC

Received: 5 July 2023 / Accepted: 30 November 2023/

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Apo2L, CD253) is a member of tumor necrosis factor (TNF)-superfamily of proteins, which has been extensively shown to induce apoptosis of sensitive cancer cells, *via* interaction with its two specific, death-domain-containing surface receptors – DR4 (TRAIL-R1) and DR5 (TRAIL-R2, KILLER) (Wiley et al, 1995; Pitti et al., 1996; Lemke et al., 2014). Characterized by its cancer-selective apoptosis-inducing potential (Ashkenazi et al. 1999; Van Dijk et al., 2013), a soluble recombinant form of TRAIL (Dulanermin) was finally tested in clinical trials, where showed a disappointing, little antitumor efficacy, due to its unstable nature and poor physicochemical properties after intravenous administration to cancer patients (Herbst et al., 2010). Besides, evident preservation of the expression of death receptors on the surface of TRAIL-resistant cancer cells gave rise to the discussion about the role of this ligand-receptor system in such cells (Azijli et al., 2013; Ozawa et al., 2001; Ganten et al., 2009; von Karstedt et al. 2015; Bavi et al., 2010). Thus, while novel formulations of this "death-ligand" have been developed and tested, a growing list of evidence is showing a non-canonical activity of TRAIL on the cancer cells (Ehrhardt et al. 2003; Varfolomeev et al., 2005). Further studies concerning the potential role of TRAIL in tumorigenesis showed, that TRAIL-triggered secretome, induced in surviving cancer cells, drives monocyte polarization to pro-tumorigenic M2-macrophages and myeloid-derived suppressor cells (Hartwig et al., 2017).
We recently showed that human soluble TRAIL (hsTRAIL), secreted intratumorally by non-pathogenic, *Lactococcus lactic* bacteria, was able to reduce the growth of subcutaneous HCT116-tumor in NOD-SCID mice and this effect could be further enhanced by oral administration of metformin (MetF) (Kaczmarek et al., 2021). However, data demonstrate that existing preclinical subcutaneous models of human colorectal cancer (CRC) markedly differ from the orthotopic transplants (Zhao et al., 2017), e.g. orthotopic CRC models follow the same metastatic patterns as in humans, while subcutaneous grafts are non-invasive (Zhang et al., 2013;

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Guilbaud et al., 2001). This may finally impact on the effectiveness of the tested treatment. For this reason, we have assessed the effectiveness of *L.lactis*(hsTRAIL+) in mono- and combined therapy with MetF, already shown to enhance the TRAIL-induced apoptosis of CRC cells (Kaczmarek et al., 2021), in mice with orthotopically developed human CRC. This was achieved by implantation of HCT116 Red-FLuc cells to cecum of SCID mice. The therapy, based on the continuous oral delivery of hsTRAIL by *L.lactis*(hsTRAIL+) to colon led to significant progression of CRC, which was accompanied by local accumulation of M2-macrophages and shortening of the colon crypts. In opposite to subcutaneous model of CRC, MetF was ineffective neither in sensitizing cancer cells to TRAIL-induced apoptosis, nor even in reducing its pro-tumorigenic effect. However, the combined therapy of *L.lactis*(hsTRAIL+) and MetF inhibited the formation of metastases in the spleen and lungs. Results from our study emphasize a significant role of CRC microenvironment in hsTRAIL activity, and indicate a potential application of *L.lactis* bacteria as vehicles for colonic production of therapeutic proteins after oral administration.

2. Materials and Methods

2.1. Cell cultures

Human colon carcinoma cell line HCT116 Red-FLuc (Bioware® Brite Cell Line BW124318) was obtained commercially (Perkin Elmer, Waltham, MA, USA) and maintained according to the distributor's instructions, in a 37°C humified atmosphere with 5% CO_2. Briefly, the cells were cultured in McCoy's 5A (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco, UK) puromycin (2 µg/mL; Cayman Chemical, Ann Arbor, MI, USA), passaged twice a week with 0.05% trypsin (PAN-Biotech GmbH, Aidenbach, Germany) in EDTA (Eurx, Gdansk, Poland) and regularly tested for *Mycoplasma* sp. contamination by PCR-ELISA test (Roche, Mannheim, Germany).

2.2. *Lactococcus lactis* bacteria

Lactococccus lactis NZ9000 host strain was obtained from MoBiTec (Goettingen, Germany). Design and preparation of genetically modified *L.lactis* clones, harbouring secretion plasmid vector pNZ8124 (MoBiTec; designated as "*L.lactis*("empty" vector)") or their counterparts with the hsTRAIL-cDNA insert, containing the optimized codons to fit the codon usage pattern (codon bias) of *L.lactis*, placed downstream of the inducible promoter PnisA ("*L.lactis*(hsTRAIL+)"), were previously described (Ciaćma et al., 2018). Bacteria were cultured without aeration at 30°C, in M17 medium (BTL, Lodz, Poland), supplemented with 0.5% glucose (POCH, Gliwice, Poland) and 10 µg/mL chloramphenicol (Cm10; Sigma Aldrich, Saint Louis, MI, USA) to maintain the plasmid. Bacteria stocks were frozen in M17 medium, supplemented with 0.5% glucose, Cm10 and sterile 20% glycerol (Eurx) and stored at –80°C until further use.

2.3. Animals for in vivo CRC model

Reinforcement of the principle of 3Rs was considered in the experimental design. For orthotopic model of human CRC, mice of the SCID (CB17/Icr-Prkdcscid/IcrlcoCrl) strain (Charles River Laboratories, Wilmington, MA, USA), homozygous for the severe combined immune deficiency spontaneous mutation *Prkdc*^{scid} with the lack of mature B and T lymphocytes, as described previously (Bosma et al., 1983; Bosma 1991), were chosen. The model was based on human HCT116 Red-FLuc cell line with luciferase activity due to their transfection with luciferase gene from *Luciola Italica*. Orthotopic implantation of human CRC cells was commercially ordered in Onco*design* (Dijon, France), according to the established protocol. 51 females at 6–8 weeks of age with orthotopically developed CRC were used for the experiment. Mice were maintained in authorized animal facility of Onco*design* and local facility at the Jagiellonian University Medical College (Krakow, Poland). Mice were housed in dedicated housing rooms under aseptic and controlled laboratory conditions: 12/12 h light/dark cycle, room temperature 20–22°C, humidity 45–55%, HEPA filtered air, group housing, access to food and water *ad libitum*. Animals were monitored daily for signs of distress and health status. All experiments were performed in accordance with the European Union Directive (2010/63/EU), Polish and French law and were approved by the local Ethical Committees (Poland: 202/2018; France: CNREEA approval no.91).

2.4. Monitoring of orthotopic human CRC tumors by in vivo bioluminescence imaging

To monitor the growth of HCT116 Red-FLuc-tumors, the mice were subjected to bioluminescence imaging (BLI). Ten minutes prior to imaging, the mice received intraperitoneal (i.p.) injections (100 µL, 125 mg/kg) of XenoLight D-luciferin (Perkin Elmer) in phosphate-buffered saline (PBS; Corning, New York, NY, USA), were anesthetized with 1-3% isoflurane and placed in the dark chamber of Ami HT. Detection and quantification of the photons, emitted from the bioluminescent tumors, were performed using Ami HT (Spectral Instruments Imaging, Tucson, AZ, USA). To optimize the signal level to avoid saturation of the light detector, the exposure time was adjusted to the amount of luciferase activity and luciferin pharmacokinetics, finally reaching 5 s. Images were analysed by manually defined regions of interest, using Aura Imaging Software (Spectral Instruments Imaging). Bioluminescence data were shown as Photon flux, defined as

average number of photons per second, that radiate from the mouse in a unit area (1 cm^2) and unit angle (1 sr). When all tumors exceeded 1.14 × 10^5 [photon/s/cm^2/sr], the mice were randomized according to the similar mean value of the signal level in each experimental group, divided into six experimental groups (*n*=8) and received the first dose of treatments (Day 0). The number of mice/experimental group was defined according to previous studies and published evidence. Mice were imaged along abdominal view at the following days: 0, 1, 4, 8, 13, 17, 22, 28, 32, 38.

2.5. Treatment of mice with *L.lactis* bacteria

The broth cultures of *L.lactis*(hsTRAIL+) and corresponding negative control (*L.lactis*("empty" vector)) were prepared, as previously described (Kaczmarek et al., 2021; Ciaćma et al., 2018). The bacteria were administered by gastric gavage, at a dose of 2 × 10^9 colony forming unit (c.f.u.), in a volume of 100 µL of lactose-free skimmed milk (10%), supplemented with $ZnSO_4$ (100 µM; Linegal Chemicals, Warsaw, Poland), nisin (50 ng/mL; MoBiTec) and aprotinin (2 µg/mL; Bioshop, Burlington, Canada), used as vehicle to increase the survival of *L.lactis* strain in the gastrointestinal tract before reaching the tumor site (colon) (Kos et al., 2000). All treatments with the bacteria were performed once a day, at the same time, for 37 consecutive days. Induced cultures of *L.lactis* were freshly prepared each day of the therapy. Simultaneously, the control group of mice ("Mock control" or "Control" (CRC)) were treated with vehiculum only, administered by gastric gavage at the same volume as bacteria to maintain the same experimental conditions.

2.6. Treatment of mice with MetF

Stock solutions of MetF (Sigma Aldrich) were prepared in PBS, sterilized by filtration through a 0.22 µm filter (Carl Roth, Karlsruhe, Germany) and stored at 4°C. The therapeutic was administered by gastric gavage at a dose of 250 mg/kg in 10% skimmed milk, once a day, at the same time for 37 consecutive days.

2.7. Immunohistochemistry analysis

When all animals developed extensive metastases and the decrease of their initial body weight reached 20% (Day 38), the experiment was terminated. After BLI measurements, animals were euthanized by over-dosage of isoflurane, followed by spinal cord dislocation. Then, colons with tumors, liver, spleen and lungs were isolated. Due to the limited activity of luciferase after the animal's death, the resected organs were immediately subjected to BLI. The colons and primary tumors were subsequently fixed in 10% formalin solution ON, for further hematoxylin-eosin (H&E) staining and immunohistochemistry (IHC) analysis. For IHC analysis, the slides (3.5 µm) of paraffin-embedded colons with CRC were treated with sodium citrate at 97°C for 35 min followed by incubation with appropriate antibodies. To examine the local production of TRAIL, slides of colon with CRC, were incubated with rabbit monoclonal anti-human TRAIL antibody (1:100; Cell Signalling Technology, Danvers, MA, USA). To verify the potential interaction between M2-macrophages and CRC cells, slides of colon and primary tumor were also incubated with rabbit anti-mouse mannose receptor (CD206) polyclonal antibody (1:4000; Abcam, Cambridge, UK) and rabbit anti-human monocyte chemoattractant protein-1 (MCP-1/CCL2) polyclonal antibody (1:1000; Thermo Fisher Scientific). All dilutions were performed in Dako Antibody Diluent (Dako, Glostrup, Denmark). Visualisation was achieved using Dako REAL™ ENVison™ Detection System (Dako) according to the manufacturer's instructions. All slides were analysed in a blinded manner using OLYMPUS IX70 microscope (Olympus, Tokyo, Japan) and the CellSens Dimension software (Olympus). Measurements and calculation of the colon crypt depth were performed using paint.net (v. 4.1.6, Rick Brewster, Pullman, WA, USA).

2.8. Viability of *L.lactis*(hsTRAIL+) bacteria in the tumor

The viability of orally administered bacteria in the location of CRC was assessed after homogenization of excised cecum with corresponding fragment of colon, followed by plating of the homogenates in the presence of selection marker (Cm10) and PCR analysis of plasmids isolated from the grown colonies. Briefly, randomly selected cecum was cut into small pieces with a sterile scalpel and transferred to the Eppendorf tubes. Homogenization was done in M17 culture medium in the presence of metal beads, at frequency 20 Hz for 2 min using a Tissue Lyser II (Qiagen, Hilden, Germany). The obtained homogenates were plated in a volume of 100 µL on M17 agar (1.5%) Petri dishes, in the presence of 0.5% glucose and Cm10. After 48 h of incubation at 30°C, the grown colonies were randomly isolated to prepare ON broth cultures. Plasmid DNA was isolated using the Miniprep DNA Purification Kit (Eurx) according to the manufacturer's instructions for isolation of plasmids from gram-positive bacteria. Concentration of the isolated plasmids was measured using a Quawell Q500 spectrophotometer (Quawell Technology, Inc., San Jose, CA, USA). To verify the presence of the hsTRAIL-cDNA insert, PCR using primers 5′-TGGTACTCGTGGTCGTAGCA-3′ sense and 5′-GAAGCTTCGTGGTCCATGTC-3' antisense, was performed. The PCR reaction products were subsequently separated on a 1.5% agarose gel with 0.5 µg/mL EtBr. Perfect ™ 100 bp DNA Ladder (Eurx) was used as a size marker. The gels were photographed using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY, USA). To create the

appropriate positive and negative controls, plasmids isolated from fresh stocks (stored at –80°C) of *L.lactis*(hsTRAIL+) and *L.lactis*("empty" vector), respectively, were also examined.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (v. 6.00) and Microsoft Excel (v. 2019). Comparisons between multiple groups were performed using analysis of variance (ANOVA test) with post-hoc multiple comparisons by Tukey's method. Data were presented as mean ± SEM. The statistically significant results were showed as $*p<0.05$, $**p<0.01$, $***p<0.001$.

2.10. Graphics

All graphics were created with BioRender.com.

3. Results

3.1. *L.lactis*(hsTRAIL+) promote growth of orthotopically developed human CRC in mice

Encouraged by the results from our previous studies with subcutaneous model of CRC (Kaczmarek et al., 2021), showing a significant retardation of the tumor growth in NOD-SCID mice after intra-tumoral secretion of hsTRAIL by *L.lactis*(hsTRAIL+) bacteria, here we examined the effect of *L.lactis*-delivered hsTRAIL in the orthotopic model of human CRC. To this end we used the model developed by implantation of human HCT116 Red-FLuc cells to cecum of SCID mice, allowing for monitoring the tumor size by BLI. **Figures 1a,b** summarizes an experimental setup for this therapy model. Simultaneous analysis of the pattern of bioluminescence distribution and number of photons emitted from the tumor showed a stimulatory effect of the *L.lactis*(hsTRAIL)-treatment on tumor development (**Figures 1c,d**). Daily administration of the hsTRAIL-expressing bacteria for 37 days significantly stimulated the growth of CRC, when compared to mock control ($p<0.001$) or animals treated with *L.lactis*("empty" vector) ($p<0.001$). In this context it was clear that the observed effect was related to biological activity of continuously delivered hsTRAIL, but not to genetically modified bacteria by themselves (**Figure 1c**). The effect of co-treatment with MetF was also different, comparing to subcutaneous model (Kaczmarek et al., 2021). In this case, MetF given in monotherapy did not affect the tumor growth ($p>0.5$ compared to mock control), neither impeded the pro-tumorigenic activity of hsTRAIL in combined therapy with *L.lactis*(hsTRAIL+) ($p>0.5$), although the dose of MetF was intentionally doubled, expecting an enhancement of its activity (comparing to subcutaneous model, where MetF was used at the concentration of 125 mg/kg) (**Figures 1c,d**).

3.2. Administration of *L.lactis*(hsTRAIL+) in the presence of MetF affects survival of the bacteria in colon

In general, *L.lactis* species are sensitive for the action of digestive enzymes (Klijn et al., 1995; Berlec et al., 2015). In order to ensure the best survival of orally administered *L.lactis*(hsTRAIL+) or corresponding negative control, the bacteria were administered in a solution of skimmed milk in a dose of 2×10^9 c.f.u. (intentionally doubled when compared to the subcutaneous CRC model, [Kaczmarek et al., 2021]) and their survival in the gut was assessed *post mortem*, by counting bacterial colonies grown from the cecum homogenates (**Figure 2a**). The presence of MetF allowed for better survival of both *L.lactis*(hsTRAIL+) and *L.lactis*("empty" vector), indicating that this effect was not related to the ability of *L.lactis*-strain to produce hsTRAIL (**Figure 2b**). To exclude the contamination of the homogenates with other bacterial strains from the colon, we performed a PCR analysis for hsTRAIL-cDNA. Results obtained for the randomly picked colonies confirmed, that they originated from orally-delivered *L.lactis*(hsTRAIL+) (**Figure 2c**).

3.3. Local secretion of hsTRAIL in the colon induces infiltration of primary CRC tumor by CD206$^+$ macrophages

IHC analysis of the colon tissue has documented a local secretion of hsTRAIL after oral administration of *L.lactis*(hsTRAIL+) bacteria, and this was not affected by MetF (**Figure 3**). It was shown for the orthotopic model of lung cancer that TRAIL can promote the tumor growth through the induction of MCP-1 secretion by cancer cells and accumulation of M2-like cells in the tumor (Hartwig et al., 2017). Therefore, in our experimental setup we examined the presence of M2-macrophages (defined as CD206-positive cells) and MCP-1 expression in the primary CRC tumor. In this context we found that CD206$^+$ macrophages infiltrated solely tumors in mice receiving the hsTRAIL-producing bacteria, although their presence in colon tissue was detected in all treatment groups (**Figure 4**). This hsTRAIL-driven tumor infiltration of M2-macrophages however, did not correlate with the production of MCP-1 by HCT116 Red-FLuc cells in the tumor tissue - an increased secretion of MCP-1 by human CRC cells in the primary tumor was observed in all groups of animals receiving *L.lactis* bacteria, regardless of their ability to secrete hsTRAIL (**Figure 5**).

3.4. Oral continuous administration of *L.lactis*(hsTRAIL+) reduces the depth of the colon crypts in mice with orthotopic CRC

To fully address the effect of the treatment on CRC-progression, we analysed changes in the colon morphology

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after 37 consecutive days of hsTRAIL administration. This was performed by H&E staining and the subsequent measurements of the colon crypts' depth **(Figure 6a).** Oral administration of *L.lactis*(hsTRAIL+) resulted in a significant reduction of the crypts' depth, when compared to healthy mice (Control (healthy); $p<0.05$), or to mock control (Control (CRC); $p<0.01$) (**Figure 6b**). The regular treatment with *L.lactis*("empty" vector) did not affect the depth of the crypts, pointing on the role of systematic delivery of hsTRAIL, but not on *L.lactis* bacteria by themselves. However, this "devastating" action of hsTRAIL was abolished by MetF, suggesting its protective role on the colon crypts in CRC mice (**Figure 6b**).

4. Discussion

For many years TRAIL has been shown to induce apoptosis of sensitive cancer cells, however this sensitivity quite often revealed to be only temporary and was lost in many cancers

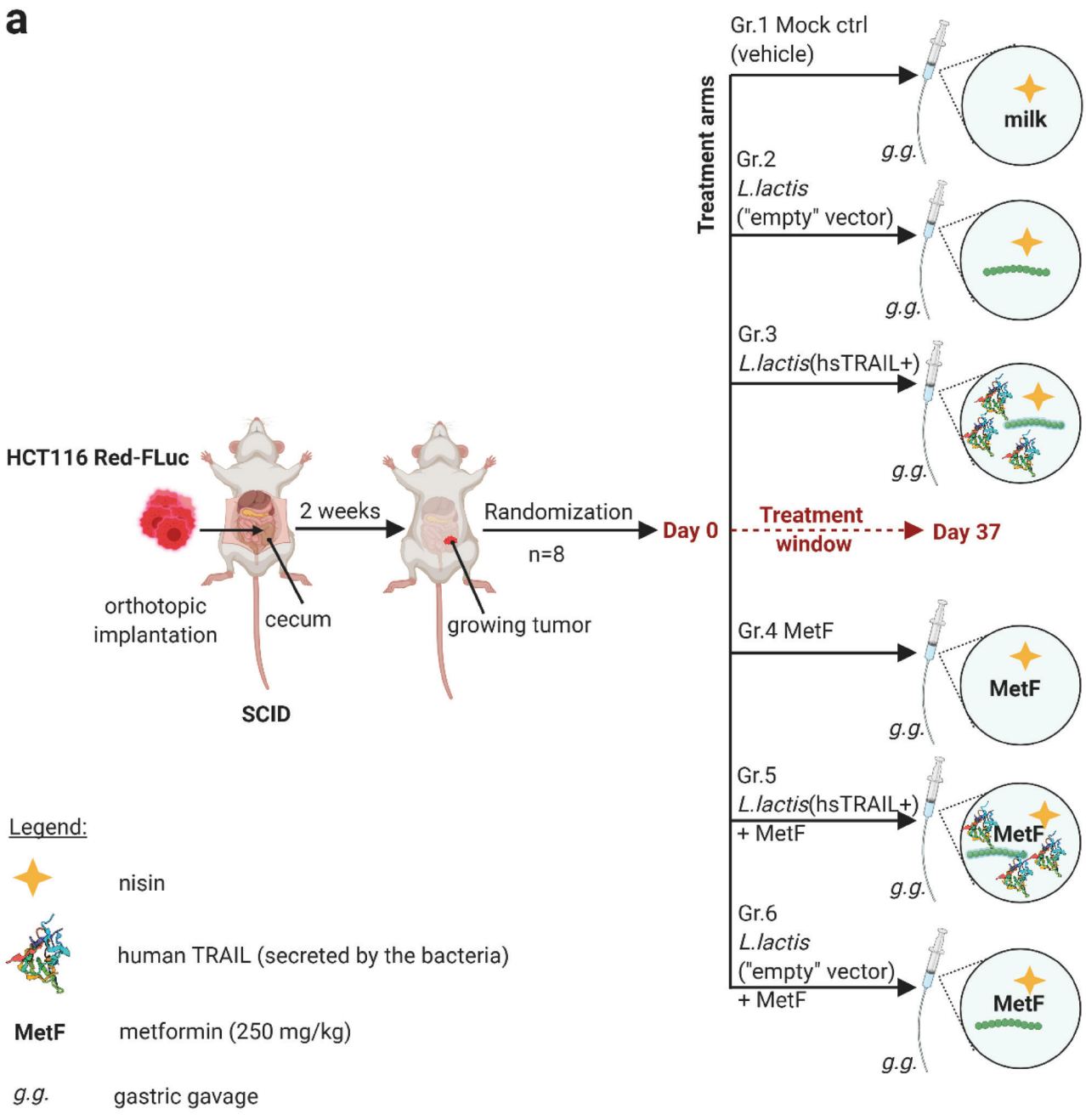

Fig 1. *Continued*

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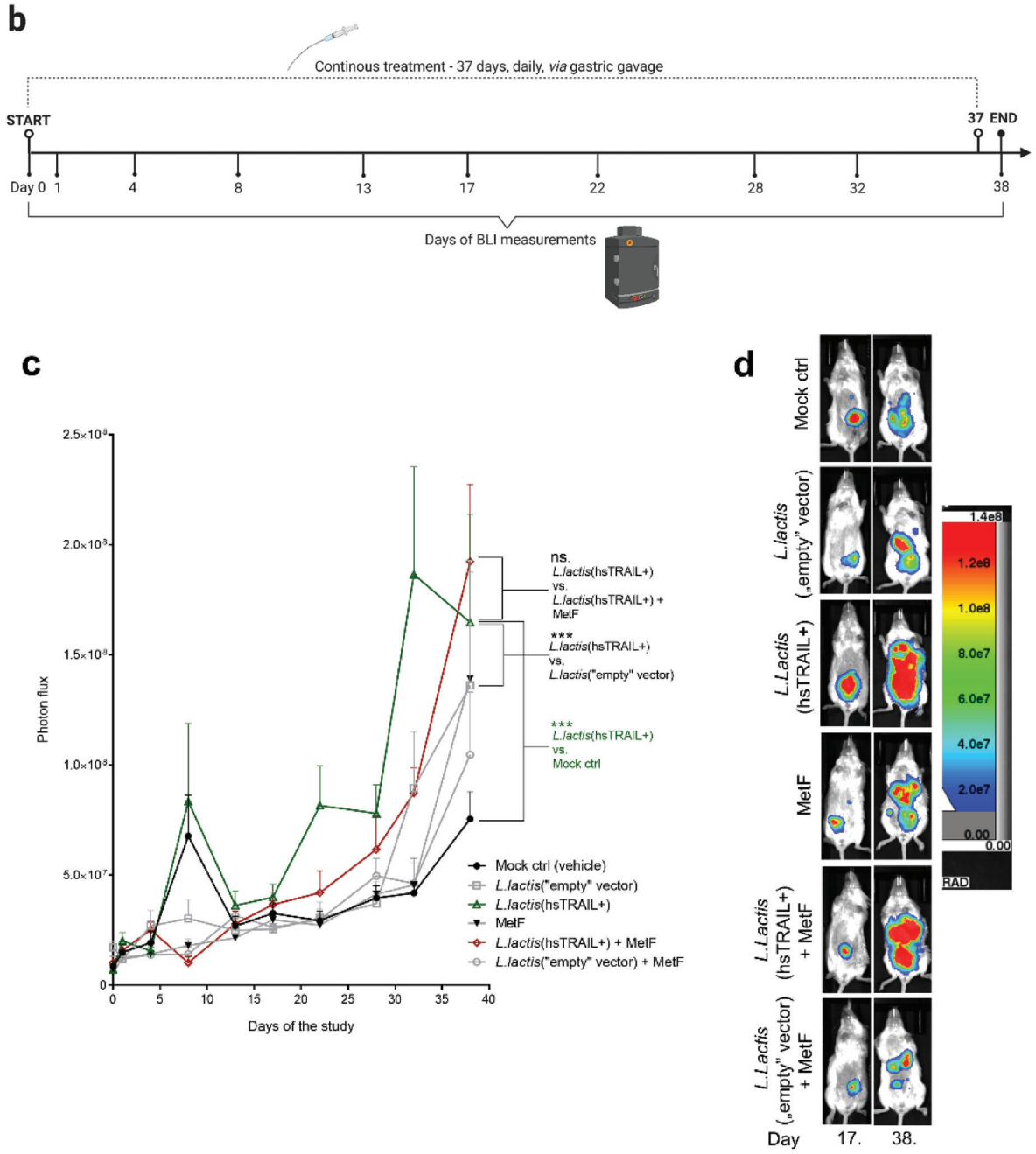

Fig 1. *L.lactis(hsTRAIL+) bacteria given orally stimulate the growth of tumor in orthotopic model of human CRC.* ***a*** *Experimental groups and treatment options. The mouse orthotopic model of human CRC was developed by engrafting of HCT116 Red-FLuc cells into the cecum of SCID mice. Two weeks after the implantation, animals were subjected to BLI to verify the presence and growth of the tumor, then divided into six experimental groups (n=8 and n=11 for mock control), treated from "Day 0" as following: Group I (Mock control) – vehicle (10% skimmed milk in PBS + $ZnSO_4$ + aprotinin + nisin, see Materials and Methods); Group II – L.lactis("empty" vector); Group III – L.lactis(hsTRAIL+); Group IV – MetF (250 mg/kg); Group V – combined therapy with L.lactis(hsTRAIL+) and MetF; Group VI – combined therapy with L.lactis("empty" vector) and MetF. All treatments were given by gastric gavage, in the presence of nisin at a dose of 50 ng/mL.* ***b*** *Timeline of the therapy model. Growth of the primary tumor and development of metastases were regularly monitored for 38 days from the beginning of the experiment ("Day 0") by BLI measurements. All treatments were administered via gastric gavage, once a day for 37 consecutive days. On Day 38, animals were subjected to BLI, then sacrificed.* ***c*** *Growth of CRC during the period of experiment. Tumor growth (BLI) in cecum was monitored at days indicated, as described in Materials and methods. Each point represents the mean for n=8–11 animals. The bars indicate the mean value ± SEM. Statistical analysis was performed using two-way ANOVA, with Tukey's multiple-comparisons post-hoc test. ***p<0.001.* ***d*** *Representative whole-body bioluminescence images of the growth of HCT116 Red-FLuc tumors, performed at indicated time points after intraperitoneal (i.p.) administration of D-luciferin. Bar: photons/second.*

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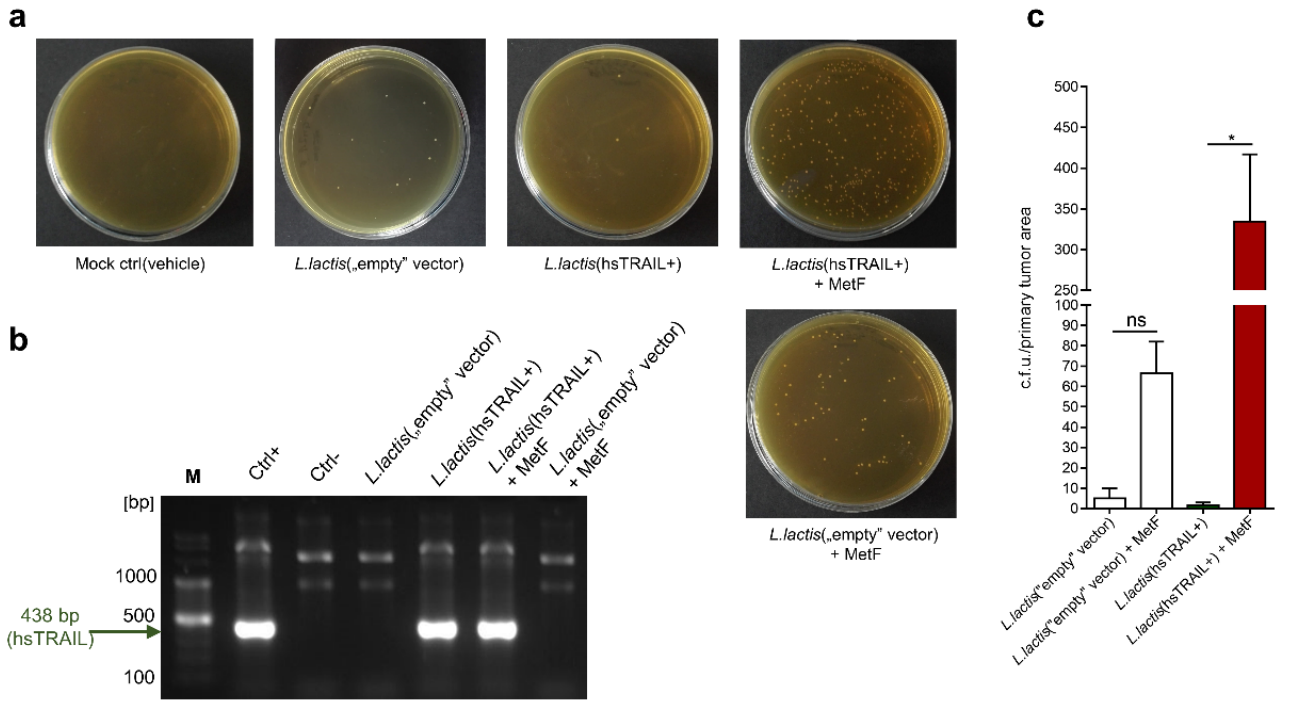

Fig 2. *L.lactis(hsTRAIL+) bacteria survive in the gut after oral administration and MetF supports the persistence of bacteria in primary CRC.* ***a*** *Cecum with corresponding fragment of colon was isolated, homogenized and plated on agar Pertri dishes in the presence of Cm10, as a selection marker. Dishes were cultured for 48 h at 30°C. Shown are bacterial colonies on agar Petri dishes.* ***b*** *After 48 h of incubation at 30°C, the number of grown colonies was counted and compared between groups.* ***c*** *L.lactis(hsTRAIL) grown from the colon homogenates was proven by PCR. Legend: An arrow – hsTRAIL-cDNA sequence insert (438 bp); M – size marker (bp); Ctrl+ – positive control; Ctrl- – negative control (details in Materials and Methods). Legend: y axis – number of bacterial colonies (c.f.u.)/primary tumor area. The bars indicate mean values ± SEM (n=6). Statistical analysis was performed using one-way ANOVA test, with Tukey's multiple comparisons post-hoc test. *p<0.05.*

during the treatment (Hernandez et al., 2001; Galligan et al., 2005; Cummins et al., 2004). Although MetF may restore sensitivity of the resistant human CRC cells to TRAIL activity in vitro (Park et al., 2016), the main concern in respect to the TRAIL-related anti-cancer therapy is still its short biological half-life, limiting its activity in clinical trials (Herbst et al., 2010; Kelley et al., 2001). The use of common probiotics targeting guts as carriers for TRAIL in CRC, could ensure its sustained release at the tumor site, and extend the duration of its activity. With this in mind, we recently proposed the probiotic *Lactococcus lactis* bacteria as carriers of human TRAIL for its local secretion at the tumor site (Ciaćma et al., 2018) and further demonstrated the positive joined effect of such hsTRAIL and MetF in elimination of human cancer cells, both in vitro and in vivo in a subcutaneous model of CRC (Kaczmarek et al., 2021).

Taking into account that orthotopic models of cancers are considered most adequate for research on the new forms of therapy (Evans et al., 2016), here we addressed if TRAIL-producing *L.lactis* after oral administration, would be similarly effective in the treatment of orthotopically developed CRC in mice, as given intratumorally in the subcutaneous model (Kaczmarek et al., 2021). Surprisingly, the therapy, which was based on the continuous colon delivery of hsTRAIL by *L.lactis*(hsTRAIL+) given orally, led to significant progression of CRC. This effect was TRAIL-dependent as was not observed in the case of *L.lactis* bacteria harboring an empty vector. In further contrast to the data from subcutaneous model, administration of MetF in monotherapy did not affect the tumor growth, while combined therapy with *L.lactis*(hsTRAIL+) resulted also in its progression.

The presence of MetF in the treatment regimen significantly increased the viability of *L.lactis* bacteria after oral administration. MetF is known to increase gastric acid secretion and participates in stimulation of bile, intestinal and pancreatic digestive juice production (Molloy et al., 1980). With this in mind, we were rather expecting a worse survival of *L.lactis* in the murine colon in the presence of MetF. In this context, a possible explanation for this observation would be an impact of MetF on the intestinal microbiome composition of the animals, enabling a transient colonization of the gut by genetically modified *L.lactis*. Interactions of

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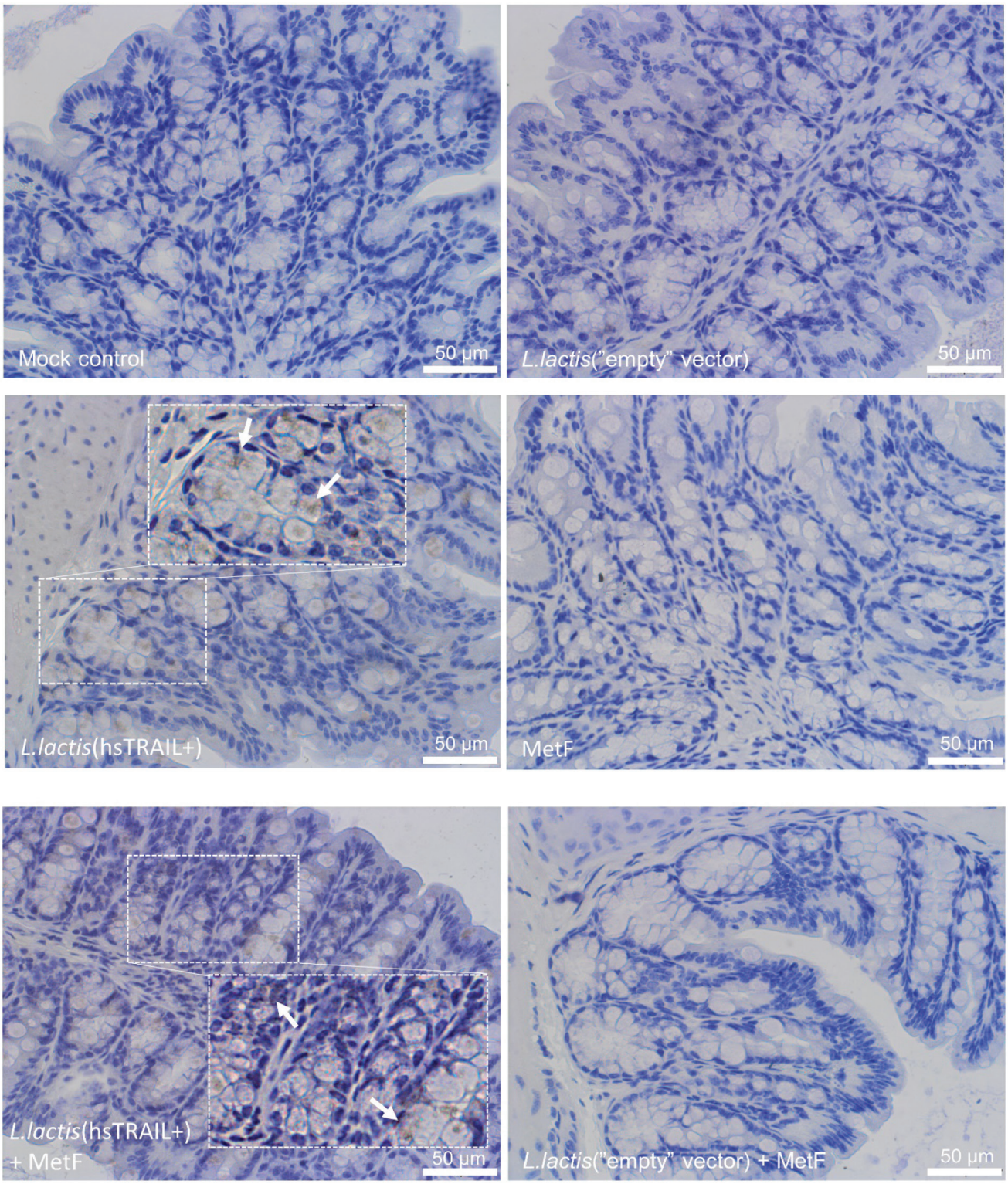

Fig 3. *Oral administration of L.lactis(hsTRAIL+) bacteria in the presence of nisin results in local secretion of hsTRAIL in the colon. The figure shows representative images from paraffin sections of colons, resected 17 h after the last treatments, subjected to IHC analysis for hsTRAIL. White arrows indicate the presence of hsTRAIL. Scale bar: 50 µm. White dashed lines denote the areas that were subsequently magnified and corrected for light intensity, sharpness, and contrast with the same parameters for all magnified areas.*

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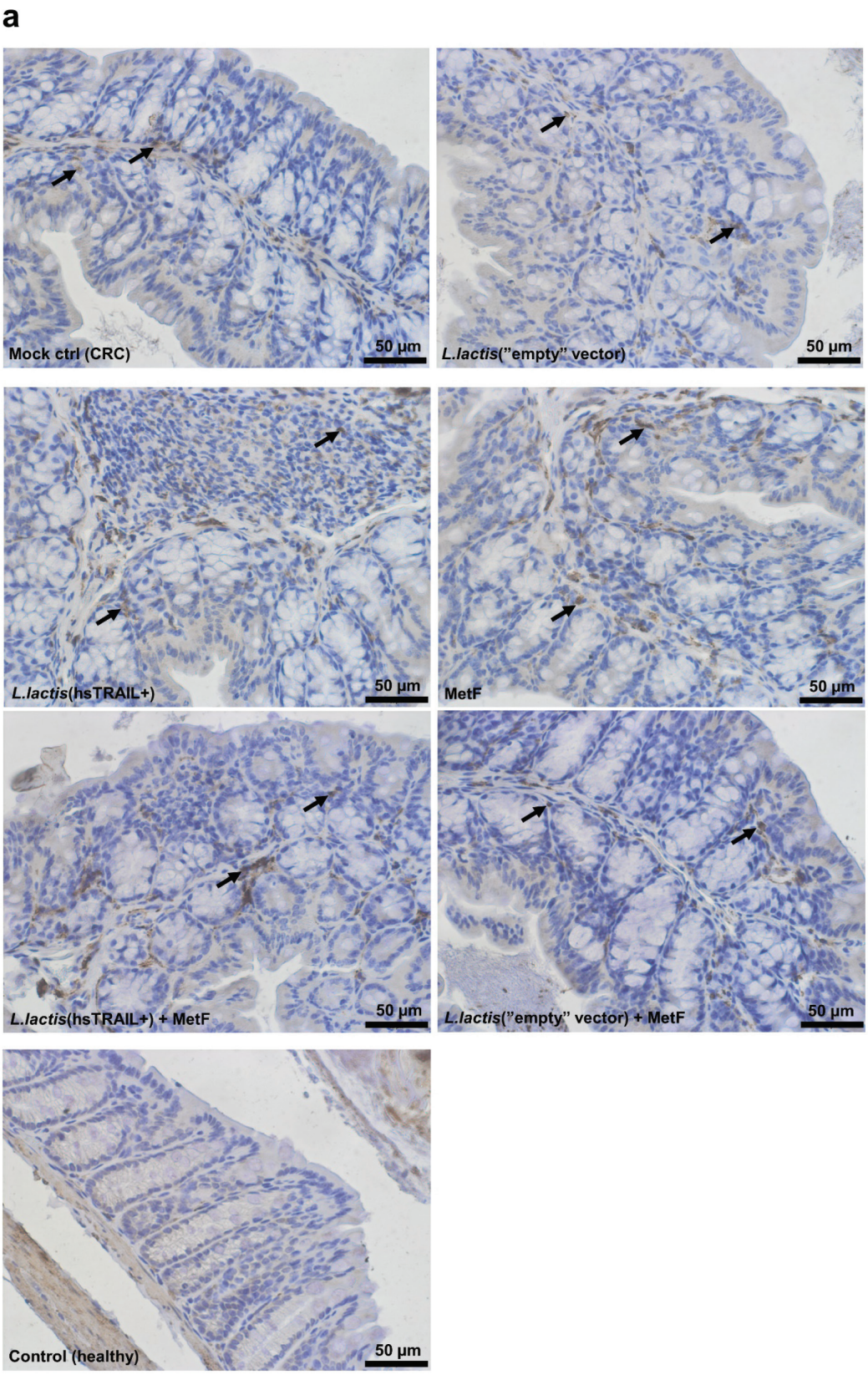

Fig 4. *Continued*

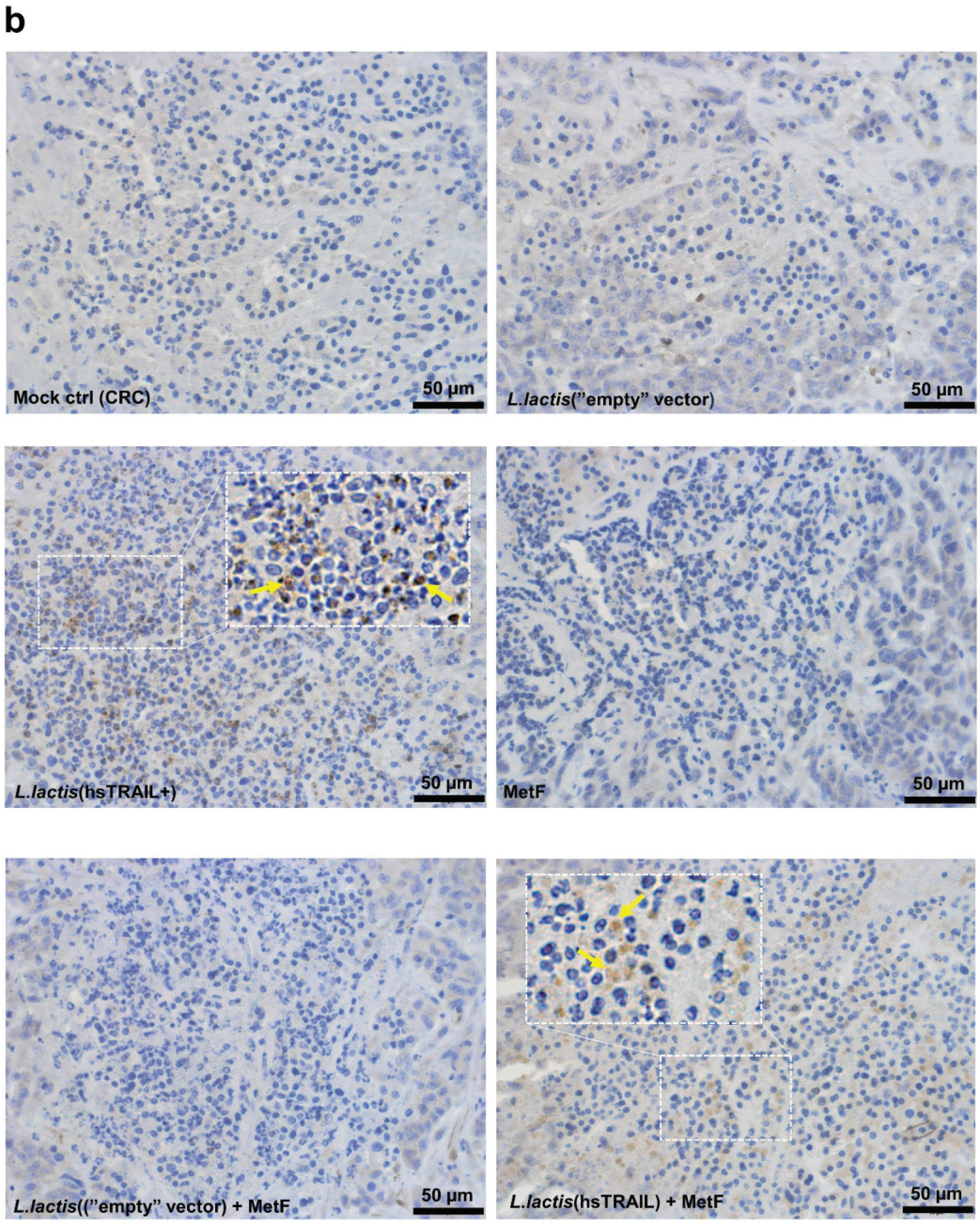

Fig 4. *Continuous production of hsTRAIL in the gut leads to infiltration of CRC by CD206⁺-macrophages.* ***a*** *Representative images of paraffin sections of colons, subjected to IHC analysis for the presence of M2-macrophages, defined as CD206⁺ cells (black arrows). Scale bar: 50 µm.* ***b*** *Representative images of paraffin sections of the primary tumor in cecum, subjected to IHC analysis for the presence of M2-macrophages (CD206⁺ cells, yellow arrows). Scale bar: 50 µm. White dashed lines denote the areas that were magnified and corrected for light intensity, sharpness, and contrast with the same parameters for all magnified areas.*

MetF with bacteria in the gut are part of its action in lowering blood glucose level (Wu et al., 2017; Ma et al., 2018). Studies by Ma et al. (2018) on healthy C57BL/6 mice receiving MetF for 30 consecutive days, at a dose of 300 mg/kg, showed an increased growth in the gut of bacteria from *Verrucomicrobiaceae* and *Prevotellaceae* sp., while decreased from *Lachnospiraceae* and *Rhodobacteraceae* sp. (Ma et al., 2018). Moreover, the abundance of bacteria from the *Lachnospiraceae* family (*Anaerostipes*, *Blautia* and *Roseburia)* in the colon, inversely correlated with colonization of the colon by bacteria from oral cavity (Wu et al., 2017), suggesting a protective role against colonization of the gut by bacteria from "external" sources, including orally administered *L.lactis.*

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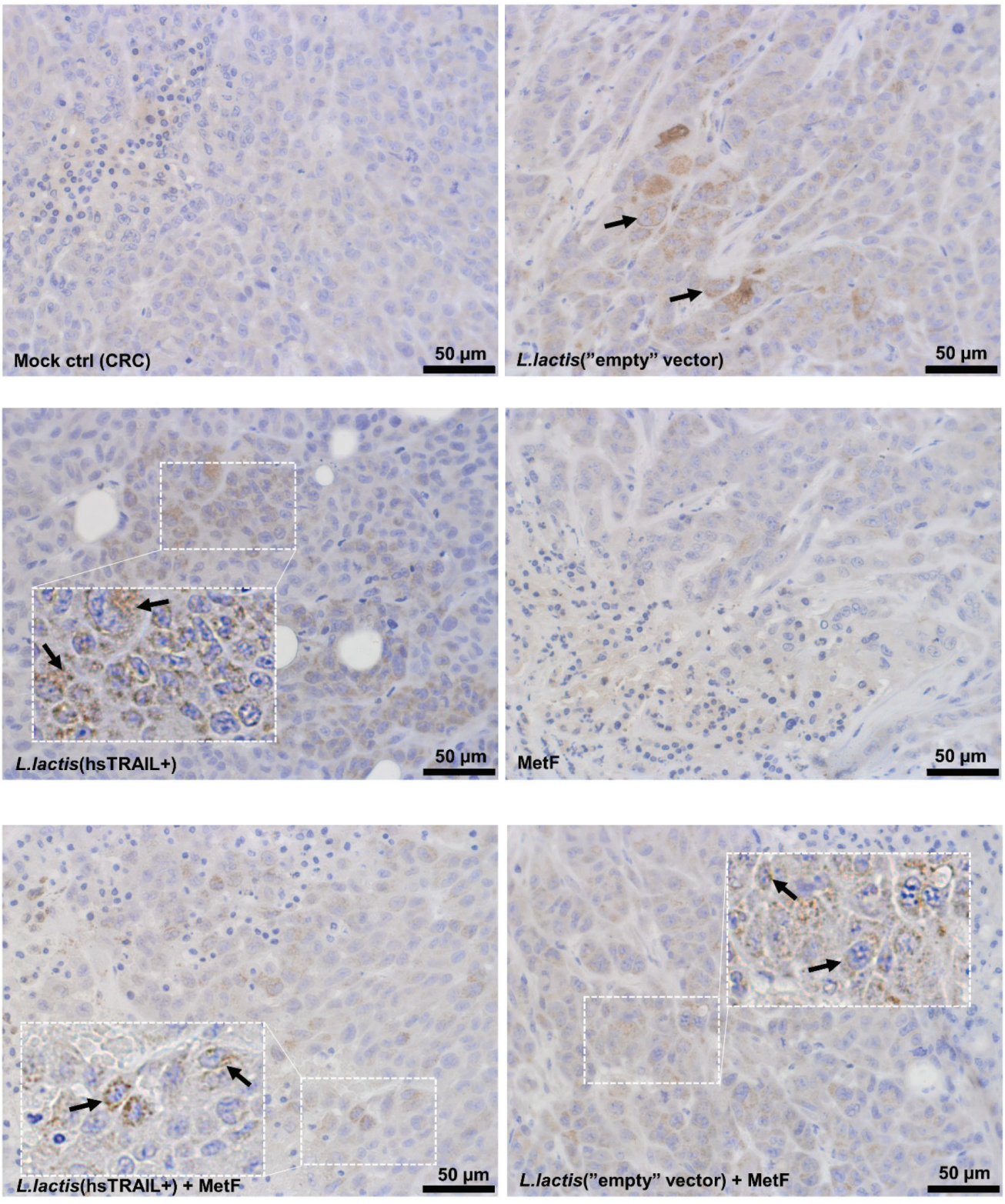

Fig 5. *hsTRAIL does not affect the MCP-1 production by CRC cells in primary tumor tissue. Representative images of paraffin sections of primary tumor in cecum subjected to IHC analysis for the presence of MCP-1. Black arrows indicate the presence of MCP-1. Scale bar: 50 µm. White dashed lines denote the areas that were subsequently magnified and corrected for light intensity, sharpness, and contrast with the same parameters for all magnified areas.*

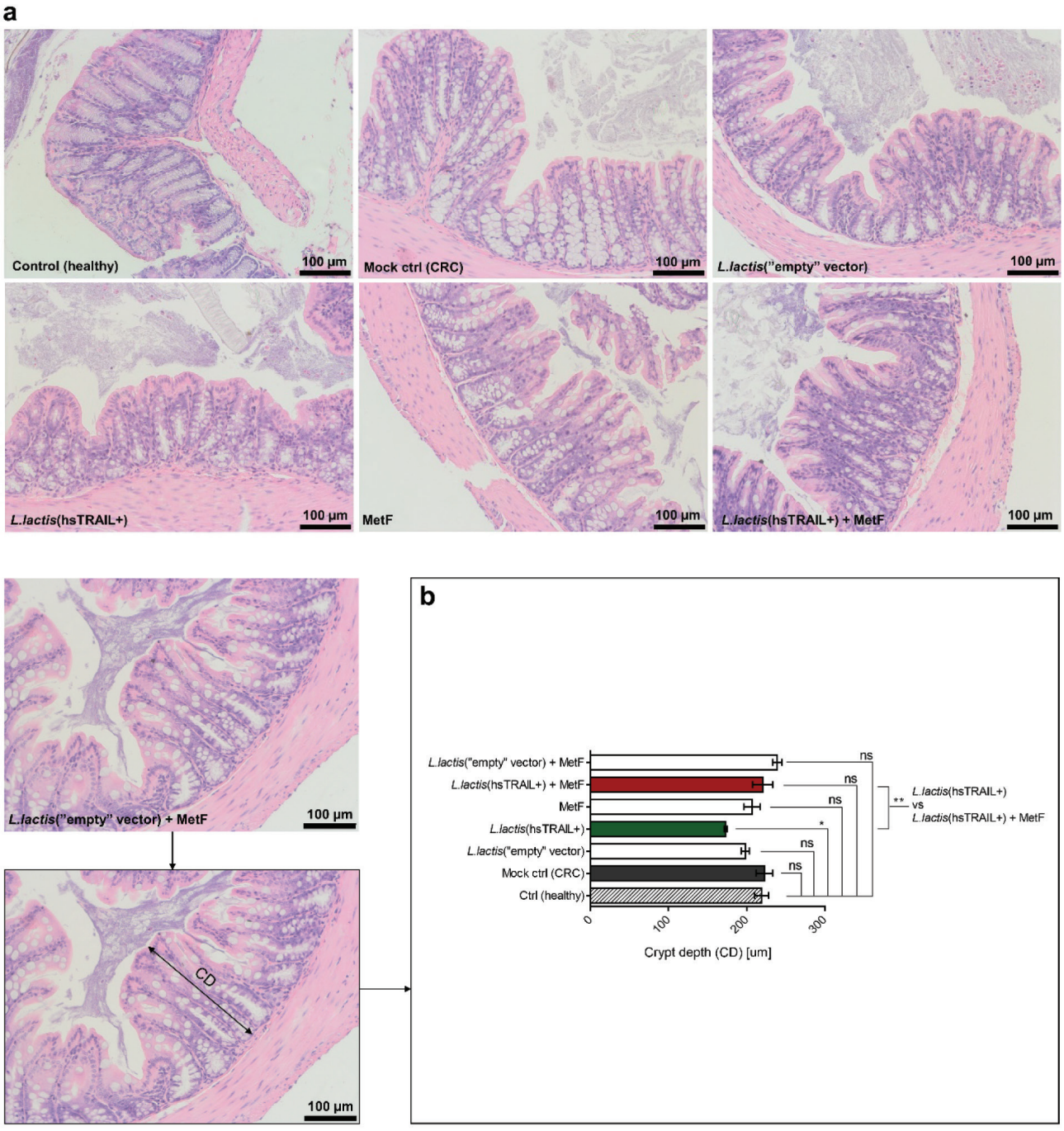

Fig 6. *hsTRAIL reduces the depth of colon crypts.* ***a*** *Representative images of paraffin sections of colons, subjected to H&E staining for the histopathological analysis. Scale bar: 100 µm.* ***b*** *Analysis of the depth of the colon crypts after 37 consecutive days of respective treatments. Crypt Depth (CD) was calculated using paint.net (version 4.1.6). The bars indicate mean values ± SEM (n=6). Statistical analysis was done using one-way ANOVA test, with Tukey's multiple comparisons post-hoc test. *p<0.05, **p<0.01.*

Structural abnormalities in colon morphology, including shortening of the crypts, are often described as a sign of the local inflammation (Dinh et al., 2016; Bai et al., 2021; Kamarudin et al., 2019). In our study, monotherapy with *L.lactis*(hsTRAIL+) significantly reduced the crypt depth, which was related to continuous secretion of hsTRAIL in the colon. This detrimental effect of hsTRAIL on the colon crypts was abrogated by metformin, a biguanide, which is widely used for treating type 2 diabetes. Recent preclinical and clinical studies have suggested that metformin not only reduces

chronic inflammation through the improvement of metabolic parameters such as hyperglycemia, insulin resistance and atherogenic dyslipidemia, but also has a direct anti-inflammatory action. Studies have suggested that metformin directly suppresses inflammatory response by inhibition of nuclear factor κB *via* AMP-activated protein kinase-dependent and independent pathways (Hosono et al., 2010). There are many studies showing a suppressive effect of metformin on tumorigenesis and cancer cell growth both in vitro and in vivo (Takayama et al., 1998). In non-diabetic subjects, it was shown that oral short-term low-dose metformin suppressed the development of colorectal aberrant crypt foci (Carvalho et al., 2017), which are considered as precursor lesions for colorectal carcinogenesis (DeRoche et al., 2014). In our model study metformin alone or in combination with hsTRAIL was able to suppress the tumor growth only at the beginning of the treatment (Day 8).

Despite the canonical activity of TRAIL leading to apoptosis of sensitive cancer cells, an increasing number of evidence also suggests its opposite, tumor-supportive and immune-modulatory nature, coming from the non-canonical pathway of TRAIL signalling (Azijli et al., 2013; Varfolomeev et al., 2005; Hartwig et al., 2017). In vitro studies performed by Varfolomeev et al. (2005) showed, that binding of TRAIL to its death-receptors in cancer cells, which display intermediate sensitivity to TRAIL, might lead to the formation of a secondary intracellular signalling complex. Such complex was suggested to support a removal of apoptotic cells, by activation of kinase pathways and promotion of the recruitment of phagocytes (Varfolomeev et al., 2005). Similar to ours, the studies performed by Hartwig et al. (2017) in orthotopic model of human lung cancer demonstrated that activation of TRAIL signalling in cancer cells surviving the treatment, induces secretion of monocyte chemoattractant protein-1 (MCP-1, CCL2), which finally drove an accumulation of M2-like macrophages in the tumor microenvironment, contributing to tumor growth (Hartwig et al., 2017). The importance of macrophages, defined as “tumor-associated macrophages” (TAMs), in cancer progression is the focus of many studies, including those related to CRC (Lin et al., 2019). In this context, it was shown that macrophages producing interleukin (IL)-1β could stimulate growth of CRC cells by activating GSK3β/Wnt signaling (Kaler et al., 2009). IL-1β production by macrophages was induced by tumor cells and resulted in protection of HCT116 cells from TRAIL-induced apoptosis (Kaler et al., 2010). In our model of human CRC, the IHC staining of the tumor slides showed an effect of hsTRAIL-delivery on the infiltration of $CD206^{+}$ macrophages into the tumor, confirming the results by Hartwig et al. (2017) and suggesting the mechanism of CRC progression in the mice receiving *L.lactis*(hsTRAIL+)-treatment. Although, the infiltration of M2-macrophages was not triggered by an increased production of MCP-1 by cancer cells, it is worth mentioning that the functional polarization of macrophages is not constant, being rather a transient state, regulated by the stimuli from the tumor environment (Mantovani et al., 2004; Wang et al., 2019). However, we cannot exclude the role of IL-1β in our experimental model – this aspect needs to be elucidated in our future studies.

One of the methods to generate clones of CRC cells resistant to the pro-apoptotic action of TRAIL is the treatment of sensitive cells with high concentrations of TRAIL, and subsequent isolation of the resistant clones (Jin et al., 2004). Although we do not have a formal proof, it seems that similar mechanism may operate in our experimental model, as HCT116 CRC cells are considered as TRAIL-sensitive (Saturno et al., 2013). In the present study, the administration pattern of *L.lactis*(hsTRAIL+) seems to play a significant role in the context of hsTRAIL effectiveness. While intratumor administration of *L.lactis*(hsTRAIL+) twice a week caused relevant anti-tumor activity (Kaczmarek et al., 2021), the continuous daily treatment resulted in its tumor-promoting action. In this case MetF was not effective in restoring CRC sensitivity to TRAIL-induced apoptosis.

In the summary, this is the first study providing evidence that *L.lactis* bacteria, harbouring a plasmid with hsTRAIL-cDNA are able for local delivery and secretion of biologically active hsTRAIL in the colon of CRC-bearing mice. Such a treatment however, reduced the depth of colon crypts, led to infiltration of M2-type macrophages into the tumor and promoted the tumor growth in SCID mice. In this context, it seems that tumorigenic action of exogenous TRAIL in CRC orthotopic mouse model is modulated by paracrine effects elicited by direct activation of TRAIL receptors on CRC cells and indirectly by activation of TAMs. Further research is necessary to fully understand the role of CRC microenvironment during *L.lactis*-delivered hsTRAIL therapy. This will be possible by including humanized immunocompetent murine models of CRC.

Declarations

Ethics approval

In vivo experiments, using orthotopic mice model of human CRC, were performed in accordance with the European Union Directive (2010/63/EU), Polish and French law and were approved by the local Ethical Committees (Poland: 202/2018, France: CNREEA approval no. 91).

Funding

This research was supported by the National Science Centre in Poland (GA no. 2014/15/B/NZ5/03484) and the European Union’s Horizon 2020 Research and Innovation Staff Exchange (RISE) programme under the Marie Skłodowska-Curie actions (GA no. 777682 [CANCER]).

Competing interest

A.C. is affiliated to Percuros B.V. as a founder and CEO. The authors declare no other conflicts of interest.

Authors' contributions

Conception of the work: J.B., K.K., J.W.; K.K., I.Q., A.G. performed the experiments; Data analysis: K.K., J.W., I.Q., A.G.; Results interpretation: K.K., J.W., I.Q., J.B.; Manuscript draft: K.K.; Final manuscript: J.B., K.K., A.C., M.S. All authors have read and agreed to the final version of the manuscript.

Data availability

The authors declare that all data supporting the results in this study are available within the paper. Source data for the figures in this study are available from the corresponding author on reasonable request.

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