Dual Functionalized Lactococcus lactis Shows Tumor Antigen Targeting and Cytokine Binding in Vitro

Abida Zahirović¹, Tina Vida Plavec¹ and Aleš Berlec¹,²*

¹Department of Biotechnology, Jožef Stefan Institute, Ljubljana, Slovenia; ²Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

Pro-inflammatory cytokines play an important role in the development and progression of colorectal cancer (CRC). Tumor-targeting bacteria that can capture pro-inflammatory cytokines in the tumor microenvironment and thus block their tumor-promoting effects might provide clinical benefits in inflammation-associated CRC. The aim of this study was to develop bacteria with dual functionality for selective delivery of cytokine-binding proteins to the tumor by targeting specific receptors on cancer cells. We engineered a model lactic acid bacterium, Lactococcus lactis, to co-display on its surface a protein ligand for tumor antigens (EpCAM-binding affitin; HER2-binding affibody) and a ligand for pro-inflammatory cytokines (IL-8-binding evasin; IL-6-binding affibody). Genes that encoded protein binders were cloned into a lactococcal dual promoter plasmid, and protein co-expression was confirmed by Western blotting. To assess the removal of IL-8 and IL-6 by the engineered bacteria, we established inflammatory cell models by stimulating cytokine secretion in human colon adenocarcinoma cells (Caco-2; HT-29) and monocyte-like cells (THP-1; U-937). The engineered L. lactis removed considerable amounts of IL-8 from the supernatant of Caco-2 and HT-29 cells, and depleted IL-6 from the supernatant of THP-1 and U-937 cells as determined by ELISA. The tumor targeting properties of the engineered bacteria were evaluated in human embryonic kidney epithelial cells HEK293 transfected to overexpress EpCAM or HER2 receptors. Fluorescence microscopy revealed that the engineered L. lactis specifically adhered to transfected HEK293 cells, where the EpCAM-targeting bacteria exhibited greater adhesion efficiency than the HER2-targeting bacteria. These results confirm the concept that L. lactis can be efficiently modified to display two proteins simultaneously on their surface: a tumor antigen binder and a cytokine binder. Both proteins remain biologically active and provide the bacteria with tumor antigen targeting and cytokine binding ability.

Keywords: cancer, probiotics, Lactococcus lactis, targeting, cytokines, IL-6, IL-8

Abbreviations: CFU, colony-forming-unit; CRC, colorectal cancer; EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; HRP, horseradish peroxidase; IL, interleukin; IRFP, infrared fluorescent protein; MCS, multiple cloning site; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline.
INTRODUCTION

Chronic, unresolved inflammation has been increasingly recognized as a key factor in the pathogenesis of many types of cancers, including liver, pancreatic, gastric, and colorectal cancers (CRC) (Terzic et al., 2010). CRC is the third most common cancer worldwide, and the second leading cause of cancer-related deaths (Sung et al., 2021). Studies have provided strong evidence that the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 (CXCL-8) have critical roles in the inflammatory processes associated with CRC (Baier et al., 2005; West et al., 2015). IL-6 has been shown to promote tumor cell growth, invasion, and migration (Becker et al., 2004; Zeng et al., 2017), while IL-8 increases proliferation, angiogenesis, and migration of malignant cells toward blood vessels, thus leading to tumor dissemination (Ning et al., 2011). Clinical data show that patients with stage IV CRC have more than 10 times higher serum level of IL-8 (1,089 pg/ml) than healthy individuals (79 pg/ml) (Lee et al., 2012). Of the pro-inflammatory cytokines involved in CRC carcinogenesis, IL-6 shows the greatest increase in patients with CRC compared to healthy controls, particularly in metastatic disease (Chung and Chang, 2003). Elevated IL-6 correlates with a bad patient prognosis and poor clinical outcome of CRC. Furthermore, pro-inflammatory cytokines have been shown to reduce the responses to cancer immunotherapy by inducing immunosuppression in the tumor microenvironment (Albini and Sporn, 2007).

A growing body of data on the critical role of pro-inflammatory cytokines at various stages of CRC development suggests that they represent new molecular targets in the treatment of CRC. Indeed, recent studies have demonstrated inhibitory effects of cytokine signaling blockers on cancer development and metastasis (Ning and Lenz, 2012; Johnson et al., 2018). These therapeutics will probably require combinatorial approaches, and might therefore serve as adjuvant therapies following standard treatment with radiotherapy, chemotherapy, or immune checkpoint inhibitors (Wattenberg and Beatty, 2020).

When considered for cancer treatment, local delivery of cytokine-binding proteins to the tumor is necessary to avoid systemic cytokine blockade, and to thus circumvent the side effects associated with down-regulation of the normal immune response. Site-specific drug delivery ensures maximal concentrations of therapeutic agents at the local site of action without negative side effects in healthy tissues. Sustained high levels of cytokine-binding proteins in tumor tissue can be achieved by targeting their delivery to the tumor using bacteria as the delivery vehicle. One of the main advantages of using bacteria for cancer treatments is their natural propensity for the central hypoxic regions of a tumor. This is a prominent feature of anerobic or facultative anaerobic bacterial pathogens such as Salmonella, Listeria, and Clostridium. However, even when these bacteria are used in an attenuated form, there is a risk of reversion to a virulent state (Toso et al., 2012).

Lactococcus lactis (L. lactis), a model lactic acid bacterium, raises no safety concerns and is considered a suitable host strain for development of biotherapeutics. L. lactis is particularly advantageous for intestinal drug delivery as it can survive the passage through the gastrointestinal tract (Plavec and Berlec, 2019; Plavec and Berlec, 2020). L. lactis can serve as a protein producer and a delivery vehicle at the same time, and it has previously been engineered for gastrointestinal delivery of various proteins, including viral antigens (Berlec et al., 2013), allergens (Zahirović and Lunder, 2018), immunomodulatory cytokines (Steidler et al., 2003) and proteins with affinity for pro-inflammatory cytokines (Ravnikar et al., 2010; Berlec et al., 2017; Kosler et al., 2017; Skrlec et al., 2018; Plavec et al., 2019) and chemokines (Skrlec et al., 2017). Moreover, alternative methods to genetic modification of bacteria have been introduced, including heterologous protein expression (Zadravec et al., 2015) and containment strategies (Steidler et al., 2003). Some applications were also aimed at the treatment of cancer (primarily CRC); these include L. lactis engineered to deliver antioxidant enzyme catalase (Del Carmen et al., 2017) or pro-apoptotic peptide kisspeptin (Zhang et al., 2016).

The wild-type lactic acid bacteria have shown protective effects against CRC through modulation of the gut microbiota, neutralization of carcinogens, and/or production of short-chain fatty acids (Uccello et al., 2012). These inherent anticancer effects can be enhanced by displaying cytokine-binding proteins on the bacterial surface, which will result in the capture of pro-inflammatory cytokines by the bacteria, and neutralization of their detrimental pro-tumorigenic effects in the tumor milieu. Furthermore, expression of specific proteins directed towards tumor antigens on the bacterial surface can enhance their selectivity for tumors (Duong et al., 2019). This was successfully achieved for attenuated Salmonella typhimurium by coating the bacterial surface with the RGD peptide that is directed against αvβ3-integrin (Park et al., 2016), or with a single-domain antibody directed against the B-lymphocyte antigen CD20 (Massa et al., 2013).

We have recently developed L. lactis strains that display small protein binders of IL-8 (Skrlec et al., 2017) and IL-6 (Zahirović et al., submitted) on their surface, and have demonstrated their binding to cytokines in vitro. Both proteins that were displayed on L. lactis, IL-8–binding evasin (Deruaz et al., 2008) and IL-6–binding affibody (Yu et al., 2014), are non-immunoglobulin binders and have high specificity and affinity for their target cytokines. We reasoned that the co-display of cytokine-binding proteins with ligands for tumor antigens can facilitate the specific interactions of the bacteria with the cancer cells, which will increase the accumulation and retention of the bacteria in the tumor tissue, and thus enable the targeted delivery of the cytokine-binding proteins to the tumors.

For tumor antigen-targeting we used epithelial cell adhesion molecule (EpCAM, CD326) (Das et al., 2015) and human epidermal growth factor receptor 2 (HER2, CD340) (Siena et al., 2018); both are up-regulated in CRC, but not in healthy cells, and therefore represent CRC markers. Small non-immunoglobulin scaffold proteins, EpCAM-binding affitin (Kalichuk et al., 2018), and HER2-binding affibody (Feldwisch et al., 2010), that possess high specificity and picomolar affinity for their receptors served as the targeting ligands. L. lactis bacteria
that displayed these tumor antigen ligands on their surface were recently shown to specifically bind to tumor surface antigens (Plavec et al., 2021b). Lectin-displaying *L. lactis* that target carbohydrate receptors on cancer cells were also developed (Plavec et al., 2021c).

In the present study, we sought to develop bacteria for targeted delivery of cytokine-binding proteins to tumors. We engineered *L. lactis* to display a combination of a tumor antigen-recognizing protein (EpCAM-binding affin AFFI or HER2-binding affibody ZHER) and a cytokine binding protein (IL-8–binding evasin EVA or IL-6–binding affibody ZIL) on their surface. The engineered bacteria were tested for their ability to remove IL-8 and IL-6 from the supernatant of cancer cells and to bind tumor antigens EpCAM or HER2.

**MATERIALS AND METHODS**

**Bacterial Strains, Media, and Growth Conditions**

The bacterial strains used in this study are shown in Table 1. *Lactococcus lactis* NZ9000 was grown at 30°C in M17 medium (MilliporeSigma, Burlington, MA, United States) supplemented with 0.5% glucose (Fluka AG, Buchs, Switzerland) (GM-17 medium) without agitation, or in the same medium solidified with 1.5% agar (Formedium, Huntstanton, United Kingdom). To maintain selection pressure on *L. lactis* transformants, 10 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, MO, United States) was added to the growth medium. Biliverdin HCl (15.5 μg/ml; Sigma-Aldrich) was added for expression of infrared fluorescent protein (IRFP).

**Construction of Dual Protein Expression Plasmids**

Restriction enzymes and T4 DNA ligase were from New England Biolabs (Ipswich, MA, United States). PCR amplifications were performed with Taq polymerase (New England Biolabs), according to the manufacturer protocols. Plasmid DNA was isolated (NucleoSpin plasmid; Macherey-Nagel, Düren, Germany), with addition of lysozyme (SigmaAldrich). Electroporation of *L. lactis* was performed according to (Holo and Nes, 1995) (Gene Pulser II apparatus; BioRad, Hercules, CA, United States). Nucleotide sequencing was performed by Eurofins Genomics (Ebersberg, Germany). Primers (Integrated DNA Technologies, Leuven, Belgium) and plasmids are listed in Table 1. Protein binders were introduced into the pNZDual plasmid using restriction enzyme-based cloning. Plasmid pNZDual contains two multiple cloning sites (MCS), each preceded by a nisin promoter, which enables expression of two proteins in *L. lactis* (Berlec et al., 2018). To construct dual expression plasmids that encode tumor antigen binder along with cytokine binder, we used pNZDual plasmids which already contained expression cassette with the tumor antigen binder gene (affi or zher) in MCS 1 and IRFP in MCS 2 [prepared

| Strain, plasmid or primer | Relevant features or sequence | Reference |
|---------------------------|-------------------------------|-----------|
| *L. lactis* NZ9000         | MAL8163 nisRK Aap3pN         | NIZO      |
| Plasmids                  |                               |           |
| pNZ8148                   | pSH71 derivative, *P*ₐₙᵦₐ, Cmᵣ, nisin-controlled expression | de Ruyter et al. (1996), Mierau and Kleerebezem. (2009) |
| pNZ-AFFI-IRFP             | pNZ8148-containing gene fusion of spUap45, affi and acmA3b on MCS1 and irfp gene on MCS2 | Plavec et al. (2021b) |
| pNZ-ZHER-IRFP             | pNZ8148-containing gene fusion of spUap45, flag-zher and acmA3b on MCS1 and irfp gene on MCS2 | Plavec et al. (2021b) |
| pNZ-IRFP                  | pNZ8148-containing irfp gene | Berlec et al. (2015) |
| pSD-EVA                   | pNZ8148-containing gene fusion of spUap45, evasin-3 and acmA3b | Škrlec et al. (2017) |
| pSD-ZIL                   | pNZ8148-containing gene fusion of spUap45, zil and acmA3b | Zahirović et al., submitted |
| pNZ-AFFI-EVA-IRFP         | pNBX-containing AFFI, EVA and IRFP cassettes | Plavec et al. (2021a) |
| pNZ-AFFI-ZIL-IRFP         | pNBX-containing AFFI, ZIL and IRFP cassettes | Plavec et al. (2021a) |
| pNZ-ZHER-EVA-IRFP         | pNBX-containing ZHER, EVA and IRFP cassettes | Plavec et al. (2021a) |
| pNZ-ZHER-ZIL-IRFP         | pNBX-containing ZHER, ZIL and IRFP cassettes | Plavec et al. (2021a) |
| pNZ-AFFI-EVA              | pNZ8148-containing gene fusion of spUap45, affi and acmA3b on MCS1 and gene fusion of spUap45, eva and acmA3b on MCS2 | This study |
| pNZ-AFFI-ZIL              | pNZ8148-containing gene fusion of spUap45, affi and acmA3b on MCS1 and gene fusion of spUap45, zil and acmA3b on MCS2 | This study |
| pNZ-ZHER-EVA              | pNZ8148-containing gene fusion of spUap45, flag-zher and acmA3b on MCS1 and gene fusion of spUap45, zil and acmA3b on MCS2 | This study |
| pNZ-ZHER-ZIL              | pNZ8148-containing gene fusion of spUap45, flag-zher and acmA3b on MCS1 and gene fusion of spUap45, zil and acmA3b on MCS2 | This study |

**Primers**

**USP-F-Nde2**

TTATTCTATATGGCTTTAAAAAAGATTATCTGAG

**A3b-R-xho2**

TTATTTCTCGAGTTATTTTATTCGTAGATACTGACC

NIZO, www.nizo.com; MCS, multiple cloning site.
FIGURE 1 | The scheme of the construction of dual expression plasmids. For construction of dual plasmids for surface display of tumor antigen binders and cytokine binders, we used previously prepared pNZDual plasmids which already contained expression cassette with the tumor antigen binder gene (ZHER or AFFI) in multiple cloning site 1 and IRFP in multiple cloning site 2. IRFP was substituted by the expression cassette containing the cytokine binder gene (EVA or ZIL). Thus, tumor antigen binder was paired with a cytokine binder, resulting in four dual plasmids pNZ-AFFI-EVA, pNZ-AFFI-ZIL, pNZ-ZHER-EVA, and pNZ-ZHER-ZIL. In each expression cassette, the binder gene was fused with the gene for Usp45 secretion signal and the gene for peptidoglycan binding domain of the AcmA protein, to enable surface display of the binder following its expression. AFFI, gene encoding EpCAM-binding affitin (183 bp); ZHER, gene encoding HER2-binding affibody (174 bp); EVA, gene encoding IL-8-binding evasin-3 (243 bp); ZIL, gene encoding IL-6-binding affibody (174 bp); Usp, gene encoding Usp45 secretion signal peptide (84 bp); AcmA, gene encoding the C-terminal portion of the AcmA surface anchoring protein containing three LysM repeats (642 bp); FLAG, epitope tag sequence; PnisA, nisin promoter.
previously in (Plavec et al., 2021b), referred to as pNZ-AFFI-IRFP and pNZ-ZHER-IRFP, respectively. We replaced IRFP in the MCS 2 with the expression cassette containing the cytokine binder gene (eva or zil) via Ndel/Xhol restriction sites. Thus, tumor antigen binder was paired with a cytokine binder, resulting in four dual plasmids pNZ-AFFI-EVA, pNZ-AFFI-ZIL, pNZ-ZHER-EVA, and pNZ-ZHER-ZIL (Table 1). A construction of dual protein expression plasmids is depicted in Figure 1. In each expression cassette, the binder gene was fused with the gene for Usp45 secretion signal and the gene for peptidoglycan binding domain of the AcmA protein, to enable its release into the growth medium and subsequent surface anchoring. The expression cassette containing cytokine binder gene eva or zil was amplified from pSD-EVA (Škrlec et al., 2017) or pSD-ZIL plasmid (Zahirović et al., submitted), respectively. For generation of pSD-ZIL plasmid, zil gene was back-translated and codon-optimized for use in L. lactis based on the amino acid sequence of IL-6-binding affibody ZIL-6_13 (Yu et al., 2014). Zil gene was cloned into a lactococcal plasmid pDBA3b in the same way as evasin 3 following the protocol described in Škrlec et al. (2017). For visualization of engineered bacteria, IRFP-encoding gene was subsequently inserted into the plasmid along with the protein binders, using BglBrick cloning, which allowed easier assembly of multiple gene cassettes (Plavec et al., 2021a).

Expression of Fusion Proteins in L. lactis

Overnight cultures of L. lactis harboring constructed plasmids were diluted (1:50) in 10 ml fresh GM-17 medium, and grown to an optical density (A600) of 0.50–0.80. Expression of the fusion proteins was induced by addition of 25 ng/ml nisin (Fluka AG) (de Ruyter et al., 1996; Mierau and Kleerebezem, 2005). After a 3 h incubation, 1 ml culture was stored at 4°C and resuspended in 250 µl incubation buffer spiked with 300 pg/ml cytokine standards (Mabtech). The optical density of the bacteria. ELISA for Determination of Cytokine Binding by L. lactis

The cytokine concentrations were determined using commercially available ELISA kits (Mabtech, Nacka Strand, Sweden), according to the manufacturer recommendations. Here, 96-well plates (Maxisorp Nunc; Thermo Fisher Scientific) were coated with cytokine-binding antibodies at 4°C overnight. The wells were washed five times with 200 µl PBS containing 0.05% Tween-20 (wash buffer) and blocked for 1 h at room temperature with PBS with 0.05% Tween and 0.1% bovine serum albumin (incubation buffer). L. lactis cells at 6 × 10⁹ colony forming units (CFU)/ml were centrifuged (5,000 × g, 5 min, 4°C) and resuspended in 250 µl incubation buffer spiked with 300 pg/ml cytokine standards (Mabtech). The optical density of bacterial suspension at 600 nm (OD₆₀₀) was used to calculate the number of L. lactis per ml using a factor determined earlier by serial dilutions (1 OD₆₀₀ = 1 ×10⁹ L. lactis/ml). Following 2 h at room temperature with gentle shaking, the bacterial cells were removed by centrifugation (7,500× g, 7 min, room temperature), and 200 µl cell-free bacterial supernatant was added to the wells and incubated for 2 h at room temperature. The remaining cytokine levels in the supernatant were determined using a calibration curves, that were generated by addition of different concentrations (0–1,200 pg/ml) of recombinant cytokine standards from the ELISA kit (Mabtech). The standards were incubated in the same buffer and under the same conditions as the samples, to ensure equal matrix effect. Following incubation and washing of the wells, 100 µl biotinylated monoclonal antibodies against the cytokines were added in incubation buffer at their recommended concentrations, and incubated at room temperature for 1 h. Following washing, 100 µl streptavidin-HRP (diluted 1:1,000 in incubation buffer) was added into the wells and incubated for 1 h at room temperature. The wells were washed again, and 50 µl 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) was added. The reaction was terminated after 20 min by addition of 50 µl 2 M sulfuric acid. Absorbances were read at 450 nm using a microplate reader (Infinite M1000; Tecan, Grödig, Austria), with wavelength correction at 650 nm. The binding is given as the proportions (%) of the cytokine removed from the solution by the bacteria.
Cell Lines and Culturing
Human embryonic kidney epithelial HEK293 cells (CRL-1573; American Type Culture Collection [ATCC], Manassas, Virginia, United States) and human colon adenocarcinoma Caco-2 cells (HTB-37; ATCC) were cultured in Dulbecco’s modified Eagle’s medium with high glucose and GlutaMAX (Gibco, Thermo Fisher Scientific). Human colon adenocarcinoma HT29 epithelial intestinal cells (HTB-38; ATCC) were maintained in McCoy’s medium with 1.5 mM L-glutamine and 2,200 mg/L sodium bicarbonate (ATCC). Human monocytic cell lines THP-1 (TIB-202; ATCC) and U-937 (CRL-1593.2; ATCC) sodium bicarbonate (ATCC). Human monocytic cell lines THP-1 (TIB-202; ATCC) and U-937 (CRL-1593.2; ATCC) were cultured in RPMI1640 medium (Gibco). Each medium was supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/ml streptomycin (Gibco). For Caco-2 cell culturing, 25 mM HEPES (Sigma-Aldrich) and 1% Eagle’s minimum essential medium nonessential amino acids solution (Sigma-Aldrich) were added. Unless otherwise stated, the cells were seeded in 24-well tissue culture plates (Corning, Thermo Fisher Scientific) at 1 ×10⁵ or 3 ×10⁵ cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Differentiation, Stimulation of Cytokine Production, and Incubation With Bacteria
THP-1 and U-937 cells were differentiated into macrophage-like cells by incubation with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 h. Before stimulation of cytokine secretion, PMA-containing medium was replaced with fresh RPMI medium without PMA, and the cells were incubated for another 48 h, to allow recovery. Cell differentiation was verified by evaluation of their morphological changes under microscope. IL-6 production was induced by treating the cells with 1 μg/ml lipopolysaccharide (LPS) from Escherichia coli serotype O55:B5 (L6529; Sigma-Aldrich) for 24 h. IL-8 secretion was induced by exposing the cells to 25 ng/ml human recombinant IL-1β (Cell Genix, Freiburg, Germany) for 6 h. Cytokine levels were measured in the conditioned media from the stimulated cells using ELISA development kits (Mabtech), as described above. For cytokine removal experiments, the engineered bacteria (6 ×10⁶ CFU/ml) were incubated with the conditioned media from the stimulated cells or were co-cultured with the stimulated cells for 2 h. Before or after incubation with bacteria, conditioned media were collected from stimulated cells, centrifuged (5 min 2,000 g at 4°C and 15 min 1,000 g at 4°C), and stored at −80°C until analysis. The contents of the remaining cytokines in the cell culture supernatants were measured with ELISA, and the proportions (%) of cytokines removed by the engineered bacteria were calculated. Conditioned media from untreated cells were used as controls.

Cell Transfection
HEK293 cells were seeded in 24-well plates at 2 ×10⁵ cells/well and transected 24 h later, at approximately 70% confluence, with 1.5 μl PolyJet (SignaGen Laboratories, Rockville, MD, United States) and 0.5 μg plasmid [for expression of the EpCAM-sfGFP fusion protein, pcDNA3-EpFL-sfGFP (Gaber et al., 2018); for expression of the HER2-mEmerald fusion protein, mEmerald-ERBB2-N-18 (Addgene plasmid #62755; http://n2t.net/addgene:62755; RRID:Addgene_62755)], according to the manufacturer protocols. The medium was replaced 24 h after transfection.

Bacterial Cell Adhesion Assay
Bacterial cell adhesion assays were carried out as reported previously (Plavec et al., 2021b). Here, the medium from transfected HEK293 cells was aspirated and 500 μl 8 ×10⁶ CFU/ml engineered L. lactis bacteria (diluted in pre-warmed RPMI; A₆₀₀ 0.8) were added to each well, and incubated for 2 h at 37°C. Following incubation, the wells were gently washed twice with PBS to remove unattached L. lactis, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed twice with PBS, and mounted on a microscope slide with DAPI-containing mounting agent (Thermo Fisher Scientific), for confocal microscopy. Images were acquired with a confocal microscope (LSM-710; Carl Zeiss, Germany) using a ×63 oil-immersion objective, with settings for bright-field, DAPI (blue channel, 405 nm), GFP/mEmerald (green channel, 488 nm), and IRFP (red channel, 633 nm). Images were processed and analyzed using the ImageJ software. The adhesion of L. lactis cells to the HEK293 cells was quantified by counting the numbers of L. lactis in 10 representative microscopy images using ImageJ (Schneider et al., 2012). The results are expressed as mean numbers of L. lactis per human cell ± standard deviation. Human cells were counted using a cell counter plugin for ImageJ (https://imagej.nih.gov/ij/plugins/cell-counter.html), where the cells on the edges were excluded. L. lactis cells were counted with the particle analysis function using the Otsu threshold algorithm. To avoid background fluorescence being included as particles, the minimum size was set to 1 μm². The area of a single L. lactis bacterium was determined by averaging areas of putative single bacteria for each set of 10 images. To determine the number of individual L. lactis cells, the areas of aggregates were divided by the calculated area of a single L. lactis bacterium.

Statistical Analysis
Statistical analyses were performed using the Graph-Pad Prism 7.00 software (San Diego, CA; United States). All of the data are presented as means ± standard deviation (SD). The concentrations of cytokines obtained by ELISA were compared using student’s two-tailed t-tests. Differences in cytokine-binding between relevant pairs of bacteria are considered significant for p < 0.05.

RESULTS
Design of Plasmids for Dual Protein Expression
Four dual plasmids containing expression cassettes for simultaneous surface display of tumor antigen binders
and cytokine binders were constructed. The expression cassette of each individual binders contained the protein binder gene fused to gene for signal peptide Usp45 and the gene for the surface anchor AcmA to enable its surface display. Based on protocol described in the Materials and methods (depicted in Figure 1), tumor antigen binder gene (zher; af) was paired with cytokine binder gene (eva; zil), resulting in dual plasmids pNZ-AFFI-EVA, pNZ-ZHER-EVA, and pNZ-ZHER-ZIL (Table 1). The L. lactis strains transformed with the constructed dual plasmids were designated as L. lactis-AFFI-EVA, L. lactis-AFFI-ZIL, L. lactis-ZHER-EVA, and L. lactis-ZHER-ZIL. The schematic of the dual gene constructs and the resulting L. lactis strains are shown in Figure 2. A FLAG-tag consensus sequence was added previously between the secretion signal and the ZHER coding sequence to facilitate detection, whereas it was excluded from the AFFI fusion protein due to negative effect on its display and functionality (Plavec et al., 2021b).

**Tumor Antigen Targeting and Cytokine Binding Proteins Are Co-expressed in L. lactis**

To examine the co-expression of the protein binders in the engineered L. lactis, whole bacterial cell lysates were analyzed by Western blotting. L. lactis transformed with the pSD plasmids that encoded the individual binders were used as positive controls. Immunoblotting revealed the bands at 35 kDa, which corresponds to the molecular weight of the full-length fusion proteins (protein binder ~7 kDa, AcmA ~25 kDa). A combination of protein binders (AFFI and EVA; AFFI and ZIL; ZHER and EVA; ZHER and ZIL) was present in the cell lysates from L. lactis strains carrying dual plasmids (Figure 3). Contrary to distinct bands representing binders ZHER, AFFI and ZIL, the

---

**FIGURE 2** | Schematic representation of the gene constructs that were cloned into the lactococcal pNZDual plasmid for co-expression and simultaneous display of tumor antigen binding proteins and cytokine binding proteins on the surface of the Lactococcus lactis bacteria (A), with the resulting recombinant L. lactis strains (B). AFFI, gene encoding EpCAM-binding af in (183 bp); ZHER, gene encoding HER2-binding af body (174 bp); EVA, gene encoding IL-8-binding evasin-3 (243 bp); ZIL, gene encoding IL-6-binding af body (174 bp); Usp, gene encoding Usp45 signal peptide for secretion into the growth medium (84 bp); AcmA, gene encoding the C-terminal portion of the AcmA protein containing three LysM repeats for surface anchoring (642 bp); FLAG, epitope tag sequence; MCS, multiple cloning site; PnisA, nisin promoter.
bands representing EVA were faint or invisible for all variants, suggesting low functionality of EVA following SDS PAGE and western blot under denaturing conditions. Successful expression of EVA from pSD-EVA has been demonstrated previously (Škrlec et al., 2017) and therefore this plasmid was used in the present study as a positive control. To facilitate detection, we tagged EVA with either cmyc or flag and confirmed EVA expression in both single and dual plasmid carrying L. lactis strains by Western blotting using anti-cmyc and anti-flag antibodies (data not shown). The fusion proteins were consistently detected as double bands, with additional slightly higher bands that probably represent their unprocessed forms (with signal sequence). In the bacterial growth medium, only one band was identified, corresponding to the mature form of the fusion proteins (data not shown). No bands were seen for lysates of control L. lactis cells carrying the empty plasmid pNZ8148.

Removal of Recombinant IL-8 and IL-6 by Engineered L. lactis as a Function of Bacterial Cell Number

The cytokine-binding ability of the engineered bacteria (i.e., L. lactis co-expressing tumor antigen binders and cytokine binders) was assessed by incubation of increasing numbers of bacterial cells with recombinant cytokines added to the solutions at 300 pg/ml. The levels of the cytokines that remained in the solution were determined by ELISA. The calculated recoveries were 102% for IL-8 and 98% for IL-6. The results showed that $6 \times 10^9$, $3 \times 10^9$, $6 \times 10^8$, and $3 \times 10^8$ CFU/ml L. lactis-AFFI-EVA removed 81, 71, 47, and 32%, respectively, of IL-8 from the solution (Figure 4A). Similarly, $6 \times 10^9$, $3 \times 10^9$, $6 \times 10^8$ and $3 \times 10^8$ CFU/ml of L. lactis-ZHER-EVA removed 75, 66, 36, and 25%, respectively, of IL-8 from the solution (Figure 4C). Thus, the extent of IL-8 removal correlated directly with the number of bacterial cells. In contrast, the extent of IL-6 removal was uniformly high (>95%) regardless of the number of bacterial cells tested (Figures 4B, D). The estimated minimal number of bacterial cells able to reduce a 50% of IL-8 was 10^8 CFU/ml, whereas the minimal number of cells able to reduce a 50% of IL-6 was at least one order of magnitude lower. L. lactis displaying only tumor antigen binder (AFFI or ZHER) were used as the negative control and showed negligible or low binding of recombinant cytokines.
**L. lactis** Co-Expressing IL-8 Binder EVA and the Tumor Antigen Binder AFFI or ZHER Removes IL-8 Secreted by Caco-2 and HT-29 Colorectal Cancer Cells

The basal secretion of IL-8 into the culture medium by untreated Caco-2 and HT-29 cells was low (<10 pg/ml) or undetectable. Following 2 h treatment with IL-1β, the concentrations of IL-8 released into the supernatant of the Caco-2 and HT-29 cells ranged from 107 to 1,221 pg/ml depending on cell number or metabolic state of the cells upon stimulation (**Figure 5**). After incubation of engineered bacteria with IL-8-conditioned medium from IL-1β–primed cells, **L. lactis**-AFFI-EVA eliminated 65% of IL-8 from the supernatant of Caco-2 cells, and 100% of IL-8 from the supernatant of HT-29 cells. High removal efficiency of IL-8 was also achieved by **L. lactis**-ZHER-EVA, which sequestered 69% of IL-8 from the supernatant of Caco-2 cells, and 71% from the supernatant of HT-29 cells (**Figures 5A,D**). Furthermore, engineered **L. lactis** were shown to retain the same levels of binding when incubated in the wells along with the producer cells (**Figures 5C,D**). **L. lactis** expressing only EVA was included for comparison, and showed the greater cytokine removal ability in most cases (bound 17–40% more IL-8) compared with **L. lactis** strains carrying dual plasmids. As for the control bacteria, **L. lactis**-ZHER eliminated negligible amounts of IL-8. On the contrary, **L. lactis**-AFFI bound surprisingly high levels of IL-8 secreted by HT-29 cells (up to 99%), which may be due to post-translational modifications of HT-29-derived IL-8 that may have led to its non-specific binding to AFFI (Vacchini et al., 2018).

**L. lactis** Co-Expressing IL-6 Binder ZIL and the Tumor Antigen Binder AFFI or ZHER Removes IL-6 Released by THP-1 and U-937 Monocyte-like Cells

To assess the removal of IL-6 by the engineered bacteria, we established a cell model of inflammation by stimulating monocyte-like THP-1 cells with LPS, thus inducing IL-6 production. After LPS treatment, the cell-conditioned media were collected and the levels of secreted IL-6 were quantified by ELISA. The basal level of IL-6 was 1 pg/ml or below the detection limit. After stimulation with LPS for 24 h, the production of IL-6 increased to 222 and 638 pg/ml in two separate experiments (**Figures 6A,D**). For cytokine binding
experiments, the engineered bacteria were incubated with IL-6-conditioned medium from LPS-stimulated cells and the residual IL-6 was determined by ELISA. As shown in Figure 6, the IL-6 binding was very efficient; *L. lactis*-AFFI-ZIL and *L. lactis*-ZHER-ZIL removed 94 and 92%, respectively, of the IL-6 from the supernatant of THP-1 cells (Figure 6A). The extent of binding was essentially equal as that of *L. lactis*-ZIL. When co-cultured with THP-1 cells, the engineered bacteria retained the same levels of IL-6 removal (Figure 6D).

In the tumor microenvironment, monocytes undergo activation and differentiation into macrophages, known as tumor-associated macrophages (Soncin et al., 2018). To examine whether the engineered bacteria can remove macrophage-derived IL-6 from their environment, we established a model of macrophages by exposing human monocytic leukemia THP-1 cells and human histiocytic lymphoma U-937 cells to PMA for 48 h. This treatment induced typical features of macrophages, such as cell adhesion, changes in morphology (from circular to spindle-shaped cells, with cellular extensions), and increased cell granularity (Schutte et al., 2009). After 24 h of stimulation with LPS, the concentration of IL-6 increased substantially. The levels of IL-6 varied in different experiments depending on the cell number, cell type (U-937 released more IL-6 than THP-1), or their differentiation status. If IL-6 concentration was above the linear range the supernatants were diluted. The supernatants that were used for incubation with bacteria contained IL-6 in the range from 469 to 1,040 pg/ml (Figures 6B,C,E,F). Similar to previous observations with undifferentiated THP-1 cells, we found that ZIL co-expressing *L. lactis* strains removed high proportion (from 96 to 99%) of the IL-6 released in the cell culture supernatant of the differentiated THP-1 cells and the differentiated U-937 cells (Figures 6B,C). When the engineered bacteria were co-cultured with the cells, the proportions of bound IL-6 remained as high as after incubation of the bacteria with conditioned media (Figures 6E,F). The control *L. lactis* strains bound a negligible amount of IL-6 (≤3%) in most cases.
Engineered *L. lactis* Strongly and Selectively Adhere to EpCAM-Overexpressing HEK293 Cells

The feasibility of tumor targeting with AFFI co-expressing bacteria was assessed using HEK293 cells transfected with the EpCAM receptor fused to fluorescent protein superfolder GFP (sfGFP). To visualize bacterial adhesion by fluorescence microscopy, we used *L. lactis* that co-expressed the IRFP together with the tumor antigen binders and cytokine binders (Plavec et al., 2021a). Surface presentation of the protein binders and intracellular IRFP expression were confirmed by flow cytometry and fluorescence measurements, respectively (Plavec et al., 2021a). Confocal microscopy showed that the engineered bacteria *L. lactis*-AFFI-EVA-IRFP and *L. lactis*-AFFI-ZIL-IRFP strongly and selectively adhered to EpCAM-overexpressing HEK293 cells, in contrast to the control strain *L. lactis*-IRFP, which showed no adhesion ability (Figure 7A). Particular adhesion pattern was observed with the clustering of the bacteria at the cell edges (Figure 7A, arrows). On average, 27 *L. lactis*-AFFI-EVA-IRFP, 48 *L. lactis*-AFFI-ZIL-IRFP, and 55 *L. lactis*-AFFI-IRFP (positive control) adhered per single EpCAM-bearing HEK293 cell, whereas only 2 *L. lactis*-IRFP (negative control) adhered per single EpCAM-bearing HEK293 cell (Figure 7C). Compared to the positive control, a 49% and a 13% reduction of the adhesion was noticed for *L. lactis*-AFFI-EVA-IRFP and *L. lactis*-AFFI-ZIL-IRFP, respectively. The average number of adhered bacteria per nontransfected HEK293 cell was <1; hence, the AFFI co-expressing bacteria showed no reactivity toward nontransfected cells (Figure 7B). These data confirm that the displayed EpCAM-targeting tumor antigen binder AFFI effectively and specifically promotes adherence of *L. lactis* to the transfected EpCAM-bearing HEK293 cells.

Engineered *L. lactis* Selectively Adhere to HER2-Overexpressing HEK293 Cells

The feasibility of tumor targeting with ZHER co-expressing bacteria was investigated using HEK293 cells transfected with
HER2 receptor fused to fluorescent protein monovalent variant of GFP mEmerald. The attached bacteria were visualized by confocal fluorescence microscopy via the IRFP reporter protein that was co-expressed in *L. lactis* with the protein binders (Plavec et al., 2021a). Confocal microscopy showed that the engineered bacteria selectively adhered to HER2-overexpressing HEK293 cells and showed no reactivity towards nontransfected HEK293 cells (Figures 8A,B). The adhesion pattern of the HER2-targeting bacteria was similar to that of the EpCAM-targeting *L. lactis* strains,
with the bacteria mostly attached to the cell edges (Figure 8A, arrows). Quantification showed that on average 6 \textit{L. lactis}-ZHER-EVA-IRFP, 3 \textit{L. lactis}-ZHER-ZIL-IRFP, 14 \textit{L. lactis}-ZHER-IRFP (positive control), and 1 \textit{L. lactis}-IRFP (negative control) adhered per HER2-bearing HEK293 cell (Figure 8C). Compared with positive control, a 57% and a 79% reduction of the adhesion was noticed for \textit{L. lactis}-ZHER-EVA-IRFP and \textit{L. lactis}-ZHER-ZIL-IRFP, respectively. ZHER co-expressing bacteria showed no reactivity towards nontransfected HEK293 cells, with no more than 1 bacterium adhering per nontransfected HEK293 cell (Figure 8B). These data confirm that the displayed HER2-targeting tumor antigen binder

FIGURE 8 | The engineered \textit{L. lactis} specifically adhere to HER2-overexpressing HEK293 cells. Representative confocal microscopy images of adhesion of ZHER co-expressing \textit{L. lactis} to HEK293 cells transfected with HER2 (A) or to nontransfected HEK293 cells (B). Images were quantified by determining the number of adhered bacteria per HEK293 cell in 10 representative micrographs for each experimental condition, using ImageJ (C). \textit{L. lactis} strains tested: \textit{L. lactis}-ZHER-EVA-IRFP (\textit{L. lactis} co-expressing ZHER, EVA, and IRFP); \textit{L. lactis}-ZHER-ZIL-IRFP (\textit{L. lactis} co-expressing ZHER, ZIL, and IRFP); \textit{L. lactis}-ZHER-IRFP (\textit{L. lactis} co-expressing ZHER and IRFP; positive control); and \textit{L. lactis}-IRFP (\textit{L. lactis} expressing only IRFP; negative control). DAPI, DAPI channel showing HEK293 cell nuclei; HEK293-HER2-mEmerald, green fluorescence channel showing mEmerald-labeled HER2 overexpressed on the surfaces of the HEK293 cells; \textit{L. lactis}, red fluorescence channel showing \textit{L. lactis}. Enlarged images show attachment of \textit{L. lactis} to transfected HEK293 with characteristic adhesion pattern (arrows). White squares indicate enlarged sections of the images. Scale bars, 20 \mu m.
ZHER effectively and specifically promotes adherence of *L. lactis* to the transfected HER2-bearing HEK293 cells. However, the adhesion level was significantly lower for the HER2-targeting *L. lactis* strains compared with the EpCAM-targeting *L. lactis* strains.

**DISCUSSION**

Tumorigenesis in CRC is driven by soluble mediators, including the pro-inflammatory cytokines IL-6 and IL-8, which orchestrate the many-fold cellular activities that underlie inflammation. Consequently, modulation of these cytokines might be a promising therapeutic strategy for cancer treatments. Here, we engineered *L. lactis* to serve as a vector for targeted delivery of cytokine-binding proteins to tumor. The *L. lactis* bacteria were constructed by displaying a tumor antigen-recognizing moiety and a cytokine-binding moiety on their surface. These engineered dual functionalized bacteria were shown to selectively target HEK293 cells that overexpress tumor antigens. Moreover, the engineered bacteria removed a high proportion of the IL-8 from the supernatants of immunostimulated Caco-2 and HT-29 colon adenocarcinoma cell lines, and depleted IL-6 from the supernatants of immunostimulated THP-1 and U-937 monocytic cells.

*L. lactis* with dual functionality was developed by simultaneously displaying a combination of a tumor antigen-recognizing protein and a cytokine-binding protein on the bacterial surface using a dual promoter plasmid (pNZDual). IL-8–binding evasin (designated as EVA) and IL-6–binding affibody (designated as ZIL) were used to achieve cytokine removal, while EpCAM-binding affitin (designated as AFFI) and HER2-binding affibody (designated as ZHER) served for tumor antigen targeting.

After confirming the co-expression of the fusion proteins in all dual plasmid carrying *L. lactis* strains, their cytokine binding properties were investigated in relation to the bacterial cell number using recombinant cytokines. The engineered bacteria removed more than 90% of recombinant IL-6 from the solution independent of bacterial cell numbers tested, whereas they removed between 25 and 81% of recombinant IL-8 in a dose-dependent manner. In agreement with previous study (Skrlec et al., 2017), the highest IL-8 removal was achieved by 6 × 10^9 CFU/ml bacterial cells, which falls within the range of therapeutic doses in current probiotic preparations for human use. Although typical doses of probiotics vary by product, most clinical studies have examined doses from 1 to 20 billion CFU per day, with higher doses generally associated with better therapeutic outcomes (i.e., >10 billion CFU per day in adults) (Kligler and Cohrssen, 2008). Therefore, we chose 6 × 10^9 CFU/ml for testing removal of IL-8 and IL-6 from the cell culture supernatants.

The inflammatory cycle in cancer is driven by the pro-inflammatory cytokines that are released in the tumor microenvironment by cancer cells, in concert with immune cells. To assess the removal of the cytokines involved in cancer inflammatory processes by the engineered bacteria, we established cell models that mimic certain elements of the inflammatory milieu within the tumor microenvironment. Under inflammatory conditions, cancer cells are a source of IL-8 in the tumor microenvironment. We used the Caco-2 and HT-29 cell lines as a model of CRC cells. To simulate inflammatory conditions, the Caco-2 and HT-29 CRC cells were primed with IL-1β, a potent inducer of IL-8 secretion. The optimal dose of IL-1β required to induce maximal IL-8 secretion in Caco-2 cells was determined to be 25 ng/ml (Skrlec et al., 2017). Autocrine secretion of IL-8 can activate intrinsic mechanism of tumor cells to promote their escape from stress-induced apoptosis (Doll et al., 2010). Cancer-cell-derived IL-8 can enhance tumor growth, invasion, angiogenesis, metastasis, and resistance to therapy (Doll et al., 2010). In breast cancer, it has been shown that expression of IL-8 can be modulated by the manipulation of EpCAM expression *in vitro* (Sankpal et al., 2013). Here, we show that cell-released IL-8 can be effectively captured and removed by the engineered bacteria both in the presence and absence of the producer cells. IL-8 removal was substantial, whereby *L. lactis* co-expressing EVA and tumor antigen binders removed 65–69% of IL-8 from Caco-2 supernatant and 71–100% of IL-8 from supernatant of HT-29 cells.

In addition to cancer-cell-derived mediators, pro-inflammatory cytokines produced by immune cells (i.e., primarily monocytes and macrophages) also drive inflammation in the CRC microenvironment (Hao et al., 2012). Monocytes are attracted to tumor tissue by chemokines such as IL-8, which is one of the major chemoattractants in CRC (Ning et al., 2011). In the tumor microenvironment, monocytes respond to paracrine stimuli from cancer cells by secreting soluble mediators, most notably IL-6, and these participate in tumor growth, invasion, intravasation, and metastasis (Soncin et al., 2018). For the analysis of IL-6 removal by the engineered bacteria, we used the immunostimulated THP-1 human acute monocytic leukemia cell line and the U937 histiocytic lymphoma cell line. PMA-differentiated THP-1 and U-937 cells were used as cell models of macrophages. It is known that the production of pro-inflammatory cytokines increases when monocytes differentiate into macrophages, which suggests that they are more likely to induce inflammation than monocytes (Soncin et al., 2018). After incubation of the engineered bacteria with stimulated cells or their conditioned medium, high levels of IL-6 were removed (>90%) by ZIL co-expressing *L. lactis* in both the undifferentiated and differentiated monocyte-like cell lines. The removal of IL-6 from cell culture supernatants by the dual functionalized bacteria was comparable to that by its single binder counterpart (*L. lactis* that expressed only ZIL). This confirms that IL-6 removal by the engineered bacteria was not affected by the presence of tumor antigen binders on their surface. Notably, IL-6 removal by the ZIL co-expressing bacteria was more pronounced than IL-8 removal by the EVA co-expressing bacteria, even though both binders have similar affinity.
constants (Kd ~500 pM for ZIL-6 (31), Kd ~430 pM for EVA (Deruaz et al., 2008)). This finding highlights that in addition to the affinities, the interactions of the ligand-displaying bacteria with the target depend on the extent of ligand display, its surface accessibility and/or spatial orientation. Overall, the cytokine removal by the engineered bacteria was high and effective across a range of cytokine concentrations (107–1,040 pg/ml). Thus, the cytokine binders expressed on *L. lactis* remained biologically active and bound the IL-8 and IL-6 in the media of four different cell lines.

Previous studies have shown that neutralization of IL-8 using monoclonal antibodies or small-interfering RNAs is not sufficient to block signaling of other chemokines in the tumor microenvironment (Ning and Lenz, 2012). Moreover, disruption of a single cytokine might trigger compensatory mechanisms that maintain pro-tumorigenic inflammation and immunosuppression (Jones et al., 2016). These studies suggest that the agent designed to attenuate the effect of a single cytokine involved in cancer inflammation is not sufficient for a complete clinical effect. Here, we have shown that multiple cytokines can be targeted by engineered *L. lactis*. The developed bacteria can be further tested for their ability to achieve additive or synergistic effects as a means to overcome cytokine redundancy.

Bacteria have a natural propensity to colonize tumor, and although they have been administered safely in clinical trials for cancer treatment (Toso et al., 2002), efficient tumor colonization requires administration of high bacterial number into the bloodstream and therefore has a narrow therapeutic index. The oral administration route is a path to selectively deliver bacteria to the CRC tumor microenvironment while minimizing systemic exposure. Oral delivery of nonpathogenic probiotic strains equipped with tumor antigen ligands for specific tumor targeting might lead to preferential accumulation of bacteria in the CRC as it allows bacteria to closely interact with tumor tissue, thus increasing drug availability in close proximity to responsive cells and limiting dilution into the luminal fluid.

The interactions between the engineered bacteria and HEK293 cells that overexpress tumor antigens EpCAM or HER-2 were investigated by fluorescent staining and confocal microscopy. We found that the engineered bacteria selectively adhered to the transfected cells, with a high level of adhesion observed for AFFI co-expressing bacteria, and a moderate level of adhesion for ZHER co-expressing bacteria. The ligand-receptor interaction is generally influenced by the affinity of the ligand for the receptor, the expression and surface accessibility of the receptor, and the rate of receptor internalization upon ligand binding. The difference in relative adhesion efficiency between AFFI and ZHER expressing strains probably reflects the lower surface accessibility of HER2 receptor compared to EpCAM rather than the affinity, considering that ZHER has a higher affinity constant (Kd = 22 pM) for HER2 than AFFI for EpCAM (Kd = 110 pM). The interactions were highly specific as non-targeted bacteria (i.e., *L. lactis* expressing only IRFP intracellularly) showed no adhesion activity towards transfected HEK-293 cells, and neither *L. lactis* strain adhered to non-transfected cells. Hence, by displaying tumor antigen binding proteins on their surface, *L. lactis* with strong cell adhesion properties were developed from a nonadhesive *L. lactis* strain. This means that the adhesion is achieved via the interaction between the tumor antigen ligand on the bacteria and the receptor on the cell surface, rather than via the nonspecific interactions between the peptidoglycans on the bacteria and the cell surface.

In general, the microscopy revealed that all of the *L. lactis* strains tested were attached to the cell edges. Such an adhesion pattern appears to be characteristic of *L. lactis*, and is consistent with previous reports of dense clusters of *L. lactis* IBB477 strain interacting with mucus secreted by HT29-MTX cells (Radziwill-Bienkowska et al., 2017). Similarly, previous studies have shown that *Lactobacillus* strains also preferentially adhere to cell edges, with the exception of highly adhesive *Lb. plantarum* C982, which adhered throughout the monolayer of the NCM460 normal human colon mucosal epithelial cell line (Garcia-Gonzalez et al., 2018).

Overall, we have demonstrated here that engineered bacteria can completely remove or markedly reduce the concentrations of IL-8 and IL-6 in the media of four different cell lines. Furthermore, these engineered bacteria exerted tumor antigen targeting in cell cultures, whereby strong and highly specific adhesion to HEK293 cells overexpressing the tumor antigens EpCAM or HER2 was achieved, particularly by the EpCAM-targeting *L. lactis* strains. When dual functionalized bacteria were compared with *L. lactis* strains that display only cytokine binders, the EVA co-expressing dual *L. lactis* strains removed less IL-8, whereas the ZIL co-expressing dual *L. lactis* strains removed the same amount of the IL-6 and no effect of tumor antigen binders was observed. As expected, the adhesion of dual *L. lactis* strains was lower (about a 10% to a 80% reduction) compared to the *L. lactis* displaying the tumor antigen binders only. Of the four *L. lactis* strains tested, *L. lactis*-AFFI-ZIL-IRFP showed the highest cytokine removal and adhesion efficiency, thus showing the greatest potential for further exploration of cytokine-targeted therapeutic strategies against cancer.

**CONCLUSION**

In this study, we have developed a set of new *L. lactis* strains with dual functionality by displaying two distinct bioactive moieties on the bacterial surface. The surface display of EpCAM- or HER2-binding proteins enabled selective targeting of the cells overexpressing tumor antigens, while the attachment of the IL-8- or IL-6-binding proteins to the *L. lactis* surface provided the bacteria with cytokine removal capacity. To the best of our knowledge, this is the first report of recombinant *L. lactis* equipped with ligands for tumor antigen targeting and cytokine neutralization. By using other therapeutic and/or diagnostic domains that bear unique functionalities, this strategy can be readily applied to
target other relevant molecules involved in the pathogenesis of cancer or other diseases.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

AZ and AB designed the methodology and conceptualization; AZ performed the experimental work and conducted data analysis; TVP assisted with the experimental work; AZ wrote the manuscript; TP and AB reviewed the final paper. All authors have read and agreed to the published version of the manuscript.

**REFERENCES**

Albini, A., and Sporn, M. B. (2007). The Tumour Microenvironment as a Target for Chemoprevention. *Nat. Rev. Cancer* 7 (2), 139–147. doi:10.1038/nrc2067

Baier, P. K., Eggstein, S., Wolff-Vorbeck, G., Baumgartner, U., and Hopt, U. T. (2005). Chemokines in Human Colorectal Carcinoma. *Anticancer Res.* 25 (5), 3581–3584.

Becker, C., Fantini, M. C., Schramm, C., Lehr, H. A., Wirtz, S., Nikolaev, A., et al. (2004). TGF-β Suppresses Tumor Progression in Colon Cancer by Inhibition of IL-6 Trans-signaling. *Immunity* 21 (4), 491–501. doi:10.1016/j.immuni.2004.07.020

Berlec, A., Malovrh, T., Zadravec, P., Steyer, A., Ravnikar, M., Sabotić, J., et al. (2013). Expression of a Hepatitis A Virus Antigen in Lactococcus Lactis and *Escherichia coli* and Evaluation of its Immunogenicity. *Appl. Microbiol. Biotechnol.* 97 (10), 4333–4342. doi:10.1007/s00253-013-4722-3

Berlec, A., Perše, M., Ravnikar, M., Lunder, M., Erman, A., Cerar, A., et al. (2017). Dextran Sulphate Sodium Colitis in C57BL/6 Mice Is Alleviated by Lactococcus Lactis and Worsened by the Neutralization of Tumor Necrosis Factor α. *Int. Immunopharmacology* 43, 219–226. doi:10.1016/j.intimp.2016.12.027

Berlec, A., Škrlček, K., Kocjan, J., Olenic, M., and Štrukelj, B. (2018). Single Plasmid Systems for Inducible Dual Protein Expression and for CRISPR-Cas9/CRISPRi Gene Regulation in Lactic Acid Bacterium *Lactococcus Lactis*. *Sci. Rep.* 8 (1), 1009. doi:10.1038/s41598-018-19402-1

Berlec, A., Zavrlinik, J., Butinar, M., Turk, B., and Štrukelj, B. (2015). In Vivo imaging of *Lactococcus Lactis, Lactobacillus Plantarum* and *Escherichia coli* Expressing Infrared Fluorescent Protein in Mice. *Microb. Cell Fact.* 14, 181. doi:10.1186/s12934-015-0376-4

Chung, Y.-C., and Chang, Y.-F. (2003). Serum Interleukin-6 Levels Reflect the Disease Status of Colorectal Cancer. *J. Surg. Oncol.* 83 (4), 222–226. doi:10.1002/jso.10269

Das, M., Duan, W., and Sahoo, S. K. (2015). Multifunctional Nanoparticle-EpCAM Aptamer Biocomjugates: a Paradigm for Targeted Drug Delivery and Imaging in Cancer Therapy. *Nanomedicine: Nanotechnology, Biol. Med.* 11 (2), 379–389. doi:10.1016/j.nano.2014.09.002

de Ruiter, P. G., Kuipers, O. P., and de Vos, W. M. (1996). Controlled Gene Expression Systems for *Lactococcus Lactis* with the Food-Grade Inducer Nisin. *Appl. Environ. Microbiol.* 62 (10), 3662–3667. doi:10.1128/aem.62.10.3662-3667.996

Del Carmen, S., de Moreno de LeBlanc, A., Levit, R., Azevedo, V., Langella, P., Bermúdez-Humaran, L. G., et al. (2017). Anti-cancer Effect of Lactic Acid Bacteria Expressing Antioxidant Enzymes or IL-10 in a Colorectal Cancer Mouse Model. *Int. Immunopharmacology* 42, 122–129. doi:10.1016/j.intimp.2016.11.017

De ruaz, M., Frauschniuk, A., Alessandri, A. L., Dias, J. M., Coelho, F. M., Russo, R. C., et al. (2008). Ticks Produce Highly Selective Chemokine Binding Proteins with Antiinflammatory Activity. *J. Exp. Med.* 205 (9), 2019–2031. doi:10.1084/jem.20072689

Doll, D., Keller, L., Maak, M., Boulesteix, A.-L., Siewert, J. R., Holzmann, B., et al. (2010). Differential Expression of the Chemokines GRO-2, GRO-3, and Interleukin-8 in colon Cancer and Their Impact on Metastatic Disease and Survival. *Int. J. Colorectal Dis.* 25 (5), 573–580. doi:10.1007/s00384-010-0911-1

Duong, M. T.-Q., Qin, Y., You, S.-H., and Min, J.-J. (2019). Bacteria-cancer Interactions: Bacteria-Based Cancer Therapy. *Exp. Mol. Med.* 51 (12), 1–15. doi:10.1088/1362-1197-0297-0

Feldwisch, J., Tolmachev, V., Lencel, C., Herne, N., Sjoberg, A., Larsson, B., et al. (2010). Design of an Optimized Scaffold for Affibody Molecules. *J. Mol. Biol.* 398 (2), 232–247. doi:10.1016/j.jmb.2010.03.002

Gaber, A., Kim, S. J., Kaake, R. M., Benchina, M., Krogan, N., Sali, A., et al. (2018). EpCam Homo-Oligomerization Is Not the Basis for its Role in Cell-Cell Adhesion. *Sci. Rep.* 8 (1), 13269. doi:10.1038/s41598-018-31482-7

Garcia-Gonzalez, N., Prete, R., Battista, N., and Corsetti, A. (2018). Adhesion Properties of Food-Associated *Lactobacillus Plantarum* Strains on Human Intestinal Epithelial Cells and Modulation of IL-8 Release. *Front. Microbiol.* 9. doi:10.3389/fmicb.2018.02392

Hao, N.-B., Lü, M.-H., Fan, Y.-H., Cao, Y.-L., Zhang, Z.-R., and Yang, S.-M. (2012). Macrophages in Tumor Microenvironments and the Progression of Tumors. *Cancer Cell.* 21 (4), 491–501. doi:10.1016/j.ccell.2012.09.015

Holc, H., and Nes, I. F. (1995). Transformation of Lactococcus by Electroporation. *Methods Mol. Biol.* 47, 195–200. doi:10.1385/0-89603-310-4:195

Johnson, D. E., O’Keefe, R. A., and Grandis, J. R. (2018). Targeting the IL-6/JAK/STAT3 Signalling axis in Cancer. *Nat. Rev. Clin. Oncol.* 15 (4), 234–248. doi:10.1038/nrclinonc.2018.8

Jones, V. S., Huang, R.-Y., Chen, L.-P., Chen, Z.-S., Fu, L., and Huang, R.-P. (2016). Cytokines in Cancer Drug Resistance: Cues to New Therapeutic Strategies. *Biochim. Biophys. Acta (Bba) - Rev. Cancer* 1865 (2), 255–265. doi:10.1016/j.bbcan.2016.03.005

Kalichuk, V., Renodon-Cornière, A., Béhar, G., Carrion, F., Obal, G., Maillison, M., et al. (2018). A Novel, Smaller Scaffold for Affinitins: Showcase with Binders Specific for EpCAM. *Biotechnol. Bioeng.* 115 (2), 290–299. doi:10.1002/bit.26463

Kliger, B., and Cohrssen, A. (2008). Probiotics. *Am. Fam. Physician* 78 (9), 1073–1078.

Kosler, S., Strukelj, B., and Berlec, A. (2017). Lactic Acid Bacteria with Concomitant IL-17, IL-23 and TNFα Binding Ability for the Treatment of Inflammatory Bowel Disease. *Curr. Pharm. Biotechnol.* 18 (4), 215–224. doi:10.2174/187220311866617020152218

Lee, Y. S., Choi, I., Ning, Y., Kim, N. Y., Khatchadourian, V., Yang, D., et al. (2012). Interleukin-8 and its Receptor CXCR2 in the Tumour Microenvironment Promote colon Cancer Growth, Progression and Metastasis. *Br. J. Cancer* 106 (11), 1833–1841. doi:10.1038/bjc.2012.177

**ACKNOWLEDGMENTS**

The authors thank Christopher Berre for the critical reading of the manuscript. We thank Emanuela Senjor for providing U-937 cells, Mila Pavšič for providing pcDNA3-EpFL-sfGFP and Michael Davidsson for providing mEmerald-ERBB2-N-18.

**FUNDING**

This research was funded by the Slovenian Research Agency, Grant Numbers P4-0127 and J4-9327.
Ning, Y., Manegold, P. C., Hong, Y. K., Zhang, W., Pohl, A., Lurje, G., et al. (2011). 
Ning, Y., and Lenz, H.-J. (2012). Targeting IL-8 in Colorectal Cancer. 
Siena, S., Sartore-Bianchi, A., Marsoni, S., Hurwitz, H. I., McCall, S. J., Penault-
Llorca, F., et al. (2018). Targeting the Human Epidermal Growth Factor 
Receptor 2 (HER2) Oncogene in Colorectal Cancer. Ann. Oncol. 29 (5), 1108–1119. 
doi:10.1093/annonc/mdy100 
Skrec, K., Pucer Janež, A., Rogelj, B., Strukelj, B., and Berlec, A. (2017). Evasion-
displaying Lactic Acid Bacteria Bind Different Chemokines and neutralize 
CXCL8 Production in Caco-2 Cells. Microb. Biotechnol. 10 (6), 1732–1743. 
doi:10.1111/1751-7915.12781 
Skrec, K., Zdravac, P., Hlavničková, M., Kuchař, M., Vánková, L., Petroková, H.,
et al. (2018). p19-targeting ILP Protein Blockers of IL-23/Th-17 Pro-
inflammatory axis Displayed on Engineered Bacteria of Food Origin. Int. J. 
Mol. Sci. 19 (7), 1933. doi:10.3390/ijms19071933 
Soncin, I., Sheng, J., Chen, Q., Foo, S., Duan, K., Lum, J., et al. (2018). The Tumour 
Microenvironment Creates a Niche for the Self-Renewal of Tumour-Foaming 
Macrophages in colon Adenoma. Nat. Commun. 9 (1), 582. doi:10.1038/s41467-
018-02344-8 
Steidler, L., Neirynck, S., Hu yghebaert, N., Snoeck, V., Vermeire, A., Goeddeers, B.,
et al. (2003). Biological Containment of Genetically Modified Lactococcus Lactis 
for Intestinal Delivery of Human Interleukin 10. Nat. Biotechnol. 21 (7), 
785–789. doi:10.1038/nbt840 
Sun, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., et al. 
(2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and 
Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J. Clin. 71 (3), 
209–249. doi:10.3322/caac.21660 
Terzic, J., Gruvennikov, S., Karin, E., and Karin, M. (2010). Inflammation and colon 
Cancer. Gastroenterology 138 (6), 2101–2114. e2105. doi:10.1053/gastro.2010.
01.058 
Toso, J. F., Gill, V. J., Hwu, P., Marincola, F. M., Restifo, N. P., Schwartzentruber, D. 
J., et al. (2002). Phase I Study of the Intravenous Administration of Attenuated 
Salmonella typhimurium to Patients with Metastatic Melanoma. J. Clin. Oncol. 
20 (1), 142–152. doi:10.1200/JCO.2002.10.1142 
Uccello, M., Malaguarnera, G., Basile, F., D’agata, V., Malaguarnera, M., 
Bertino, G., et al. (2012). Potential Role of Probiotics on Colorectal 
Cancer Prevention. BMC Surg. 12, S35. Suppl 1. doi:10.1186/1471-2428-
12-S35 
Vacmini, A., Mortier, A., Proost, P., Locati, M., Metzemaekers, M., and Borroni, E. 
(2018). Differential Effects of Posttranslational Modifications of CXCL8/
Interleukin-8 on CXCR1 and CXCR2 Internalization and Signaling 
Properties. Int. J. Mol. Sci. 19 (12), 3768. doi:10.3390/ijms19123768 
Wattenberg, M. M., and Beaty, G. L. (2020). Overcoming Immunotherapeutic 
Resistance by Targeting the Cancer Inflammation Cycle. Semin. Cancer Biol. 65, 
38–50. doi:10.1016/j.semcancer.2020.01.002 
West, N. R., McCuig, S., Franchini, F., and Powrie, F. (2015). Emerging Cytokine 
Networks in Colorectal Cancer. Nat. Rev. Immunol. 15 (10), 615–629. doi:10. 
1038/nri3896 
Yu, F., Gudmundsdottor, L., Akal, A., Gunneriussson, E., Fredj, F., and Nyrén, P.- 
Å. (2014). An Affibody-Adalimumab Hybrid Blocks Combined IL-6 and TNF-
Triggered Serum Amyloid A Secretion In Vivo. Mabs 6 (6), 1598–1607. doi:10. 
4161/mabs.36089 
Zdravac, P., Strukelj, B., and Berlec, A. (2015). Heterologous Surface Display on 
Lactic Acid Bacteria: Non-GMO Alternative. Bioengineered 6 (3), 179–183. 
doi:10.1080/21655997.2015.1040956 
Zahirović, A., and Lunder, M. (2018). Microbial Delivery Vehicles for 
Allergens and Allergen-Derived Peptides in Immunotherapy of Allergic 
Diseases. Front. Microbiol. 9, 1449. doi:10.3389/fmicb.2018.01449 
Zeng, J., Tang, Z.-H., Liu, S., and Guo, S.-S. (2017). Clinicopathological 
Significance of Overexpression of Interleukin-6 in Colorectal Cancer. 
World J. Gastroenterol. 23 (10), 1780–1786. doi:10.3748/wjg.v23.i10.1780 
Zhang, B., Li, A., Zuo, F., Yu, R., Zeng, Z., Ma, H., et al. (2016). 
Recombinant Lactococcus Lactis NZ9000 Secretes a Bioactive 
Kisspeptin that Inhibits Proliferation and Migration of Human colon 
Carcinoma HT-29 Cells. Microb. Cel Fact 15 (1), 102. doi:10.1186/
51.03157394-016-0506-7 
Conflict of Interest: The authors declare that the research was conducted in the 
absence of any commercial or financial relationships that could be construed as a 
potential conflict of interest. 
Publisher’s Note: All claims expressed in this article are solely those of the authors 
and do not necessarily represent those of their affiliated organizations, or those 
of the publisher, the editors and the reviewers. Any product that may be evaluated in 
this article, or claim that may be made by its manufacturer, is not guaranteed or 
endorsed by the publisher. 
Copyright © 2022 Zahirović, Plavec and Berlec. This is an open-access article 
distributed under the terms of the Creative Commons Attribution License (CC BY). 
The use, distribution or reproduction in other forums is permitted, provided the original 
author(s) and the copyright owner(s) are credited and that the original publication in 
this journal is cited, in accordance with accepted academic practice. No use, distribution 
or reproduction is permitted which does not comply with these terms.