Engineered bacteria secreting cytokines for use in cancer immunotherapy

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Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 August 2023 (31.08.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/161371 A1

(51) International Patent Classification:
C07K 14/55 (2006.01) *A61P 35/00* (2006.01)
C12N 15/62 (2006.01) *A61K 38/20* (2006.01)

(21) International Application Number:
PCT/EP2023/054604

(22) International Filing Date:
23 February 2023 (23.02.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
22158950.0 25 February 2022 (25.02.2022) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

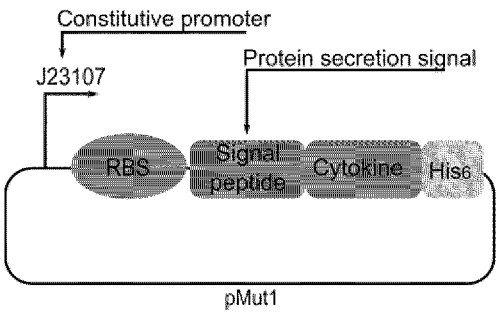
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) Title: ENGINEERED BACTERIA SECRETING CYTOKINES FOR USE IN CANCER IMMUNOTHERAPY

FIGURE 1



(57) Abstract: The invention provides engineered bacteria that secretes cytokines, such as interleukin 2 (IL-2) cytokine, for use in cancer immunotherapy, pharmaceutical compositions comprising the engineered bacteria, methods for producing recombinant therapeutical polypeptides, and methods for using the engineered bacteria for therapeutic purposes.

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TITLE: Engineered bacteria secreting cytokines for use in cancer immunotherapy

FIELD OF INVENTION

The technology described herein relates to engineered bacteria, pharmaceutical compositions comprising the engineered bacteria, methods for producing recombinant therapeutical polypeptides, and methods for using the engineered bacteria for therapeutic purposes. Specifically, the invention provides an engineered bacterium that secretes cytokines for use in cancer immunotherapy.

BACKGROUND

Using the immune system to combat cancer by means of immunotherapy is a promising strategy for cancer treatment, which is getting more popular as a first-line treatment worldwide for several types of cancers. Immunotherapy drugs are not directly toxic to cancer cells; instead they act by activating the immune cells that are then able to eliminate the cancerous cells. There are several types of immunotherapy currently in use, including CAR-T, checkpoint blockade, cancer vaccines as well as cytokine therapy. CAR-T therapy works by retrieving T-cells from the patient, genetically modifying them to recognize cancer cells and inserting them back into the patient. This method can work quite well, but is a personalized, laborious, and a quite expensive method. Checkpoint blockade acts by introducing antibodies that inhibit the immune-suppressive mechanisms of cancer cells, thereby allowing T-cells to recognize and kill cancer cells. Checkpoint blockade is the most widely used type of immunotherapy today with multiple clinical trials ongoing in combination with chemotherapy and other forms of cancer therapy. However, some cancer types are not responsive to checkpoint blockers due to low infiltration of immune cells into the tumor microenvironment. Cancer vaccines supply cancer antigens; this induces the generation of immune cells, which can detect cancer cells and eliminate them. Cytokine therapy uses immune-activating signalling proteins, but it has the drawback of high systemic toxicity.

The present invention addresses the problem of how to provide a therapeutic agent for use as a medicament in the treatment of a subject in need thereof, that is capable of providing a targeted therapeutic dose of a cytokine and whose administration has a reduced risk of toxicity side effects.

SUMMARY OF INVENTION

In a first aspect, the present invention provides a **recombinant bacterial cell** or a population derived therefrom, comprising

- a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2-type cytokine having an N-terminal LamB signal peptide for secretion of said cytokine.

In one embodiemnt, the encoded recombinant fusion polypeptide of the recombinant bacterial cell or population derived therefrom further comprises

- an InfB solubility tag fused respectively to the C-terminal residue of the signal peptide and the N-terminal residue of the interleukin 2-type cytokine, or
- an InfB solubility tag fused to the C-terminal residue of the interleukin 2-type cytokine.

In a second aspect, the present invention provides a recombinant bacterial cell of the invention or a population derived therefrom **for use as a medicament,** wherein the cell secretes the interleukin 2-type cytokine.

In one embodiment, the recombinant bacterial cell or population derived therefrom for use as a medicament is for use in prevention and/or treatment of cancer.

In a third aspect, the present invention provides a **kit of parts** for use as a medicament comprising (i) a recombinant bacterial cell of the invention, and (ii) a second part selected from the group: CAR-T cells, adoptive T cells, immune checkpoint blockers and cancer drugs, or a combination of any thereof.

DESCRIPTION OF THE INVENTION

Definitions and abbreviations

Disorder/Disease: A disease is a pathophysiological response to internal or external factors; while a disorder is a disruption to regular bodily structure and function. For the purpose of the present application the term "disorder" is to be understood to be an umbrella term that encompasses both a disease and a disorder in a mammalian subject that may be treated by the recombinant bacterial cells of the present invention.

Immunotherapy is a medical term defined as the "treatment of disease by inducing, enhancing, or suppressing an immune response".

Adoptive t-cell therapy is a type of immunotherapy in which T cells are given to a patient to help the body fight diseases, such as cancer. Specifically, tumor-specific

cytotoxic T cells are isolated from the patient to be treated, expanded ex vivo, and then infused back into cancer patients with the goal of recognizing, targeting, and destroying tumor cells.

CAR-T cell therapy is a therapy, where a patient's own immune cells are engineered to treat their cancers. Specifically, in CAR T-cell therapies, T cells are taken from the patient's blood and are engineered by adding a chimeric antigen receptor (CAR). This helps them better identify specific cancer cell antigens. The CAR T-cells are then given back to the patient.

Secretion tag and **signal peptide** are used interchangeably. This is a short peptide (usually 16-30 amino acids long) present at the N-terminus of the primary translation product of a synthesized protein in a cell, functioning to direct the cell to secrete the protein.

Solid tumor is an abnormal mass of tissue that usually does not contain cysts or liquid areas. Different types of solid tumors are named after the type of cells that form them. Examples of solid tumors are sarcomas, carcinomas, and lymphomas.

Figures

Figure 1: Illustrative sketch of plasmid used in the present invention. The native E.coli Nissle 1917 pMut1 plasmid was modified by inserting the cytokine-coding IL-2 gene flanked by nucleotide sequences encoding an N-terminal signal peptide sequence for protein secretion and a C-terminal His6 tag. Expression was mediated by a constitutive medium strength promoter from the Anderson promoter library (J23107) and a strong ribosome binding site (RBS1) sequence. A kanamycin resistance gene for selection and the hok/sok toxin-antitoxin system was inserted for plasmid stability (not indicated).

Figure 2: Cytokine (IL-2) expression by recombinant E. coli strains. **(A)** IL-2 concentration (pg IL-2/ml /OD cell culture) measured in cell lysates from strains expressing IL-2 fused to an OmpA signal peptide, but having different RBS sequences. **(B)** IL-2 concentration (pg IL-2/ml /OD cell culture) measured in supernatants from strains expressing IL-2 fused to an OmpA signal peptide, but having different RBS sequences. **(C)** IL-2 concentration (pg IL-2/ml /OD cell culture) measured in supernatants from strains expressing IL2 fused to an OmpA or DsbA signal peptide. **(D)** IL-2 concentration (ng IL-2/ml /OD cell culture) measured in supernatants from E.coli Nissle 1917 and 5 different E.coli Symbioflor strainsexpressing IL-2 fused to an OmpA signal peptide. Samples were analyzed using a commercial ELISA kit. IL-2 values, given as pg IL-2/ml (lysate or supernatant)/OD cell culture or ng IL-2/ml supernatant /OD cell

culture, and standard deviations are based on three separate colonies of each cytokine-expressing bacteria.

Figure 3: Cytokine activity (RUF). Supernatants from IL-2 expressing recombinant E.coli strains showed biological activity using a CTLL-2 assay. Mouse cytotoxic T-cells (CTLL-2) require IL-2 for their viability and growth. The cells were washed with PBS and starved for IL-2 for 5 hours before the experiment and then cultured for 16h in media supplemented with (i) 10 % T-Cell Culture Supplement with ConA and comprising IL-2 for supporting growth of the cells (positive Ctrl,), or (ii) 5-20% supernatant from a strain expressing IL-2 (IL-2 strain) or (iii) supernatant from a control strain comprising a negative plasmid (negative Ctrl). Cell viability was assessed using Alamar blue cell viability stain. RFU = relative fluorescent units. Error bars represent the standard deviation from three biological replicates.

Figure 4: PBMCs activated by supernatants from cytokine-expressing E.coli Nissle lead to HT29 tumor spheroid destruction. **(A)** 4-day old HT29 spheroid and PBMC ($1x10^5$ cells) co-cultures were exposed to supernatants from non-expressing (Neg.Ctrl) or IL-2-expressing E.coli Nissle at 30% (V/V) concentration. Cytotox green dye (Essen BioScience) was added to measure cell death in real time using the Incucyte live cell imaging system (Sartorius). Media with cytokine-containing supernatants was replaced after 3 days. Graphs show average values with standard deviation from three spheroids per condition. **(B)** Representative microscope images of HT29 spheroids after 6 days of co-culture with the indicated conditions. RFU = relative fluorescence unit; Untreated = untreated tumor spheroids with no PBMCs; IL-2 supernatant = IL-2-expressing E.coli Nissle at 30% (V/V) concentration; PBMCs = tumor spheroid and PBMC co-culture; IL-2 10 ng/ml = co-cultures treated with commercial IL-2.

Figure 5: Bacterial colonization (CFU) in BALB/C and CB6F1mice. 3 BALB/C and 7 CB6F1 mice were intravenously injected with 3x10^6 CFU of E.coli Nissle. **(A)** CFUs were plated from 10µl of blood at the indicated timepoints. **(B)** Mice were sacrificed after 48h and bacterial colonization was measured in liver, spleen, kidneys and lungs. CFU = colony forming unit; Liv = liver; Spl = spleen; Kid = kidneys; Lun = lungs.

Figure 6: E.coli Nissle 1917 colonization of CT26 tumors in CB6F1 mice. **(A)** Body weight monitoring during the study. **(B)** Tumor size (volume) monitoring during the study. **(C)** Bacterial CFU counts per ml blood after bacterial injection. 10µl of blood was mixed with 90ul of cold PBS and plated on agar plates. **(D)** and **(E)** Bacterial CFU counts per g tumor and organ tissue after 48h and 6 days respectively. Tumors and organs were excised and homogenized using GentleMacs C tubes. Serial dilutions were performed and spotted on LB agar plates. In addition, 100ul of non-diluted homogenates

were plated on petri dishes in order to increase the detection limit. CFU = colony forming unit; TL = left side tumor; TR = right side tumor; Liv = liver; Spl = spleen; Kid = kidneys; Lun = lungs.

Figure 7: Bacterial colonization in CT26 tumors and organs in CB6F1 mice. 10 CB6F1 mice harboring CT26 tumors were treated with PBS or $1x10^7$ CFU bacteria intravenously. Mice were sacrificed when tumors reached 2000mm^3 volume. Tumors and organs were homogenized and serial dilutions were plated. Individual values of each tumor and organ and the median was plotted as bacterial CFU counts per g tumor or organ tissue. TL = left side tumor; TR = right side tumor; Liv = liver; Spl = spleen; Kid = kidneys; Lun = lungs.

Figure 8: CT26 tumor volumes in CB6F1 mice. **(A)** Means of tumor sizes (mm^2) were plotted for each treatment group. Dead mice were removed from the plot in later time points. Mean tumor values with S.E.M. were plotted. Error bars indicate S.E.M. **(B), (C) and (D)** Individual tumor volume values were plotted for all tumors in the PBS group, and only for colonized tumors in bacteria-treated groups (identified as PBS=Phosphate-Buffered Saline; IL-2 = IL-2 expressing bacteria; Ctrl=empty-plasmid bacteria).

Figure 9: Cytokines in colonized tumors in CB6F1 mice. **(A)** Colonized tumors were homogenized, centrifuged and ELISA was performed to measure cytokine levels in the supernatant (pg IL-2/ml supernatant) from ctrl treatment group (empty-plasmid bacteria) and IL-2 treatment group (IL-2 expressing bacteria). Individual values and the mean are plotted. Error bars show the S.E.M. One-way ANOVA was performed to assess statistical significance. **(B)** Tumor size (mm^3) of Il-2 group (same as in Figure 8D), where tumors that had the highest cytokine levels are indicated in gray.

Figure 10: RT-qPCR analysis of cytokine response genes in CB6F1 mice tumors. RNA extraction was performed from a small piece of tumor from all tumors in the PBS group and only colonized tumors in bacteria-treated groups (identified as PBS=Phosphate-Buffered Saline; Ctrl=empty-plasmid bacteria and IL-2=IL-2 expressing bacteria). RT-qPCR analysis was performed to measure relative gene expression of known target genes. Bcl2, Nfkb1, Il2Ra, Tnfsf10, Mapkapk3 and Dusp5 gene expression was normalized to GAPDH expression level. The graph indicates mean expression values with S.E.M. One-way ANOVA was performed to assess statistical significance between PBS and bacteria-treated groups. Only statistically significant differences between samples are indicated.

Figure 11: Cytokine levels and activity produced by recombinant E. coli strains using nucleotide sequences encoding different secretion tags cloned upstream of the IL2-His6 gene. **(A)** Supernatants from the different strains were collected and IL-2 secretion was

measured by ELISA and plotted as ng IL-2/ml supernatant /OD cell culture. **(B)** Activity of IL-2 (RFU) in the supernatants was measured using the IL-2 activity assay. IL-2 values from three biological replicates with standard deviations are shown. His6 = Hexahistidine tag, Neg.Ctrl = supernatant from non-expressing strain; Positive Ctrl = Commercial human recombinant IL-2; supernatant from control strain comprising an empty plasmid (Neg.Ctrl); OmpA-IL-2_His6 and tested new IL-2_His6 identify IL-2 proteins expressed by recombinant strains.

Figure 12: Recombinant bacterial strains after secretion tag optimization produced cytokines that activated PBMCs and lead to tumor spheroid destruction. Cytotoxicity was monitored in HT29 spheroid and PBMC ($1x10^5$ cells) co-cultures exposed to supernatants from the recombinant strains listed in Figure 11. **(A)** Cytotoxicity from co-cultures exposed to supernatants from IL2-His6 strains at 10% V/V concentration. **(B)** Representative images of spheroid integrity after 3 days of co-culture with IL-2 supernatants. Cytotox green dye (Essen BioScience) was used to measure cell death in real time using the Incucyte live cell imaging system (Sartorius). Mean values with standard deviation from 3 spheroids per condition were plotted. RFU = relative fluorescence unit; Neg.Ctrl = supernatant from non-expressing bacteria; Untreated = untreated tumor spheroids with no PBMCs; PBMCs = tumor spheroid and PBMC co-culture; IL-2 10 ng/ml = co-cultures treated with commercial IL-2.

Figure 13: IL-2 secretion with InfB solubility tag by recombinant E. coli strains. The InfB solubility tag was fused to the N- or C-terminus of the cytokine. **(A)** IL-2 secretion by the tested strains, measured in supernatants by ELISA (given as ng IL-2/ml supernatant /OD cell culture). **(B)** Activity of IL-2 (RFU) in the supernatants of the tested strains measured using the IL-2 activity assay using CTLL-2 cells. **(C)** IL-2 activity (RFU) in supernatantsof strains comprising LamB-IL2-His6 and LamB-IL2-InfB. Values from three biological replicates with standard deviations are shown. RBS = ribosome binding site; Sec.tag = secretion tag; His6 = Hexahistidine tag, Neg.Ctrl = supernatant from non-expressing strain; Positive Ctrl = Commercial human recombinant IL-2.

Figure 14: Testing different promoter and RBS combinations upstream of the LamB-IL2-InfB construct. **(A)** Growth profile (OD) of the IL-2 expressing recombinant E. coli strains for evaluating the metabolic burden caused by cytokine expression. **(B)** IL-2 secretion by the strains, measured in supernatants by ELISA (given as ng IL-2/ml supernatant /OD cell culture). **(C)** Activity of IL-2 in the supernatants, measured using the IL-2 activity assay using CTLL-2 cells. MS8 is a strong promoter, MS6 is comparable to the Anderson promoter, J23107. Strong RBS is the same RBS1 as used in figure 2, medium RBS is 2-fold weaker. Values from three biological replicates with standard

deviations are shown. RBS = ribosome binding site; Sec.tag = secretion tag; His6 = Hexahistidine tag, Neg.Ctrl = supernatant from non-expressing strain.

Figure 15: (A) Cytotoxicity in HT29 spheroid and PBMC co-cultures exposed to recombinant IL-2 or supernatant from LamB-IL2-InfB. Cytotox green dye (Essen BioScience) was used to measure cell death in real time using the Incucyte live cell imaging system (Sartorius). Mean values with standard deviation from 3 spheroids per condition were plotted. RFU = relative fluorescence unit. **(B)** Representative images of spheroid integrity after 3 days of co-culture. **(C)** and **(D)** Supernatant from LamB-IL2-INfB induces gene expression associated with immune cell-mediated cytotoxicity. RT-qPCR analysis of IL-2 response genes from tumor spheroid + PBMC co-culture and PBMC monoculture, respectively. Gene expression was normalized to GAPDH expression. Mean values of relative gene expression and S.E.M. from three independent experiments are shown. Two-way ANOVA with multiple comparisons was performed to determine statistical significance relative to Neg.Ctrl in B and untreated PBMCs in C. Only statistically significant differences are shown. Neg.Ctrl = supernatant from non-expressing bacteria; IL-2 10 ng/ml and 1ng/ml = co-cultures treated with commercial IL-2.

Figure 16: IFNγ production by tumor spheroid and PBMC co-culture or PBMC monoculture induced for 24h by IL-2 secreted by LamB-IL2-InfB strain compared to commercial IL-2. ELISA was performed to quantify secreted IFNγ levels in conditioned media from **(A)** tumor spheroid and PBMC co-culture. **(B)** PBMC monoculture. Mean values with S.E.M. from three independent experiments are shown. One-way ANOVA was performed to determine statistical significance.

Figure 17: Tumor growth of mice treated with the optimized IL-2 producing E. coli strain (LamB-IL2-InfB). CB6F1 mice harboring CT26 tumors were treated with PBS or $1x10^8$ CFU of bacteria intravenously. Mice were sacrificed when tumors reached $2000mm^3$ volume. **(A)** Tumor volumes measured throughout the study. Mean tumor volumes with S.E.M. are shown. Two-way ANOVA with multiple comparisons was performed to determine statistical significance relative to PBS group. Statistical differences between PBS and IL-2 are shown. Differences between PBS and Ctrl groups were not significant. **(B)**, **(C)** and **(D)** Individual tumor volumes from each treatment group: PBS, Ctrl (supernatant from non-expressing bacteria) and IL-2 (LamB-IL2-InfB strain).

Figure 18: IL-2 concentration in colonized CT26 tumors in CB6F1 mice. The optimized IL-2 producing E. coli strain (LamB-IL2-InfB) produces higher levels of cytokines in the tumor microenvironment. Colonized tumors were homogenized and ELISA was performed to measure human IL-2 levels. Individual IL-2 values and the mean was

plotted. Error bars represent the S.E.M. One-way ANOVA was performed to determine statistical significance.

Figure 19: Recombinant bacteria producing IL-2 shift $CD4^+$ T cell differentiation to central memory-type in spleens and tumors of CB6F1 mice harboring CT26 tumors. Analysis of immune cell composition performed from tumors and spleens. The organs were homogenized, stained with cell-specific antibodies and analysed by flow cytometry. **(A)-(B)** Immune cell composition of lymphocytes ($CD45^+$ $CD11b^-$ cells) in spleens and tumors. CD19 and CD335 were used to gate B and NK cells, respectively. $CD3^+CD4^+$ cells are labelled as $CD4^+$ and $CD3^+CD8^+$ cells are labelled as $CD8^+$. **(C)-(D)** Differentiation of $CD4^+$ T cells in spleens and tumors into T_{CM} ($CD44^{high}CD62^{high}$), T_{EM} ($CD44^{high}CD62L^-$) and T_{Naive} ($CD44^-CD62L^{high}$). **(E)-(F)** Differentiation of $CD8^+$ T cells in spleens and tumors with the same parameters as for $CD4^+$ T cells. Means and S.E.M are plotted. One-way ANOVA was performed to assess statistical significance. Only significant differences are shown.

Detailed description

Commonly used laboratory bacterial species, such as *Escherichia coli* can accumulate in tumors. Advances in synthetic biology provide the means of modifying these tumor-homing bacteria for diagnostic or drug delivery applications. An advantage of using modified bacteria is that they can specifically accumulate in the tumor site and act locally, whereas classical drug treatments suffer from systemic exposure of the body.

Using the immune system to combat cancer by means of immunotherapy is a potent strategy for cancer treatment. The purpose of cytokine therapy is to manipulate the immune response in such a way as to generate the appropriate immune effector cells to eradicate solid tumors.

The present invention concerns recombinant cytokine-expressing bacteria, or a population derived therefrom, which when injected into the body will accumulate and proliferate at the tumor site due to its hypoxic and immune-supressed microenvironment. These bacteria will via their cytokine expression promote immune cell activation in the tumor microenvironment, while minimizing systemic toxicity. The invention exploits the property of some types of bacteria to specifically accumulate at tumor sites, where a localized secretion of the immune-activating agent minimizes its toxicity.

As evidenced herein, the use of pro-inflammatory cytokine-expressing tumor-homing *E.coli* Nissle 1917 bacteria is a promising strategy to help our body recognize and combat cancer, avoiding systemic exposure of the body to the cytokines by efficient colonization

at the tumor microenvironment and locally providing sufficient levels of cytokines to activate the immune cells.

The targeted delivery of innume-activating agents (cytokines) into an immune-suppressed tumor mocroenvironment enhances the treatment of tumors in a patient mediated by the patient's own native adaptice immune response, adoptive T-cell, CAR-T therapy, and/or cancer drugs.

I. A recombinant bacterial cell

Some types of bacteria have the property of specifically accumulating at tumor sites when injected into a cancer patient - such bacteria are exploited in the present invention for recombinant engineering to make them cytokine-producing bacteria.

In one aspect, the recombinant bacterial cell of the invention is a live bacterium having the property of accumulating at tumor sites (such as demonstrated in example 3.3). In one embodiment, the recombinant bacterial cell of the invention is a live bacterium having the property of accumulating at solid tumors, where by secreting IL-2 it acts as a therapeutic agent in treatment of the solid tumor (such as demonstrated in example 3.3 and 5.4). Though the recombinant bacterial are mostly restricted to the tumor sites, the cells may be engineered to be auxotropic to ensure control of their growth and enhance safety. Higher amounts of auxotrophic bacteria can potentially be safely injected into the bloodstream than what is deemed safe for non-auxotrophic replicating strains. In one embodiment, the cells may be designed to contain DAP (diaminopimelic acid) auxotrophy (Δ*dapA*, deletion of chromosomal copies of the *dapA* gene (SEQ ID NO.: 104) encoding 4-hydroxy-tetrahydrodipicolinate synthase (SEQ ID NO.: 105)) which will inhibit growth in environments such as the bloodstream where DAP is not present. In one embodiment, the recombinant bacterial cell of the invention is a species of a genus selected from among *Escherichia, Listeria, Salmonella, Bifidobacterium, Streptococcus, Lactobacillus, Fusobacterium, Corynebacterium, Sphingomonas, Paracoccus, Staphylococcus, Enterobacter, Klebsiella, Citrobacter, Roseomonas, Sphingomonas, Staphylococcus, Sphingomonas, Actinomyces, Pseudomonas, Acinetobacter, Neisseria, Enterobacter*. Preferably, the recombinant bacterial cell of the invention is a species of a genus selected from among *Escherichia, Listeria, Salmonella, and Bifidobacterium.*

In a most preferred embodiment, the recombinant recombinant bacterial cell is a strain of *E. coli*, where members of this species have the added advantage of being easily engineered. In one embodiment, the recombinant strain of the species E.coli is selected from among E. coli Nissle strains (such as E. coli Nissle 1917) and E. coli Symbioflor

strains (such as E. coli Symbioflor G1/, G4/9, 5, G6/7, and G8). In a preferred embodiment, the recombinant bacterial cell is E. coli Nissle 1917, which further is a well-characterized probiotic strain, classified as a risk group I organism

I.i *Heterologous expression of cytokines*

In one aspect, the recombinant bacterial cell of the invention is engineered to express one or more genes encoding a cytokine for promoting immune cell activation.

IL2 (Interleukin 2) is a cytokine. IL-2 is produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. IL-2 is normally produced by the body during an immune response.

IL2 is conserved in chimpanzee, Rhesus monkey, dog, mouse, and rat. The gene produces a 17628 Da protein composed of 153 amino acids. IL2 is a powerfully immunoregulatory lymphokine that is produced by lectin- or antigen-activated T cells.

IL2 stimulates the growth, differentiation, and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes. IL2 can further stimulate B-cells, monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells.

In one example, the recombinant bacterial cell of the invention comprises one of more genes encoding an interleukine-2 (IL-2) cytokine, where the expression of said IL-2 cytokine confers on the cell the ability to promote immune cell activation and thereby provide treatment against cancer, on administration to a cancer patient.

In one aspect, the amino acid sequence of said IL-2 cytokine may be one having at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 1 from human (Homo sapiens), SEQ ID NO: 2 from mouse (mus musculus), or SEQ ID NO: 3 from rat (ratus norvegicus).

In a preferred embodiment, the recombinant bacterial cell comprises a recombinant nucleic acid molecule encoding a IL-2 cytokine whose amino acid sequence has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 1 from human (Homo sapiens).

In a further aspect, the recombinant bacterial cell of the invention comprises on one or more plasmids that comprise the one or more recombinant nucleic acid molecules encoding the therapeutic agent. The coding sequence for the therapeutic cytokine agent in each of the one or more recombinant nucleic acid molecules is operatively linked to a

promoter, RBS, and signal peptide in the prokaryotic cell, these being selected to provide a desired expression level of the cytokine(s) in the recombinant bacterium of the invention.

In one embodiment, the recombinant cell of the invention is E. coli Nissle 1917 comprising native plasmid pMUT1 (SEQ ID NO 4). In a preferred embodiment, the native pMUT1 plasmid of E. coli is engineered to comprise the recombinant nucleic acid molecule encoding the therapeutic cytokine for expression in E. coli.

IL-2 secreted by the recombinant bacterial cell of the invention, and as described above, is characterized by having cytokine activity that can be measured by methods known in the art, for example by use of the method set out in Example 1.5, Furthermore, the amount of IL-2 secreted by the recombinant bacterial cell of the invention can be quantitated using IL-2 specific antibodies, for example by ELIZA assay as set out in example 1.4.

I.ii Transcription and translation of the cytokine(s)

Expression of heterologous proteins may negatively affect expression hosts, because resources that could be used for growth are directed for synthesis of the product protein. Protein expression strength is largely determined by how much mRNA is produced and how much mRNA is translated into proteins. These processes can be controlled by modifying promoter strength and the strength of the ribosome binding site (RBS). However, transcription and translation are not the only factors, which determine how much active protein is produced. The capacity of the cell to perform correct protein folding, disulphide bond formation and translocation can be a limiting factor for protein secretion. Therefore, fine-tuning the level of recombinant cytokine protein expression may be needed to obtain optimal conditions for active protein production.As a means for regulating the expression of cytokines in the recombinant cell, different promoters may be used.

In one embodiment, the recombinant bacterial cell of the invention comprises a heterologous nucleic acid sequence encoding IL-2 which is operably linked to a constitutive prokaryotic promoter.

For example, where the recombinant bacterium is a strain of *E. coli*, the nucleic acid sequence of the promoter operatively linked to the IL-2 cytokine is selected from the Anderson promoter collection (http://parts.igem.org/Promoters/Catalog/Anderson), such as any of SEQ ID NO 5-24.

Members of the Anderson promoter collection are suitable for general protein expression in E. coli. The collection is known to cover a range of activities - i.e. different promoter

strengths - so by testing different promoters it should be possible for a person skilled in the art to find a promoter activity that suits a specific application. The relative strengths of these promoters has been measured and reported by Anderson et al 2006.

In one embodiment, the recombinant bacterium is a strain of E. coli, and the nucleic acid sequence of the promoter operatively linked to the IL-2 cytokine is a promoter having a relative strength of between 0.2-0.5, preferably between 0.3-0.4, compared to the Anderson promoter J23119 (SEQ ID NO 5).

In a preferred embodiment, the recombinant bacterium is a strain of E. coli, comprising a recombinant nucleic acid sequence encoding cytokine IL-2, and wherein the nucleic acid sequence of the promoter operatively linked to the IL-2 cytokine coding sequence is the Anderson promoter J23107: SEQ ID NO: 13.

In another embodiment, the recombinant bacterium is a strain of *E. coli*, the nucleic acid sequence of the promoter operatively linked to the IL-2 cytokine coding sequence is selected from SEQ ID NO: 25 (MS8 promoter) and SEQ ID NO: 26 (MS6 promoter).

As a further means of regulating the expression of cytokines in the recombinant cell, different ribosomal binding sites (RBSs) having different strengths may be used to modify the translational strength of the cytokine gene. In bacteria, translational strength is defined by the Shine-Dalgarno/ribosome binding site (RBS) sequence directly upstream of the start codon.

In one embodiment, the recombinant bacterial cell of the invention comprises a heterologous nucleic acid sequence encoding IL-2 which is operably linked to an optimized ribosomal binding site sequence.

RBSs in *E. coli* conferring a broad range of translational strengths are provided in Table 3, while further examples can be found in the literature (e.g. Bonde et al, 2016). A skilled person in the art can optimize the translational strength of the cytokine gene by testing different RBS variant (see example 2.1).

For example, where the recombinant bacterium is a strain of *E. coli*, the nucleic acid sequence of the RBS operatively linked to the IL-2 cytokine is selected from RBS1, RBS2, RBS3, RBS4, RBS5, RBS6, RBS7, RBS8, and RBS9 as defined in table 3 in section 2.1.

In a most preferred embodiment, the recombinant cell of the invention expressing IL-2 cytokine(s) is a strain of E. coli, comprising the Anderson promoter J23107 (SEQ ID NO 13) and RBS1 (see table 3) for regulating transcriptopn and translation of the cytokine(s).

I.iii Secretion of the cytokine(s)

It is essential for the invention that the cytokine is released from the bacteria to act as the therapeutic agent for treatment of cancer tumors. Therefore, in one aspect, a signal peptide sequence is fused to the IL-2 cytokine to ensure secretion of the IL-2 peptide from the recombinant cell. For example, where the recombinant bacterium is a strain of *E. coli*, then the signal peptide sequence for secretion of the IL-2 is selected from one having at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 27 (OmpA), SEQ ID NO: 28 (LamB), SEQ ID NO: 29 (OmpF), SEQ ID NO: 30 (NSP4), SEQ ID NO: 31 (OmpC), SEQ ID NO: 32 (PhoA), SEQ ID NO: 33 (G1M5), SEQ ID NO: 34 (PelB), SEQ ID NO: 35 (PhoE), SEQ ID NO: 36 (Lpp), or SEQ ID NO: 37 (OmpT).

In one embodiment, the signal peptide sequence for secretion of the IL-2 cytokine has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 28 (LamB).

In a preferred embodiment, the signal peptide sequence for secretion of the IL-2 cytokine has at least 90 % sequence identity to SEQ ID NO: 28 (LamB).

The signal peptide is located at the N-terminal of the IL-2 cytokine - it may be directly coupled to the IL-2 or indirectly via a solubility tag, as further disclosed herein.

In a preferred embodiment, the invention provides a recombinant bacterial cell or a population derived therefrom, comprising a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2-type cytokine having an N-terminal signal peptide for secretion of said cytokine, wherein said signal peptide has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 28 (LamB).

In a preferred embodiment, the recombinant bacterium is a strain of E. coli comprising a recombinant nucleic acid sequence encoding IL-2 fused to a signal peptide for secretion of the IL-2 cytokine; wherein the signal peptide is fused to the N-terminal of the IL-2, and wherein the amino acid sequence of said signal peptide has at least 90 % sequence identify to SEQ ID NO: 28 (LamB).

In a most preferred embodiment, the recombinant cell of the invention expressing IL-2 cytokine(s) is a strain of E. coli, comprising the Anderson promoter J23107 (SEQ ID NO 13) and RBS1 (see table 3) for regulating transcription and translation of the cytokine(s), further comprising a nucleic acid sequence encoding the LamB signal peptide (SEQ ID NO: 28) for secretion of the cytokine(s), wherein the LamB signal peptide is fused to the N-terminal of the expressed IL-2 cytokine.

In another embodiment, the signal peptide sequence for secretion of the IL-2 cytokine has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 32 (PhoA).

In a preferred embodiment, the signal peptide sequence for secretion of the IL-2 cytokine has at least 90 % sequence identity to SEQ ID NO: 32 (PhoA).

I.iv Protein solubility tag

Protein solubility is a common issue when expressing heterologous proteins. Hydrophobic or insoluble proteins are notoriously hard to express, especially in their active form. Furthermore, protein misfolding and aggregation can be toxic to the cell. Solubility tags can be used to increase the solubility of the protein, therefore, preventing formation of insoluble aggregates and retaining protein activity. Commonly used solubility tags are typically medium or large size hydrophilic proteins, such as the maltose-binding protein (MBP, 43kDa) or Glutathione-S-transferase (GST, 26kDa). These solubility tags can help by shielding hydrophobic residues in micelle-like structures, attracting molecular chaperones or inhibiting protein aggregation by stabilizing the protein structure while folding.

IL-2 is a small (15kDa) hydrophobic protein, with a high propensity for aggregation (Fatima et al 2012). However, the commonly used solubility tags (e.g. MBP) are 2-3 times larger than IL-2, and attaching such a large solubility tag might interfere with the activity.

To overcome these problems, the present invention uses a much smaller tag for improving solubility. Specifically, a small tag derived from the InfB gene of E. coli is demonstrated to enhance solubility of IL2 and improve active levels of IL2 expression (see examples 4.2).

The solubility tag may be located at the N-terminal or C-terminal of the IL-2 cytokine.

In one embodiment, a nucleic acid sequence encodes a solubility tag, whose amino acid sequence has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 38 (InfB), where the tag is fused to IL-2 such that either the N-terminal or C-terminal of the expressed protein will comprise the tag. The optimal position of the tag (N- or C-terminal) may depend on which other expression components are used (such as promoter, RBS, signal peptide).

In one embodiment, the amino acid sequence of the solubility tag has at least 90% sequence identity to SEQ ID NO: 38 (InfB). In a preferred embodiment, the amino acid sequence of the solubility tag has at least 90% sequence identityy to SEQ ID NO: 38 (InfB) and is fused respectively to the C-terminal residue of the signal peptide and the N-terminal residue of the interleukin 2-type cytokine.

In a preferred embodiment, the invention provides a recombinant bacterial cell, preferably a strain of E. coli, or a population derived therefrom, comprising a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2-type cytokine having a signal peptide for secretion of said cytokine, wherein the amino acid sequence of said signal peptide has at least 90 % sequence identify to SEQ ID NO: 28 (LamB), said cell further comprising (i) a solubility tag fused to the C-terminal residue of the signal peptide and the N-terminal residue of the interleukin 2-type cytokine, or (ii) a solubility tag fused to the C-terminal residue of the interleukin 2-type cytokine, wherein the amino acid sequence of said solubility tag has at least 90% sequence identity to SEQ ID NO: 38 (InfB).In a most preferred embodiment, the recombinant cell of the invention expressing IL-2 cytokine(s) is a strain of E. coli, comprising the Anderson promoter (SEQ ID NO 13) and RBS1 (see table 3) for regulating transcription and translation of the IL-2 cytokine(s), further comprising a nucleic acid sequence encoding the LamB signal peptide (SEQ ID NO: 28) fused to the N-terminal of the expressed IL-2 cytokine, and further comprising a nucleic acid sequence encoding the InfB secretion signal (SEQ ID NO 38) fused to the C-terminal of the expressed IL-2 peptide.

II. Cancer treatment by the recombinant bacterial cell

The recombinant bacterial cell of the invention comprises one or more nucleic acid molecules encoding and secreting cytokines for use in treatment of cancer, wherein the expression of said cytokine(s) may be regulated as discussed above. In one aspect, the invention provides a recombinant cell or a population derived therefrom as disclosed herein for use as a medicament, wherein the cell secretes the active agent interleukin 2-type cytokine.

As discussed previously, the recombinant bacterial cell of the invention is a bacterium having the property of accumulating at tumor sites, especially solid tumors, where it by secreting IL-2 acts as a therapeutic agent in treatment of the solid tumor by stimulating T cells, such as via the activation of the expression of specific genes of the cells located in or around the tumor (see example 3.3.4 and 5.3).

In one embodiment, the invention provides a recombinant bacterial cell as disclosed herein for use in treatment of cancer, prefereably for use in treatment of solid tumors.

In one aspect, those cancers for which the recombinant bacterial cells of the invention may be used in providing therapeutic treatment may be selected from the group:

Adrenocortical Carcinoma; AIDS-Related Cancer; AIDS-Related Lymphoma; Lymphoma; Anal Cancer; Gastrointestinal Carcinoid Tumor; Astrocytomas; Atypical Teratoid/Rhabdoid Tumor, Basal Cell Carcinoma; Bile Duct Cancer; Bladder Cancer; Bone Cancer; Ewing Sarcoma; Osteosarcoma; Malignant Fibrous Histiocytoma; Brain Tumors; Lung Cancer; Burkitt Lymphoma; Non-Hodgkin Lymphoma; Carcinoid Tumor; Cardiac Tumor, Medulloblastoma; Cervical Cancer; Cholangiocarcinoma; Chordoma; Myeloproliferative Neoplasm; Rectal Cancer; Craniopharyngioma; Cutaneous T-Cell Lymphoma; Mycosis Fungoides; Ductal Carcinoma In Situ; Endometrial Cancer; Uterine Cancer; Ependymoma; Esophageal Cancer; Esthesioneuroblastoma; Fallopian Tube Cancer; Gallbladder Cancer; Gastric Cancer; Gastrointestinal Carcinoid Tumor; Gastrointestinal Stromal Tumor; Gestational Trophoblastic Disease; Hepatocellular Cancer; Hodgkin Lymphoma; Hypopharyngeal Cancer; Intraocular Melanoma; Islet Cell Tumor, Pancreatic Neuroendocrine Tumor; Langerhans Cell Histiocytosis; Laryngeal Cancer; Lip and Oral Cavity Cancer; Liver Cancer; Lung Cancer, Pleuropulmonary Blastoma, Tracheobronchial Tumor; Lymphoma; Melanoma; Melanoma, Merkel Cell Carcinoma; Mesothelioma, Metastatic Squamous Neck Cancer; Midline Tract Carcinoma With NUT Gene Changes; Mouth Cancer; Multiple Endocrine Neoplasia Syndromes; Multiple Myeloma; Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Neoplasm; Myeloproliferative Neoplasm; Nasal Cavity cancer; Paranasal Sinus Cancer; Nasopharyngeal Cancer; Non-Hodgkin Lymphoma; Non-Small Cell Lung Cancer; Pleuropulmonary Blastoma; Oropharyngeal Cancer; Osteosarcoma; Ovarian Cancer; Pancreatic Cancer; Papillomatosis; Paraganglioma; Parathyroid Cancer; Penile Cancer; Pheochromocytoma; Pituitary Tumor; Plasma Cell Neoplasm; Breast Cancer; Lymphoma; Peritoneal Cancer; Prostate Cancer; Recurrent Cancer; Renal Cell Cancer; Retinoblastoma; Rhabdomyosarcoma, Salivary Gland Cancer; Sarcoma;; Skin Cancer; Small Intestine Cancer; Soft Tissue Sarcoma; Squamous Cell Carcinoma; T-Cell Lymphoma, Testicular Cancer; Thymoma and Thymic Carcinoma; Thyroid Cancer; Urethral Cancer; Uterine Cancer, Endometrial; Uterine Sarcoma; Vaginal Cancer; Vascular Tumors; Vulvar Cancer; Chondrosarcoma; Osteosarcoma; Rhabdomyosarcoma; Heart cancer; Carcinoid tumor, gastrointestinal; Colon cancer; Extrahepatic bile duct cancer; Gastrointestinal stromal tumor; Hepatocellular cancer; Pancreatic cancer; Endometrial cancer; Renal cell carcinoma; transitional cell cancer; Gestational trophoblastic tumor; Wilms tumor; Oral cancer; Paranasal sinus and nasal

cavity cancer; Pharyngeal cancer; Salivary gland cancer; AIDS-related lymphoma; Anaplastic large cell lymphoma; Angioimmunoblastic T-cell lymphoma; Burkitt's lymphoma; Cutaneous T-cell lymphoma; Diffuse large B-cell lymphoma; Follicular lymphoma; Hepatosplenic T-cell lymphoma; Hodgkin's lymphoma; Hairy cell leukemia; Intravascular large B-cell lymphoma; Lymphoplasmacytic lymphoma; Lymphomatoid granulomatosis; Mantle cell lymphoma; Marginal zone B-cell lymphoma; Mediastinal large B cell lymphoma; Myelodysplastic syndromes; Mucosa-associated lymphoid tissue lymphoma; Mycosis fungoides; Nodal marginal zone B cell lymphoma; Primary central nervous system lymphoma; Primary cutaneous follicular lymphoma; Primary cutaneous immunocytoma; Primary effusion lymphoma; Plasmablastic lymphoma; Splenic marginal zone lymphoma; Skin adnexal tumors; sebaceous carcinoma; Merkel cell carcinoma; Sarcomas of primary cutaneous origin; dermatofibrosarcoma protuberans; Bronchial adenoma and carcinoid; Mesothelioma; Pleuropulmonary blastoma; Kaposi sarcoma; Epithelioid hemangioendothelioma; Desmoplastic small round cell tumor; and Liposarcoma.

In one preferred embodiment, the recombinant bacterial cell of the invention may be used in providing therapeutic treatment of soft tissue sarcoma, melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour, haemangiosarcoma, lingual SCC, osteosarcoma, nasal adenocarcinoma or fibrosarcoma

In one aspect, the invention provides a recombinant bacterial cell for use in treatment of cancer, wherein said cell accumulates at a cancerous tumor site, wherein said cell comprises one or more nucleic acid molecules encoding cytokines whose expression may be regulated as disclosed herein.

Another aspect of the invention provides a method of treating a patient suffering from a cancerous disease – such as selected from the above mentioned lists – said method comprising administering a recombinant cell according to the ivention to said patient.

III Administration of the recombinant cell to the patient

The recombinant bacterial cell of the invention for use in the prevention and/or treatment of an immune-related disorder in a subject in need thereof, is suitable for administration to the subject by a mode of administration selected from the group: intravenous, intra-arterial, intraperitoneal, intralymphatic, sub-cutaneous, intradermal, intramuscular, intraosseous infusion, intra-abdominal, oral, intratumor, intravascular, intravenous bolus; and intravenous drip.

Preferably the mode of administration is either intravenous, or intralymphatic, or intratumoral, or intraperitoneal administration.

In one embodiment, the recombinant bacteria of the invention are administered as a population of bacterial cells capable of secreting a therapeutic dose to the patient in need thereof. Such population may comprise at least 10^5bacterial cells, such as at least 10^6, 10^7, or at least 10^8 bacterial cells. In one embodiment, a therapeutic dose of between 10^3 to 10^10, preferably between 10^4 to 10^9, most preferably between 10^5 to 10^8 recombinant bacterial cells is administered to the patient.

In one embodiment, the administered recombinant population provides an intertumoral concentration of IL-2 of at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 pg/ml.

The recombinant cell of the invention may be administered together with other cancer treaments, such as adaptive T-cell therapy, CAR-T therapy, and/or cancer drugs.

In one aspect, the invention provides a kit of parts comprising (i) the recombinant bacterial cell expressing IL2 as disclosed herein and (ii) cells for CAR-T cell therapy.

The administration of the recombinant cell of the present invention in combination with other cancer treaments may be carried out as one combined dosage or as separate dosages, wherein (i) the recombinant bacterial cell expressing IL2 as disclosed herein is administered before or after (ii) cells for CAR-T cell therapy.

In another aspect, the invention provides a kit of parts comprising (i) the recombinant bacterial cell expressing IL2 as disclosed herein and (ii) other cancer drugs, such as immune checkpoint blockers, chemotherapeutics or radiotherapy. Examples of immune checkpoint blockers comprise anti-PD1; anti-PD-L1; anti-CTLA4; anti-CD40L; anti-CD-137; anti-IL-10; anti-IL-10R; CCL21; anti-OX40; Anti-B7-H4; LIGHT; anti-LAG3; and anti-GITR. Examples of hemotherapeutics comprises doxorubicin; paraplatin; cyclophosmamide; epirubicin, 5-fluoro uracil; gemcitabine; eribulin; mutamycin; paclitaxel; and docetaxel.

The administration of the recombinant cell of the present invention in combination with these other cancer treaments may be carried out as one combined dosage or as separate dosages, wherein (i) the recombinant bacterial cell expressing IL2 as disclosed herein is administered before or after (ii) other cancer drugs, such as immune checkpoint blockers, chemotherapeutics or radiotherapy. Examples of immune checkpoint blockers comprise anti-PD1; anti-PD-L1; anti-CTLA4; anti-CD40L; anti-CD-137; anti-IL-10; anti-IL-10R; CCL21; anti-OX40; Anti-B7-H4; LIGHT; anti-LAG3; and anti-GITR. Examples of hemotherapeutics comprises doxorubicin; paraplatin; cyclophosmamide; epirubicin, 5-fluoro uracil; gemcitabine; eribulin; mutamycin; paclitaxel; and docetaxel.

Numbered embodiments of the invention

Numbered embodiment 1: A recombinant bacterial cell or a population derived therefrom, comprising

- a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2-type cytokine having an N-terminal LamB signal peptide for secretion of said cytokine.

Numbered embodiment 2: The recombinant bacterial cell or population derived therefrom according to Numbered embodiment 1, wherein the encoded recombinant fusion polypeptide further comprises

- an InfB solubility tag fused respectively to the C-terminal residue of the signal peptide and the N-terminal residue of the interleukin 2-type cytokine, or
- an InfB solubility tag fused to the C-terminal residue of the interleukin 2-type cytokine.

Numbered embodiment 3: The recombinant bacterial cell or population derived therefrom according to Numbered embodiment 1 or 2, wherein the heterologous nucleic acid sequence is operably linked to a constitutive prokaryotic promoter and an optimized ribosomal binding site sequence.

Numbered embodiment 4: The recombinant bacterial cell or population derived therefrom according to any one of Numbered embodiments 1-3, wherein said bacterial cell is Escherichia coli, such as strain Nissle or Symbioflor.

Numbered embodiment 5: The recombinant bacterial cell or population derived therefrom according to any one of Numbered embodiments 1-4, wherein the amino acid sequence of said Interleukin 2 cytokine has at least 80% sequence identity to SEQ ID NO.: 1.

Numbered embodiment 6: The recombinant bacterial cell or population derived therefrom according to any one of Numbered embodiments 1-5, wherein the amino acid sequence of said LamB signal peptide has at least 80% sequence identity to SEQ ID No.: 28.

Numbered embodiment 7: The recombinant bacterial cell or population derived therefrom according to any one of Numbered embodiments 1-6, wherein the amino acid sequence of said InfB solubility tag has at least 80% sequence identity to SEQ ID No.: 38.

Numbered embodiment 8: A recombinant bacterial cell or a population derived therefrom according to any one of Numbered embodiments 1-7 for use as a medicament, wherein the cell secretes the interleukin 2-type cytokine.

Numbered embodiment 9: The recombinant bacterial cell or population derived therefrom for use as a medicament according to Numbered embodiment 8, for use in prevention and/or treatment of cancer.

Numbered embodiment 10: The recombinant bacterial cell or population derived therefrom for use in prevention and/or treatment of cancer according to Numbered embodiment 9, wherein said cancer is a solid tumor cancer.

Numbered embodiment 11: The recombinant bacterial cell or population derived therefrom for use in prevention and/or treatment of cancer according to Numbered embodiment 9 or 10, wherein said cancer is selected from soft tissue sarcoma, melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour, haemangiosarcoma, lingual squamous-cell carcinoma, osteosarcoma, nasal adenocarcinoma and fibrosarcoma.

Numbered embodiment 12: A kit of parts for use as a medicament comprising

(i) a recombinant bacterial cell according to any one of Numbered embodiments 1 to 8 as a first part, and (ii) a second part selected from the group: CAR-T cells, adoptive T cells, immune checkpoint blockers and cancer drugs, or a combination of any thereof.

Numbered embodiment 13: The kit of parts according to Numbered embodiment 12, for use in prevention and/or treatment of cancer, wherein said cancer is a solid tumor cancer.

Numbered embodiment 14: The kit of parts for use in prevention and/or treatment of cancer according to Numbered embodiment 13, ,wherein said cancer is selected from soft tissue sarcoma, melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour, haemangiosarcoma, lingual squamous-cell carcinoma, osteosarcoma, nasal adenocarcinoma and fibrosarcoma

Numbered embodiment 15: The kit of parts according to Numbered embodiment 13 or 14 for use in treatment of cancer in a patient, wherein the components (i) and (ii) are administered to the patient separately.

EXAMPLES

1. General methodology

1.1 *Cloning of cytokine-expressing strains*

The native E.coli Nissle 1917 plasmid pMut1 (SEQ ID NO. 4) was used for expression of the cytokines. The plasmid has been previously modified to insert a gene encoding Kanamycin resistance (SEQ ID NO. 41) for antibiotic selection. Plasmid backbone was PCR amplified with Phusion high fidelity PCR master mix with primers, that would produce 20-25nt overhangs with the DNA sequences coding the cytokines. DNA sequences coding the cytokine IL-2 was obtained from UniProt, codon optimized for E.coli (SEQ ID NO. 42), and synthesized by IDT. The constructs were cloned into the pMut1 plasmid using Gibson assembly according to manufacturer's recommendations. After Gibson assembly, the plasmids were transformed into E.coli TOP10 strain either using electro competent or chemically competent cells. Correct strains were selected by colony PCR and Sanger sequencing. Plasmids were purified from E. coli TOP10 bacteria and were transformed into electro competent E.coli Nissle 1917 bacteria to produce the final cytokine-expressing strain. A list of strains is found in section 1.13.

1.2 *Cell culture*

Mammalian cells were maintained in T75 flasks and sub cultured every 3-4 days. Complete growth media contained 10% FBS and 1% antibiotic cocktail. **CT26** mouse colon carcinoma cells were cultured in complete RPMi media. **CTLL-2** mouse cytotoxic T-lymphocytes cells were cultured in complete RPMi media supplemented with 10% T-cell culture supplement with ConA). **HT29** human colorectal carcinoma cells were cultured in complete McCoy's5A media. The cells were grown in a humidified incubator at 37°C with 5% CO2 atmosphere.

1.3 *Preparation of bacterial supernatants*

The media was supplemented with 10% FBS and 50µg/ml of Kanamycin. RPMi media was used for making supernatants for IL-2 activity assay. McCoy's 5A media was used for making supernatants for 3D tumor spheroid cytotoxicity assay. A single bacterial colony was inoculated into RPMi or McCoy's 5A media and grown overnight at 37°C with shaking 250 RPM. Overnight cultures were diluted 100-times into fresh media and grown until stationary phase. Cultures were centrifuged at 4000G for 10 minutes and supernatants were filter-sterilized using a 0,22µm syringe filter. Supernatants were stored at -20°C for further use.

1.4 *Quantification of cytokines in supernatants*

Bacterial or cell culture supernatants were diluted in ELISA dilution buffer and cytokine levels were measured with a commercial ELISA kit according to the manufacturer`s recommendations.

1.5 *IL-2 activity assay*

CTLL-2 cells were centrifuged 200G for 5min and washed twice with PBS to remove the IL-2 culture supplement from the media. The cells were starved for IL-2 by incubating them in complete RPMi media without any IL-2 for 5-6 hours. $2x10^4$ cells in a 45µl of volume were placed into each well of a 96-well black, clear-bottom plate. 45µl of diluted supernatants were added to the cells at a final concentration 10% (V/V), unless specified otherwise. After 16 hours 10µl of Alamar blue was added and incubated for 30 minutes. Fluorescence intensity was measured at 530nM excitation and 560nM of emission wavelengths.

1.6 *PBMC isolation*

Fresh human buffy coats were bought from the blood bank of Rigshospitalet, Denmark. PBMCs were isolated by density gradient centrifugation using Lymphoprep and SepMate-50 tubes. Isolation was performed according to manufacturer's recommendations. Frozen PBMCs were stored for later use. PBMCs were used immediately after thawing.

1.7 *3D tumor spheroid cytotoxicity assay*

5000 HT-29 cells were seeded in a 96-Well Clear Ultra Low Attachment Microplate and centrifuged at 1000G for 10 minutes and were placed into the incubator to form spheroids. 4-day old tumor spheroids were co-cultured with $1x10^5$ human PBMCs and the supernatants from bacteria (30% or 10% concentration V/V) in a volume of 100µl. 1µl of 30X diluted Cytotox green dye was added per well and cytotoxicity was measured up to 72h using the Incucyte S3 live cell imaging system. Spheroid integrity images were taken after 72 hours of incubation. Co-cultures were gently pipetted up/down 5 times to disturb the pelleted cells and pictures were acquired immediately with the Incucyte system.

1.8 *Analysis of target gene expression in PBMCs and tumor spheroid + PBMC co-cultures*

Human PBMCs or tumor spheroid + PBMCs were incubated with bacterial supernatants or recombinant IL-2 for 24 hours. The assay was performed using 12 wells of a 96-well plate for each condition. Cells were pooled into a 1,5ml Eppendorf tube and the cells were pelleted by centrifugation at 200G for 10min. The supernatant was frozen and used to assay INFγ secretion and the pelleted cells were resuspended in TRIzol. RNA was extracted and reverse-transcription reaction was performed according to manufacturers' recommendations. cDNA was diluted 6-times with MiliQ water. 3-step qPCR reactions were performed by mixing 6µl of the 2x qPCR master mix, 2ul of cDNA and 2ul of primers (3µM).

1.9 *Animal experiments*

All animal experiments were done according to the experimental license nr. 2017-15-0201-01209. CB6F1 mice were bought from Envigo. CT26 cells were washed 3 times in PBS and $5x10^5$ cells were inoculated into each flank of the mice. Sizes of the tumors were measured and the volume was calculated according to the formula 3,14/6× 〖D1×D2〗 ^(3/4), where D1 and D2 are dimensions in millimeters. After 10-12 days when tumors have formed and reached at least 100mm3 size mice were injected i.v. with PBS or 1x107 CFU of bacteria in a 100µl volume. Tumor volumes and mouse weight were measured 3 times per week. Mice were sacrificed when tumors reached 2000mm3 volume. Mice were killed by cervical dislocation and tumors and organs were dissected. Dissected tumors were cut into small pieces and divided into three tubes to analyze tumor colonization and cytokine levels, RNA expression of target genes and immune cell composition in the tumors.

1.10 *Assessing colonization and cytokine levels in tumors and organs*

After dissection even-sized parts of the tumors, whole spleen or whole liver were placed into GentleMACS C tubes and homogenized using the m_Imptumor_01_01 program. Cell clumps were removed by forcing the homogenized samples through a 70µm cell strainer. Serial dilutions of the organ homogenates were plated on LB plates containing Kanamycin and bacterial CFUs were counted the next day. In order to quantify expressed cytokine levels in the tumors – the same organ homogenates were centrifuged at 5000G for 10 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged at 17000G for 15 minutes at 4°C. The supernatant was stored at -80°C. ELISA was performed with 2-fold diluted supernatants to measure cytokine levels in the tumors.

1.11 *RT-qPCR of the cytokine target genes from tumor tissue*

After dissection a piece of tumor tissue was placed into a tube with RNAlater stabilization solution. The samples were stored at -80°C until use. A small piece of tumor tissue was placed into a tube with TRIzol and the tissue was homogenized using glass beads. RNA was purified and RT-qPCR was performed as previously described.

1.12 *List of consumables*

Table 1

Name	**Manufacturer**	**Catalog number**
RPMi media	ThermoFisher Scientific	A1049101
McCoy's 5A media	ATCC	30-2007
TrypLE select	Thermofisher Scientific	11588846
Antibiotic cocktail	Sigma-Aldrich	P4083-20ML
FBS	Sigma-Aldrich	F0804-500ML

T-75 flask	Thermofisher Scientific	10364131
T-Cell Culture Supplement with ConA (IL-2 Culture Supplement), Rat	Thermofisher Scientific	11513540
HT-29 human colorectal carcinoma cells	ATCC	HTB-38
CT26 mouse colon carcinoma cells	ATCC	CRL-2638
CTLL-2 mouse cytotoxic T-lymphocytes	ATCC	TIB-214
0,2µm syringe filter	Frisenette	03CP020AS
96-well black, clear-bottom plate	Sigma-Aldrich	CLS3603-48EA
96-Well Clear Ultra Low Attachment Microplates	Fisher Scientific	10023683
Cytotox Green dye	Essen Biosciences	ESS4633
Human IL-2 ELISA kit	AbCam	ab46033
Human IL-15 ELISA kit	AbCam	ab100554
Human INFγ ELISA kit	StemCell technologies	02002
TRIzol	Thermofisher Scientific	15596018
Direct-Zol RNA MiniPrep Plus extraction kit	Nordic Biosite	BioSite-R2072
Superscript IV first-strand synthesis system	Thermofisher Scientific	18091050
Human recombinant IL-2	Thermofisher Scientific	PHC0026
Human recombinant IL-15	Thermofisher Scientific	PHC9154
Gibson assembly master mix	BioNordika	NEB-E2611L
Phusion high-fidelity PCR Master mix	Thermofisher Scientific	M0531L
Chemically competent TOP10 cells	ForteBio	C404006
RNA later stabilization solution	Thermofisher Scientific	AM7020
Lymphoprep	StemCell Technologies	07811
SepMate-50 tubes	StemCell Technologies	85460
Kapa SYBR fast universal 2x qPCR master mix	Roche	KK4602
GentleMACS C tube	VWR	130-096-334
70µm cell strainer	Thermofisher Scientific	10788201

1.13 *List of strains*

E. coli TOP10: F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK λ− rpsL(StrR) endA1 nupG (ForteBio cat # C404006.

E. coli Nissle 1917 (from a commercial probiotic Mutaflor, ArdeyPharm)

E. coli Symbioflor G1/2 (from a commercial probiotic SymbioFlor 2, SymbioPharm)*E. coli* Symbioflor G4/9 (from a commercial probiotic SymbioFlor 2, SymbioPharm).

E. coli Symbioflor 5 (from a commercial probiotic SymbioFlor 2, SymbioPharm).

E. coli Symbioflor G6/7 (from a commercial probiotic SymbioFlor 2, SymbioPharm).

E. coli Symbioflor G8 (from a commercial probiotic SymbioFlor 2, SymbioPharm).

Table 2. Overview of strains and their IL-2 constructs

Strain name	Plasmid back-bone*	IL-2 constructs and the relative order of the construct features						
		promoter	RBS	Secre-tion tag	N-term. InfB	Il-2 gene	C-term. His6 or InfB	SEQ ID NO.
OmpA-IL2-His6	pMUt1	Anderson	RBS1	OmpA	-	Il-2	His6	43
LamB-IL2-His6	pMUt1	Anderson	RBS1	LamB	-	Il-2	His6	44
OmpF-IL2-His6	pMUt1	Anderson	RBS1	OmpF	-	Il-2	His6	45
NSP4-IL2-His6	pMUt1	Anderson	RBS1	NSP4	-	Il-2	His6	46
OmpC-IL2-His6	pMUt1	Anderson	RBS1	OmpC	-	Il-2	His6	47
PhoA -Il2-His6	pMUt1	Anderson	RBS1	PhoA	-	Il-2	His6	48
G1M5-IL2-His6	pMUt1	Anderson	RBS1	G1M5	-	Il-2	His6	49
PelB-IL2-His6	pMUt1	Anderson	RBS1	PelB	-	Il-2	His6	50
PhoE-IL2-His6	pMUt1	Anderson	RBS1	PhoE	-	Il-2	His6	51
Lpp-IL2-His6	pMUt1	Anderson	RBS1	Lpp	-	Il-2	His6	52
OmpT-IL2-His6	pMUt1	Anderson	RBS1	OmpT	-	Il-2	His6	53
OmpT-InfB-IL2	pMUt1	Anderson	RBS1	OmpT	InfB	Il-2	-	54
OmpC-InfB-IL2	pMUt1	Anderson	RBS1	OmpC	InfB	Il-2	-	55
PhoA-InfB-IL2	pMUt1	Anderson	RBS1	PhoA	InfB	Il-2	-	56
LamB-InfB-IL2	pMUt1	Anderson	RBS1	LamB	InfB	Il-2	-	57
OmpA-IL2-InfB	pMUt1	Anderson	RBS1	OmpA	-	Il-2	InfB	58
OmpT-IL2-InfB	pMUt1	Anderson	RBS1	OmpT	-	Il-2	InfB	59
OmpC-IL2-InfB	pMUt1	Anderson	RBS1	OmpC	-	Il-2	InfB	60
PhoA-IL2-InfB	pMUt1	Anderson	RBS1	PhoA	-	Il-2	InfB	61
LamB-IL2-InfB	pMUt1	Anderson	RBS1	LamB	-	Il-2	InfB	62
MS8 strong RBS	pMUt1	MS8	RBS1	LamB	-	Il-2	InfB	63
MS6 strong RBS	pMUt1	MS6	RBS1	LamB	-	Il-2	InfB	64
MS6 medium RBS	pMUt1	MS6	RBS3	LamB	-	Il-2	InfB	65

*Plasmid backbone used in all examples is modified pMut1 (SEQ ID NO 40), where nucleotide "X" in the plasmid designates the location of the IL-2 construct.

The negative control plasmid (SEQ ID NO 39) lacks the IL-2 gene.

2. Initial cloning and expression of IL-2 cytokines

A first generation of cytokine IL-2 harboring a C-terminal His6 tag were cloned into a modified native E.coli Nissle 1917 plasmid (modified pMut1, SEQ ID NO 40) and were transformed into E.coli Nissle 1917 (see Figure 1). Cytokines are signaling molecules that are produced and secreted by various human cells. Therefore, only production of cytokines in E.coli is not sufficient, because they need to reach and interact with the immune cells in order to exert their signaling effects. Therefore, the cytokine-producing strains were optimized for cytokine expression as well as secretion into the supernatant. Cytokine expression and secretion was optimized by testing different conditions in cytokine translation and signal peptide optimization, and further analyzing different E.coli probiotic strains for highest cytokine production.

2.1 *RBS optimization*

Different RBS sequences (Table 3) with varying translation strengths were tested for the secretion of IL-2 (comprising OmpA signal peptide and His6-tag). The constructs harboring different RBS sequences resulted in variation of IL-2 expression in the cell lysate (Figure 2A), which corresponded to the relative translation strength. However, only the strongest RBS (original RBS1) showed high levels of IL-2 in the supernatant (Figure 2B), compared with other RBS sequences. This RBS1 sequence was chosen as the most suitable RBS for further experiments.

Table 3: List of RBS sequences tested

RBS	Relative strenght	Sequence	Comment
1	1	AAAGGAGGA	Strong RBS
2	0.55	ACAGGAGGT	
3	0.49	ACAGGAGGA	Medium RBS
4	0.16	ACAGGAGGG	
5	0.08	ACAGGAGAG	
6	0.07	AAAGGAGCA	
7	0.06	ACAGGAGAA	
8	0.06	ACAGGAGAT	
9	0.02	ACAGGAGAC	

2.2 *Secretion tag optimization*

Secretion pathway can have a big impact on protein secretion efficiency. Protein secretion tags from two different secretion pathways were tested: the Sec-dependent secretion pathway (OmpA signal peptide), and the SRP-dependent pathway (DsbA signal peptide). In the Sec-dependent pathway the protein is exported from the cytoplasm after translation, whereas in the SRP-dependent pathway the protein is exported co-translationally. The tested strains comprised RBS1. The OmpA signal peptide showed higher secretion efficiency, compared to the DsbA signal peptide (Figure 2C). The OmpA signal peptide was therefore chosen as most suitable signal peptide for further experiments. Using the OmpA secretion signal the expression was around 1 ng/(ml*OD) for IL-2.

2.3 *E.coli strain optimization*

Different probiotic E.coli strains exist, which all have shown to be safe and exhibit robust tumor colonization (Kocijancic et al., 2016). Protein expression and secretion can be strain-dependent. Therefore, we tested cytokine expression in E.coli Nissle 1917, as well as 5 different Symbioflor strains. All tested strains comprised the Anderson promoter, RBS1, OmpA signal peptide, IL-2 and His6-tag. Similar levels of IL-2 were expressed in Nissle 1917, Symbioflor G6/7 and Symbioflor G8, while other Symbioflor strains had very low levels of IL-2 in the supernatant (Figure 2D). E.coli Nissle 1917 was chosen for further experiments. However, E.coli Symbioflor G6/7 and G8 may also be good candidates for cytokine expression in the tumors.

Cytokines in the following examples were expressed using the OmpA secretion signal and the strongest RBS1 in E.coli Nissle 1917.

3. Cytokine activity testing

In the following, the optimized E. coli Nissle 1917 stain (OmpA-IL2-His6) is used, comprising backbone vector pMUT1 (SEQ ID NO 40) with IL-2 construct "OmpA-IL2-His" as defined in Table 2,

The cytokines produced by the recombinant strains are secreted, and the bacterial supernatants were tested for cytokine activity using different assays disclosed here.

3.1 CTLL-2 viability assay

IL-2 Cytokine activity was tested by using a CTLL-2 viability assay. CTLL-2 are mouse T-cells that require IL-2 to remain viable. CTLL-2 cells incubated with supernatants from IL-2 expressing bacteria showed high viability after 16 hours of culture, while CTLL-2 cells incubated with supernatant from non-expressing bacteria (Neg. Ctrl) showed

decreased viability (Figure 3). This supports that the biological effect comes from the secreted cytokines in the supernatant and not the bacterial supernatant itself.

3.2 *3D tumor spheroid model*

To test whether the expressed IL-2 cytokines can activate human immune cells and lead to tumor destruction, a 3D tumor spheroid model was established. Tumor spheroids were formed using human colorectal cancer cells HT29. The spheroids were co-cultured with human immune cells in the form of peripheral blood mononuclear cells (PBMCs) and supernatants from cytokine-expressing or non-expressing bacteria. Commercial human recombinant IL-2 was used as a positive control. Cytotoxicity was measured as a marker for induction killing of tumor cells. Co-cultures exposed to cytokine-containing supernatants had higher induction of cytotoxicity, compared to supernatant not containing any cytokines (Figure 4A). IL-2 supernatants was further shown to lead to complete destruction of the tumor spheroid after 6 days of co-culture (Figure 4B). Co-cultures with either PBMCs alone or together with supernatant from non-expressing bacteria (Neg.Ctrl) did not show any tumor spheroid destruction, while co-cultures with commercial IL-2 cytokines lead to complete spheroid destruction, as expected. It has to be noted, that the commercial cytokines were used in higher concentration (10ng/ml) than the cytokine concentration found in the supernatants, therefore they should not be compared directly.

3.2 *In vivo – estabilising a mouse model*

In order to test the therapeutic efficacy of the cytokine-expressing strains in vivo, a mouse model was established. CB6F1 mice were used herein, which are a crossbreed between BALB/C and C57BL/6J, allowing the use of any mouse cancer cell line to establish tumors in the same mouse strain.

First, the difference between BALB/C and CB6F1 mice in bacterial clearance from blood and bacterial colonization in vital organs, such as liver and spleen, was compared (Figure 5). Healthy BALB/C and CB6F1 mice were injected intravenously with 1x10^7 CFU (colony forming units) of E.coli Nissle 1917 and CFU in blood was measured for up to 6 hours post infection. BALB/C mice cleared the bacteria from the blood circulation faster than CB6F1 mice. Bacteria fell below the detection limit after 30minutes in BALB/C mice. Whereas, most bacteria were cleared from CB6F1 mice after 3 hours (with some outliers being detectable even at 6h post injection). Even though bacterial half-life in blood was different between mouse strains, the colonization of liver and spleen was similar between the strains after 48h. Therefore, it was decided to use CB6F1 mice for further experiments.

An experiment to measure tumor colonization was performed on CB6F1 mice harbouring CT26 (mouse colon carcinoma) tumors. The tumors were implanted on both flanks and 1x10^7 bacteria were injected i.v. when all tumors were above 100mm^3 volume (12 days after implantation). Tumor size during bacterial infection is important, because too small tumors may not have a big enough hypoxic core for the bacteria to survive. Bacterial injection into the mice lead to a decrease in body weight of about 10% (Figure 6.A), which is expected and has been reported in the literature (Kocijancic et al., 2016). The body weight was recovered after a couple of days and the mice seemed healthy upon visual inspection. No mice died in the experiment. Injected bacteria had no effect on tumor growth (Figure 6.B). Successful bacterial i.v. administration was confirmed by measuring CFU in blood after injection (Figure 6.C). Mice were sacrificed after 48h or after 6 days post infection and bacteria were counted in tumors and organs (Figure 6. D and E). 3/5 mice had high bacterial loads (above 10^7 CFU/g) in at least one of the tumor after 48h. Bacteria were present in the vital organs too, but in around 1000-fold lower numbers. After 6 days all mice had high bacterial loads in at least one of the tumors. Bacteria could be detected in vital organs as well, but now the differences between bacterial levels in tumors and organs was around 10000-fold.

E.coli Nissle 1917 successfully colonized CT26 tumors in CB6F1 mice, therefore making it a good mouse cancer model to test cytokine-expressing strains. Bacteria were detected in high amounts in the tumors and the colonization persisted at least for 6 days. Even though there was colonization in the vital organs, it was 1000-10000x lower than in the tumors.

3.3 *Effect of IL-2 strains in vivo*

The therapeutic effect of IL-2-expressing strains was tested in the established mouse model. Non-expressing bacteria were used as a control (Ctrl). After the mice received a single i.v. injection of PBS or bacteria, their tumor volumes were monitored for up to 16 days. Due to inherent variability in tumor growth, some mice had to be sacrificed earlier if the size of their tumors reached 2000mm^3.

3.3.1 Location of colonization

Different strains of bacteria can have different efficiencies in colonizing the tumors and organs. This can be affected by decreased fitness of the strain, due to increased metabolic burden from expressing cytokines. Furthermore, producing immune-activating cytokines can affect the immune cell composition in the surrounding microenvironment. This might also affect the ability of bacteria to evade clearance by the immune cells. Therefore, colonization of the strains was measured in excised tumors,

liver, spleen, kidneys and lungs (Figure 7). Measuring colonization is an endpoint measurement, when the organs are plated after the mouse has been sacrificed. Bacteria carrying an empty plasmid (Ctrl) displayed the highest overall colonization of tumors (16/18 tumors colonized), compared to the cytokine-expressing strains (11/20 for IL-2) (Figure 7). However, the Ctrl strain also showed the highest unspecific colonization in the vital organs. The fact, that cytokine-expressing strains show lower unspecific colonization is a clear benefit. It makes them safer for systemic administration and local cytokine production in the tumor is preferred.

3.3.2 Tumor volumes

Tumor volumes were measured to assess the therapeutic response of the treatment. No clear difference in means tumor volume were seen between the groups treated with PBS, non-expressing and cytokine-expressing bacteria (Figure 8A). Most of the mice were still alive 9 days after bacterial infection. On day 11 and later days the mice had to be sacrificed due to tumor size. Removal of dead mice affected the overall mean of tumor size, which can be seen after day 11. PBS-treated mice showed a high range of variability when individual tumors were plotted (Figure 8B). Similar variability was observed when individual tumors were plotted from tumors colonized by the Neg.Ctrl bacteria (Figure 8C). Colonized tumors from the IL-2-expressing bacteria group showed a diverging split between tumors that grew faster and those that grew slower (Figure 8D).

3.3.3 Cytokine levels

Production of active cytokines in the tumor microenvironment is important in order to observe a therapeutic effect. Tumors that had bacterial colonization were homogenized and cytokine levels were measured by ELISA. The expressed cytokines are human origin and should be specifically detected by the human ELISA antibodies. Tumors from the IL-2-expressing bacteria group had significantly higher levels of IL-2 detected, compared to tumors treated with Ctrl bacteria (Figure 9A). Furthermore, the tumors that had the highest levels of IL-2 also seemed to grow slower (Figure 9A&B, indicated in white circles).

3.3.4 Activation of gene expression by IL-2

IL-2 is a signaling protein, which regulate activation of gene expression in immune cells. Kovanen et al 2003 have performed a global gene expression analysis of human PBMCs and have shown upregulation of multiple genes upon IL-2 stimulation. Their study confirmed upregulation of genes associated with IL-2 signal transduction (Il2Ra, Bcl2), transcription factors (Nfkb1), pro-apoptotic ligands (Tnfsf10) MAPK signaling pathway (Mapkapk3) and regulation of MAPK signaling (Dusp5). Gene expression of these genes

in PBS-treated and colonized tumors treated with bacteria was measured by RT-qPCR analysis using primers specified in table 4. A significant increase in Dusp5 expression was found in IL-2 groups, compared to PBS group (Figure 10).

Table 4. qPCR primers

Gene	Forward primer	Sequence	Reverse primer	Sequence
Bcl2	mouse_Bcl2_fw (SEQ ID NO 66)	ATGCCTTTGTGGA ACTATATGGC	mouse_Bcl2_rw (SEQ ID NO 67)	GGTATGCACCCA GAGTGATGC
Nfkb1	mouse_Nfkb1_fw (SEQ ID NO 68)	ATGGCAGACGAT GATCCCTAC	mouse_Nfkb1_rw (SEQ ID NO 69)	TGTTGACAGTGGT ATTTCTGGTG
Il2ra	mouse_Cd25_fw (SEQ ID NO 70)	AACCATAGTACCC AGTTGTCGG	mouse_Cd25_rw (SEQ ID NO 71)	TCCTAAGCAACGC ATATAGACCA
Tnfsf10	mouse_Trail_fw (SEQ ID NO 72)	ATGGTGATTTGCA TAGTGCTCC	mouse_Trail_rw (SEQ ID NO 73)	GCAAGCAGGGTC TGTTCAAGA
Mapkapk3	mouse_Mapkapk3_fw (SEQ ID NO 74)	TGAAGCTCCTGTA TGACAGCC	mouse_Mapkapk3_rw (SEQ ID NO 75)	CTTGCCGTGGTG CATATTCTC
Dusp5	mouse_Dusp5_fw (SEQ ID NO 76)	GGGGTATGAGAC CTTCTACTCAC	mouse_Dusp5_rw (SEQ ID NO 77)	GCGTGGTAGGCA CTTCCAA

4 Optimization of cytokine-expressing strains

The initial prepared cytokine-expressing strains (comprising OmpA-IL2-His6) did not show any clear therapeutic effect, when it comes to tumor growth. However, the produced cytokines were detected in colonized tumors and seemed to decrease the growth of tumors, which had the highest levels of IL-2. Furthermore, IL-2 produced in the tumors lead to upregulation of the IL-2 response gene Dusp5.

Further optimization of cytokine production was carried out in order to obtain even better performing strains.

First, it was tried to remove the His-tag from the construct. However, it was not possible to clone IL-2 constructs without the C-terminal His_6 tag. All constructs exhibited frame shift mutations, which indicated, that removing the C-terminal makes IL-2 toxic to the bacteria (data not shown).

4.1 *Optimization of secretion tag*

In the first initial strains it was shown that the SecB secretion pathway using OmpA secretion tag was superior for secretion of IL-2, compared to the SRP secretion pathway using the DsbA signal. However, only 2 different tags were compared, therefore, leaving many possibilities for further optimization. As mentioned previous, there are several protein secretion systems in E.coli, including SRP-dependent, SecB-dependent, and TAT-dependent. More secretion tags belonging to the SecB and TAT pathways were now tested: OmpA, LamB, OmpF, NSP4, OmpC, PhoA, G1M5, PelB, PhoE, Lpp, OmpT – see

table 5.

Table 5. **Secretion tags**			
Secretion tag	**Amino acid sequence**	**SEQ ID No**	**Source**
OmpA	MKKTAIAIAVALAGFATVAQA	27	Choi et al 2004
LamB	MMITLRKLPLAVAVAAGVMSAQAMA	28	Choi et al 2004
OmpF	MMKRNILAVIVPALLVAGTANA	29	Choi et al 2004
NSP4	MKKITAAAGLLLLAAQPAMA	30	Han et al 2017
OmpC	MKVKVLSLLVPALLVAGAANA	31	Choi et al 2004
PhoA	MKQSTIALALLPLLFTPVTKA	32	Choi et al 2004
G1M5	MNDLNDFLKTISLSFGFFLLLSLPTVAEA	33	Jonet et al 2012
PelB	MKYLLPTAAAGLLLLAAQPAMA	34	Zhang et al 2018
PhoE	MKKSTLALVVMGIVASASVQA	35	Choi et al 2004
Lpp	MKATKLVLGAVILGSTLLAG	36	Choi et al 2004
OmpT	MRAKLLGIVLTTPIAISSFA	37	Choi et al 2004

Only two of the new constructs (comprsing LamB and OmpF) as well as the previous strain (comprsing OmpA) showed detectable levels of total IL-2 in the supernatant (based on ELISA assay data) (Figure 11A). Only LamB showed higher IL-2 protein secretion than the previous strain. Active IL-2 levels (based on CTLL-2 viability assay data) from OmpA-IL2-His_6 and OmpF-IL2-His_6 correlated well with the higher levels of total IL-2 (Figure 11B). However, LamB strain showed lower levels of active IL-2, even though the total IL-2 amount was the highest, indicating that some of the produced protein is inactive. Conversely, OmpC strain showed low amount of total IL-2, but had comparable levels of active protein to OmpA strain.

To further characterize the activity of the secreted cytokines – a 3D spheroid assay was performed. Supernatants of IL2-His6 from OmpA, LamB, OmpF, OmpC, PhoA and OmpT strains were incubated with human immune cells (PBMCs) and a HT29 tumor spheroid. Supernatant from non-expressing bacteria (Neg.Ctrl) was used as negative control. Supernatants from OmpA, LamB, OmpF and OmpC activated the human immune cells, which lead to increased cytotoxicity and destabilization of the tumor spheroid (Figure 12A&B). This correlated well with higher levels of active IL-2 detected in these samples. On the other hand, supernatants from OmpT and PhoA showed much lower cytotoxicity and no tumor destabilization, which also correlated with low levels of IL-2 in those supernatants. These results show, that the best performing strains secrete biologically active cytokines that can activate human immune cells and make them cytotoxic against

the tumor spheroid.

Secreted IL-2 levels were increased by about 2-fold, however, some of the secreted protein seemed to be inactive. Poor protein solubility and aggregation might be the cause for production of inactive IL-2. Increasing protein solubility might be beneficial to obtain more active protein.

4.2 *Optimization of protein solubility*

It was previously observed that, in the present expression setup, removing the C-terminal His6 tag from the IL-2 protein lead to frame shift mutations of the IL-2 gene. The hydrophilic residue of the His6 tag might help to stabilize the protein and make it more soluble. Therefore, increasing IL-2 solubility could help in obtaining higher secretion of the protein. Hansted et al 2011 disclose a small (21nt) expressivity tag derived from the InfB gene of E.coli, which can enhance protein solubility. This InfB solubility tag was tested in combination with several of the best performing IL-2 strains.

The InfB solubility tag was cloned either on the N or the C-terminus of the cytokine gene. Addition of the InfB tag had a beneficial effect for total IL-2 secretion in some of the strains (Figure 13A). OmpC-InfB-IL2 and LamB-InfB-IL2 with the solubility tag on the N-terminus and OmpA-IL2-InfB with the solubility tag on the C-terminus, exhibited a much higher secretion of total IL-2, compared to the previous strains LamB-IL2-His_6 (which showed highest secretion in Figure 11A). The strain OmpA-IL2-Infb with the solubility tag on the C-terminus showed an 8-fold increase in total IL-2 protein secretion. Therefore, the InfB solubility tag greatly improves the secretion of IL-2. However, not all of the secreted IL-2 is in its active form, as evidenced by the Il-2 activity measured (Figure 13B). The samples with highest total IL-2 levels (OmpC-InfB-IL2, LamB-InfB-IL2, and OmpA-IL2-Infb) did not show higher IL-2 activity when compared to the previous strains OmpA-IL2-His_6 and LamB-IL2-His_6. In fact, the strain, which had the highest amount of total IL-2 (OmpA-IL2-InfB) showed very low IL-2 activity, indicating that most of the produced cytokine is inactive. Strains that showed increased active IL-2 production were PhoA-InfB-IL2 and LamB-IL2-InfB. Supernatants from these strains had very similar levels of total IL-2 protein to the previous strains, but higher IL-2 activity. This means, they produce more active protein. For the previous LamB-IL2-His6 strain, exchanging the C-terminal His_6 tag for the InfB tag increased active protein secretion by more than 50% (Figure 13C).

4.3 *Expression optimization*

The LamB-IL2-InfB construct was cloned under the expression of two different promoters (MS8 and MS6 – see table 6) and two different RBS strengths (RBS1 (strong) and RBS3 (medium) – see table 6). The MS8 and MS6 promoters were developed by the

present inventors, where the MS8 is a much stronger and MS6 is comparable to the Anderson J23107 promoter used previously.

Table 6. Promoters

Name	Sequence	SEQ ID No.
J23107 Anderson promoter	TTTACGGCTAGCTCAGCCCTAGGTATTATGCTAGC	13
MS8 promoter	TGCTTGACTCGTCGTTATCCTACGTGTATAATTGGC	25
MS6 promoter	TGCTGGACTCGTCGTAATCCTGCGTGTATAATTGGC	26

Expressing a heterologous protein will increase the metabolic burden of the cell, which can be evaluated by measuring the growth kinetics. Strains that have high metabolic burden can have decreased growth. The growth profiles of LamB-IL2-InfB was compared with the new promoter/RBS combinations (Figure 14A). Also, the previous strain OmpA-IL-2-His6 and a non-expressing strain (Neg.Ctrl) was used as controls. As expected, higher expression strength increased the metabolic burden, which resulted in slower growth of the strains harboring the stronger promoters (MS8 and MS6), compared to the LamB-IL2-InfB. The strain OmpA-IL2-His6 had the worst growth profile, whereas the growth of LamB-IL2-InfB was very similar to the non-expressing bacteria. Decreased growth rate is a disadvantage, but it can be tolerated if higher expression increases protein production. Indeed, the total IL-2 secretion was increased up to 2-fold when using the stronger promoters and strong RBS, compared to the weaker Anderson promoter (LamB-IL2-InfB) (Figure 14B). However, higher expression did not increase active IL-2 levels in the supernatant (Figure 14C). Our results show that stronger expression systems do not increase the amount of active IL-2, even though total IL-2 production is higher. The results indicate, that increasing LamB-IL2-InfB expression increases the metabolic burden of bacteria without producing more active IL-2.

5. Optimized strain LamB-IL2-InfB

Strain LamB-IL2-InfB (comprising Anderson promoter and RBS1) was chosen for further experiments, because it had the highest active IL-2 secretion and the best growth profile.

IL-2 is an important cytokine, which participates in activation of cancer-killing immune cells, such as CD8+ T cells and NK cells. In response to infection or recognition of tumor antigens – these cells start production of pro-inflammatory cytokines like INFγ and acquire a cytotoxic phenotype. Their immunological response is also regulated by IL-2. IL-2 induces the production of proteins, that participate in direct contact-mediated cytotoxicity, such as granzymes, perforin and FasL. Expression of other pro-

inflammatory proteins like IFNγ, TNFα, TNFβ, TRAIL can also be increased by IL-2.

Previously, it was shown, that supernatants from bacteria expressing IL-2 can activate human immune cells. To gain more information about the immune cell activation mechanism – cytotoxicity, spheroid destabilization, as well as expression of IL-2 target genes by supernatant of the LamB-Il2-InfB strain was measured in tumor spheroid + PBMC co-cultures, or PBMCs monoculture.

5.1. *Cytotoxicity and spheroid destabilization*

Tumor spheroid + PBMC co-cultures were incubated with supernatants from LamB-IL2-InfB, Neg.Ctrl, or commercial IL-2, and cytotoxicity and spheroid destabilization was measured (Figure 15A&B). Commercial IL-2 (IL-2 10ng/ml) showed a dose-dependent activation of immune cell cytotoxicity, and spheroid destabilization was seen only with 10ng/ml after 3 days of co-culture. Supernatant from LamB-IL2-InfB also induced cytotoxicity, although it was lower in earlier time points, compared to the commercial cytokine. The supernatants were incubated in a 30% concentration (V/V), therefore, the final IL-2 concentration in the co-culture should be around 1ng/ml or lower. This is in agreement with the lower cytotoxicity observed in the LamB-IL2-InfB in earlier time points. However, LamB-IL2-InfB induced cytotoxicity reaches the same levels as the commercial IL-2 after 72h. Furthermore, tumor spheroid integrity was similar to the 10ng/ml IL-2.

5.2 *Activation of genes associated with immune cell-mediated cytotoxicity*

Next, it was determined if the IL-2 supernatants of the LamB-Il2-InfB strain induce cytotoxicity via the same mechanism as the commercial IL-2. Therefore, gene expression analysis was performed using PBMCs or tumor spheroid + PBMC co-culture. Samples were collected after 24h of exposure. Gene expression was measured by RT-qPCR analysis using primers specified in table 7. GAPDH was used as a reference gene to normalize gene expression of all the other genes.

Table 7. qPCR primers

Gene	Forward primer	Sequence	Reverse primer	Sequence
GAPDH	Human_GAPDH_fw (SEQ ID NO 78)	CGCTCTCTGCTCC TCCTGTT	Human_GAPDH_rw (SEQ ID NO 79)	CCATGGTGTCTG AGCGATGT
IFNG	Human_IFNγ_fw (SEQ ID NO 80)	GAGTGTGGAGAC CATCAAGGAAG	Human_IFNγ_rw (SEQ ID NO 81)	TGCTTTGCGTTG GACATTCAAGTC
BLIMP1	Human_BLIMP1_fw (SEQ ID NO 82)	CAGTTCCTAAGAA CGCCAACAGG	Human_BLIMP1_rw (SEQ ID NO 83)	GTGCTGGATTCA CATAGCGCATC
GZMA	Human_GrA_fw (SEQ ID NO 84)	TCTCTCTCAGTTG TCGTTTCTCT	Human_GrA_rw (SEQ ID NO 85)	GCAGTCAACACC CAGTCTTTTG
GZMB	Human_GrB_fw (SEQ ID NO 86)	TACCATTGAGTTG TGCGTGGG	Human_GrB_rw (SEQ ID NO 87)	GCCATTGTTTCG TCCATAGGAGA
GZMM	Human_GrM_fw (SEQ ID NO 88)	ACACCCGCATGT GTAACAACA	Human_GrM_rw (SEQ ID NO 89)	GGAGGCTTGAAG ATGTCAGTG

PRF1	Human_Perforin_fw (SEQ ID NO 90)	GGCTGGACGTGA CTCCTAAG	Human_Perforin_rw (SEQ ID NO 91)	CTGGGTGGAGGC GTTGAAG
FASLG	Human_FasL_fw (SEQ ID NO 92)	GGTTCTGGTTGC CTTGGTAGGA	Human_FasL_rw (SEQ ID NO 93)	CTGTGTGCATCT GGCTGGTAGA
TNFB	Human_TnfB_fw (SEQ ID NO 94)	ACACCTTCAGCTG CCCAGACTG	Human_TnfB_rw (SEQ ID NO 95)	TCCGTGTTTGCT CTCCAGAGCA
TNFA	Human_TNFa_fw (SEQ ID NO 96)	CCTCTCTCTAATC AGCCCTCTG	Human_TNFa_rw (SEQ ID NO 97)	GAGGACCTGGGA GTAGATGAG
TRAIL	Human_TRAIL_fw (SEQ ID NO 98)	CAGAGGAAGAAG CAACACATT	Human_TRAIL_rw (SEQ ID NO 99)	TGATGATTCCCA GGAGTTTATTTTG
IL2RA	Human_CD-25_fw (SEQ ID NO 100)	ACGGGAAGACAA GGTGGAC	Human_CD-25_rw (SEQ ID NO 101)	TGCCTGAGGCTT CTCTTCAC
DUSP5	Human DUSP5 fw (SEQ ID NO 102)	GCGACCCACCTAC ACTACAAA	Human DUSP5 rw (SEQ ID NO 103)	CTTCATAAGGTAA GCCATGCAGA

In tumor spheroid + PBMC co-culture the expression of genes-associated with immune cell killing was increased with commercial IL-2 in a dose-dependent manner (Figure 15C). Exposure to 10ng/ml of commercial IL-2 significantly increased expression of IFNG, FASLG, GZMA and PRF1, whereas exposure to 1ng/ml of IL-2 lead to a much lower upregulation of these genes. Co-cultures exposed to supernatant from LamB-IL2-InfB lead to a target gene activation, which was more similar to the 10ng/ml of commercial IL-2. This is surprising, because the total concentration of IL-2 in the supernatant was only around 1ng/ml, but co-cultures exposed to it showed a higher upregulation of gene expression, especially IFNG. Similar results were observed in gene expression from PBMC monoculture (Figure 15D). In PBMC monoculture LamB-IL2-InfB lead to a higher IFNG upregulation than 10ng/ml of commercial IL-2. There was no significant upregulation of genes participating in immune cell killing (FASLG, granzymes or perforin). This can be explained by the absence of direct contact between immune cells and tumor cells, which is needed for activation of these genes. Overall, supernatant from LamB-IL2-InfB strain shows higher target gene activation than would be expected based on the concentration of IL-2 in the supernatant.

5.3 *IFNγ expression activation*

IFNγ is an important cytokine, which mediates antitumor responses. IFNγ can directly affect tumor cells by exerting anti-proliferative and anti-angiogenic effects, as well as indirectly by increasing macrophage tumoricidal activity and attracting other immune cells.

INFG gene expression was increased upon stimulation with IL-2 (Figure 15A&B). The secreted INFγ levels were quantified in the conditioned media from the PBMC + tumor spheroid co-culture and PBMC monoculture. Similar to the gene-expression results, secreted INFγ levels increased with IL-2 stimulation in a dose-dependent manner (Figure 16A). Cells stimulated with supernatant from LamB-IL2-InfB secreted 3 times more

INFγ, compared to the highest commercial IL-2 condition. The differences in INFγ secretion between LamB-IL2-InfB and commercial IL-2 were even higher in the conditioned media from PBMC monoculture (Figure 16B). These results indicate, that IL-2 secreted from LamB-IL2-InfB has a very strong immune activation effect.

It has been shown, that bacterially secreted IL-2 can activate immune cells and lead to killing of the tumor spheroid and further that the same target genes were activated in secreted and commercial IL-2, indicating, that the mechanism of action is dependent on IL-2. However, IL-2 secreted by LamB-IL2-InfB strain showed a stronger effect in activation of immune cells than the commercial IL-2, even though the secreted levels were 10-fold lower. Supernatant from bacteria contain not only secreted IL-2, but also other immunogenic molecules derived from bacteria, such as lipopolysacharide (LPS) that can act as an adjuvant. LPS has been reported to work synergistically with IL-2 in enhancing INFγ production and cytotoxicity. Therefore, the additive effect of secreted IL-2 together with LPS and other immunogenic molecules could give good therapeutic responses.

5.4 *In vivo testing*

The optimized IL-2 strain (LamB-IL2-InfB) was used in another animal study to determine if the new strain has a therapeutic effect. Mice bearing CT26 tumors were treated with a single i.v. injection of phosphate-buffered saline (PBS), non-expressing bacteria (Ctrl) or the optimized IL-2 strain.

Mice treated with IL-2 bacteria exhibited significantly slower tumor growth in the first week, compared to PBS or Ctrl groups (Figure 17A). Looking at the individual tumor sizes, tumor growth was quite uniform in the PBS and Ctrl groups (Figure 17B&C). However, in the IL-2 group two populations could be seen that were responding and non-responding to the therapy (Figure 17D). The tumors not responding to IL-2 therapy grew as fast as in PBS, therefore, by day 9 the significant differences were lost. Half of the mice were sacrificed from PBS and IL-2 groups on day 11 in order to gain information about colonization, IL-2 levels and immune cell composition in the tumors while some of them were still responding to the treatment. The rest of the mice were sacrificed on day 16. Overall, the results indicate that the higher secretion of IL-2 from the optimized strain had a therapeutic effect. However, not all tumors responded to the therapy.

The optimized IL-2 strain (LamB-IL2-InfB) produces more cytokine than previously tested strains, higher amounts of IL-2 in the tumors were therefore expected. IL-2 levels were measured from all PBS tumors and colonized Ctrl and IL-2 tumors. As expected, IL-2 levels between PBS and Ctrl groups were not different and they represent

background levels (Figure 18). IL-2 levels were significantly higher in tumors from the IL-2 group.

Overall, bacteria expressing LamB-IL2-InfB could be used in bacterial cancer therapy. Tumor-bearing mice treated with these bacteria exhibited delayed tumor growth, which was caused by local IL-2 delivery in the tumor microenvironment.

5.5 *Immune-cell phenotyping of tumors and spleens*

IL-2 is a growth factor for T- and NK cells and can increase their expansion and cytotoxicity. $CD8^+$ T cells and NK cells are key effector cells that participate in direct killing of cancer cells. However, there is increasing evidence that $CD4^+$ T cells also play a key role in supporting activity of $CD8^+$ T cells by producing cytokines, as well as directly inhibiting tumor growth.

In order to assess whether bacteria secreting IL-2 had an impact on the immune cell composition, immuno-phenotyping in spleens and tumors was performed.

5.5.1 Preparation of tumor and spleen cells for immuno-phenotyping

Tumors and spleens were excised from mice and kept on ice until processing. For tumor digestion 50 µl of DNAase I (stock 600 U/ml) and 0.5 ml of collagenase IV (stock 10 mg/ml) were incubated with small pieces (1-2mm) of excised tumors (around 500mg) in complete DMEM in a total 5ml volume. The digestion was carried out at 37°C for 45 minutes with thorough shaking. Whole spleens were homogenized with GentleMACS C tubes using the program m_spleen_01. Cell clumps were removed by forcing the homogenized samples through a 70µm cell strainer. The cells were washed with PBS 3 times and cells were frozen at 1x10^7 viable cells per cryotube in 1ml volume. The media was supplemented with 10% DMSO. RPMi media was used for spleens and DMEM media for tumor cells.

5.5.2 Immuno-phenotyping phenotyping of tumors and spleens

Frozen cells from tumors or spleens were quickly thawed with warm complete RMPi media and immediately transferred into a 15ml falcon tube with 9ml of warm RPMi media. The cells were centrifuged at 1500RPM for 5min at 4°C and washed with selection buffer (1x PBS, 2% FBS, 1mM EDTA). Cells were filtered through a filter FACS tube and CD45 positive selection was performed only on the tumor cells according to manufacturer's recommendations. After selection, cells were washed with FACS buffer (1xPBS, 2% FBS) and transferred to a 96-well plate. Cells were incubated with 10µl Fc block on ice for 10 minutes. Then 20µl of antibody panel A (Table 9) was added and incubated for 15 minutes at 37°C in the dark. After the incubation the cells were washed

with FACS buffer and incubated with panel B (Table 9) for 30 minutes on ice. Cells were washed twice with FACS buffer and fixed with 50µl PFA overnight at 4°C. The next day cells were washed with FACS buffer twice and filtered through a filtering plate before running on the flow cytometer.

Table 8. Materials

	Manufacturer	Cat#
70µm cell strainer	Thermofisher Scientific	10788201
5mL Round Bottom Polystyrene FACS Tubes With 35um Strainer Cap	Stellar scientific	FSC-9005
EasySep™ Mouse CD45 Positive Selection Kit	StemCell Technologies	18945
CCR7 (BV421 Rat Anti-Mouse CD197)	BD Biosciences	566291
CD28 (PE/Cyanine7 anti-mouse CD28)	BioLegend	102126
CD62L (BV510 Rat Anti-Mouse CD62L)	BD Biosciences	563117
CD44 (FITC Rat Anti-Mouse CD44)	BD Biosciences	553133
CD45.2 (Alexa Fluor® 700 Mouse Anti-Mouse CD45.2)	BD Biosciences	560693
CD11b (BV786 Rat Anti-CD11b)	BD Biosciences	740861
CD39 (PE-CF594 Rat Anti-Mouse CD39)	BD Biosciences	567268
CD19 (PerCP-Cy™5.5 Rat Anti-Mouse CD19)	BD Biosciences	551001
CD335(NKp46) (BV711 Rat Anti-Mouse CD335 (NKp46))	BD Biosciences	740822
CD279 (PD-1) (PE Hamster Anti-Mouse CD279 (PD-1))	BD Biosciences	561788
CD3 (BUV395 Hamster Anti-Mouse CD3e)	BD Biosciences	563565
CD4 (BV605 Rat Anti-Mouse CD4)	BD Biosciences	563151
CD8 (BUV737 Rat Anti-Mouse CD8a)	BD Biosciences	564297
CD183 (CXCR3) (APC Hamster Anti-Mouse CD183)	BD Biosciences	562266
Collagenase IV	Thermofisher Scientific	2525
DNAse I	Sigma-Aldrich	11284932001

5

Table 9. Antibody panels for immuno-phenotyping

Panel A		
Antibody	**Fluorochrome**	**Dilution**
CCR7	BV-421	50
CXCR3	APC	50
Panel B		

Antibody	Fluorochrome	Dilution
CD11b	BV786	100
CD45.2	A700	50
CD3	BUV-395	20
CD4	BV-605	200
CD8	BUV-737	100
CD19	PerCp/Cy5.5	50
CD28	PE/Cy7	50
PD-1	PE	50
NKp46	BV-711	25
CD44	FITC	200
CD39	PE-CF594	50
CD62L	BV-510	200
L/D NIR	APC/Cy7	1000

5.5.3 Results

There were no differences in B or NK cell population between groups in both spleen and tumors (Figure 19A and B). However, $CD4^+$ T cell numbers in tumors were higher in both Ctrl and IL-2 groups, compared to PBS (Figure 19B). Furthermore, there was a decrease in $CD8^+$ T cell numbers in tumors in the Ctrl group (Figure 19B).

Upon antigen-stimulated activation, Naïve T cells (T_{Naive}) differentiate into effector-memory T (T_{EM}) cells or central memory T cells (T_{CM})[3]. T_{EM} cells have stronger cell killing capacity *in vitro*, however, it has been shown that T_{CM} cells give more favorable antitumor responses *in vivo* (Gattinoni et al 2005). High IL-2 stimulation increases differentiation to the T_{EM} phenotype, whereas lower stimulation leads to T_{CM} phenotype (Kaartinen et al 2017). Since the IL-2 levels detected in tumors were in pg range, low-level T cell stimulation was expected, which could give rise to T_{CM} cells. Indeed, increased levels of $CD4^+$ T_{CM} and T_{EM} cells were seen in spleens from IL-2 group (Figure 19C). In the tumors, $CD4^+$ T cell levels were similar between PBS and IL-2 groups, but were decreased in Ctrl group (Figure 19D).

$CD8^+$ T cell differentiation was not different between groups in both spleen and tumors (Figure 19E and F).

The results indicate that bacteria secreting IL-2 in the tumor microenvironment have a local and systemic effect on $CD4^+$ T cell stimulation by increasing levels of $CD4^+$ T_{CM}

cells. Increased levels of T_{CM} cells are associated with a better antitumor response, which we have observed in the IL-2 group.

$CD4^+$ TCM cell levels were increased in these mice, which are associated with a better antitumor response. Although $CD8^+$ T cells are usually the main tumor-killing cells, $CD4^+$ T cells are also able to differentiate into cytotoxic effector cells during chronic or acute infection (Hunder et al 2008, Kennedy et al 2008). $CD4^+$T cell cytotoxic capacity may be lower than that of $CD8^+$ T cells, and this might explain why tumor growth was reduced, but the tumors were not eliminated. Increase in $CD4^+$T cells could also indicate higher population of Tregs in the tumors. Although FOXP3 or CD25 antibodies were not part of the tested panel, highly immunosuppressive Tregs are reported to have $CD44^{high}CD62L^{low}$ phenotype (Levine et al 2014), which corresponds to TEM in our profiling. Since these cells were not upregulated in the tumors, it can be assumed that the $CD4^+$ T cell increase in the tumor microenvironment was not caused by accumulation of immunosuppressive Tregs.

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CLAIMS

1. A recombinant bacterial cell or a population derived therefrom for use as a medicament, wherein the cell or each cell of the derived population comprises a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2 cytokine fused to a solubility tag and having an N-terminal LamB signal peptide for secretion of said cytokine fusion protein having IL2 activity,
wherein the amino acid sequence of the Interleukin 2 cytokine has at least 70% sequence identity to SEQ ID NO.: 1.

2. The recombinant bacterial cell or population derived therefrom for use as a medicament according to claim 1, wherein the amino acid sequence of said LamB signal peptide has at least 80% sequence identity to SEQ ID No.: 28.

3. The recombinant bacterial cell or population derived therefrom for use as a medicament according to claim 1 or 2, wherein the amino acid sequence of the solubility tag has at least 80% sequence identity to InfB of SEQ ID No.: 38.

4. The recombinant bacterial cell or population derived therefrom for use as a medicament according to claim 3, wherein the infB solubility tag is fused to the C-terminal residue of the interleukin 2 cytokine.

5. The recombinant bacterial cell or population derived therefrom for use as a medicament according to anyone of claims 1-4, for use in prevention and/or treatment of cancer.

6. The recombinant bacterial cell or population derived therefrom for use in prevention and/or treatment of cancer according to claim 5, wherein said cancer is a solid tumor cancer.

7. The recombinant bacterial cell or population derived therefrom for use in prevention and/or treatment of cancer according to claim 5 or 6, wherein said cancer is selected from soft tissue sarcoma, melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour, haemangiosarcoma, lingual squamous-cell carcinoma, osteosarcoma, nasal adenocarcinoma and fibrosarcoma.

8. A kit of parts for use as a medicament comprising
(i) a recombinant bacterial cell according to any one of claims 1 to 4 as a first

part, and (ii) a second part selected from the group:
CAR-T cells, adoptive T cells, immune checkpoint blockers and cancer drugs, or a combination of any thereof.

9. The kit of parts according to claim 8, for use in prevention and/or treatment of cancer, wherein said cancer is a solid tumor cancer.

10. The kit of parts for use in prevention and/or treatment of cancer according to claim 9, ,wherein said cancer is selected from soft tissue sarcoma, melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour, haemangiosarcoma, lingual squamous-cell carcinoma, osteosarcoma, nasal adenocarcinoma and fibrosarcoma

11. The kit of parts according to claim 9 or 10 for use in treatment of cancer in a patient, wherein the components (i) and (ii) are administered to the patient separately.

12. A recombinant bacterial cell or a population derived therefrom, comprising a heterologous nucleic acid sequence encoding a recombinant fusion polypeptide comprising an interleukin 2 cytokine fused to an InfB solubility tag and having an N-terminal LamB signal peptide for secretion of said cytokine fusion protein,
wherein the amino acid sequence of the Interleukin 2 cytokine has at least 70% sequence identity to SEQ ID NO.: 1, and wherein the infB solubility tag is fused to the C-terminal residue of the interleukin 2-type cytokine.

13. The recombinant bacterial cell or population derived therefrom according to claim 12, wherein said bacterial cell is *Escherichia coli*.

14. The recombinant bacterial cell or population derived therefrom according to claim 12 or 13, wherein said bacterial cell is *Escherichia coli* strain Nissle or strain Symbioflor.

15. The recombinant bacterial cell or population derived therefrom according to any one of claims 12-14, wherein the heterologous nucleic acid sequence is operably linked to a constitutive prokaryotic promoter and an optimized ribosomal binding site sequence.

FIGURE 1

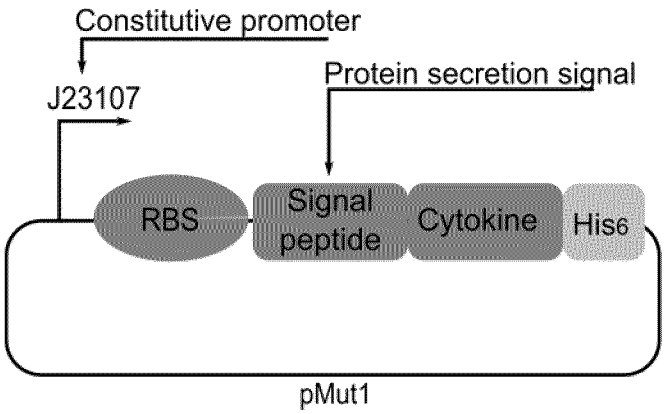

FIGURE 2

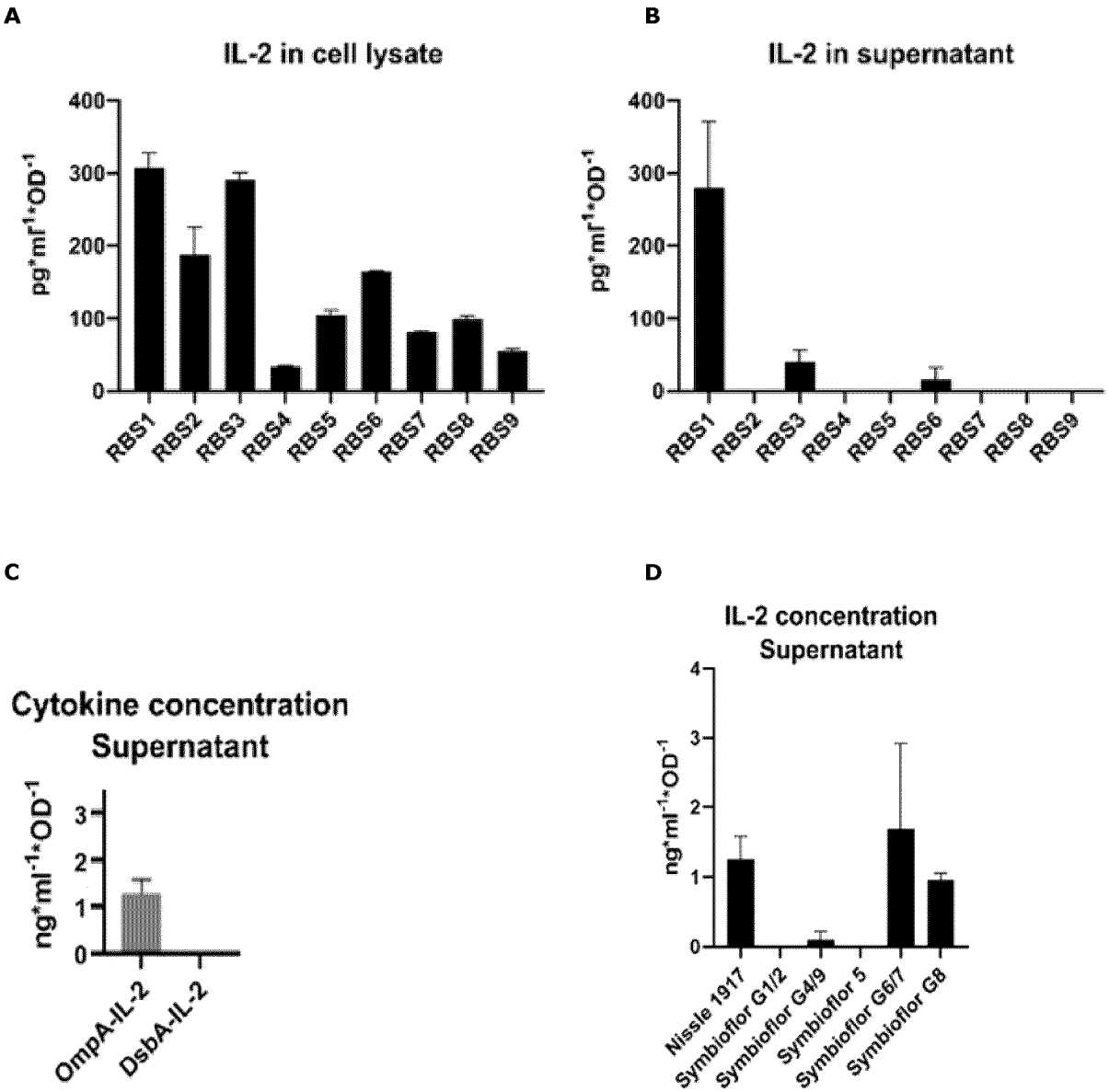

FIGURE 3

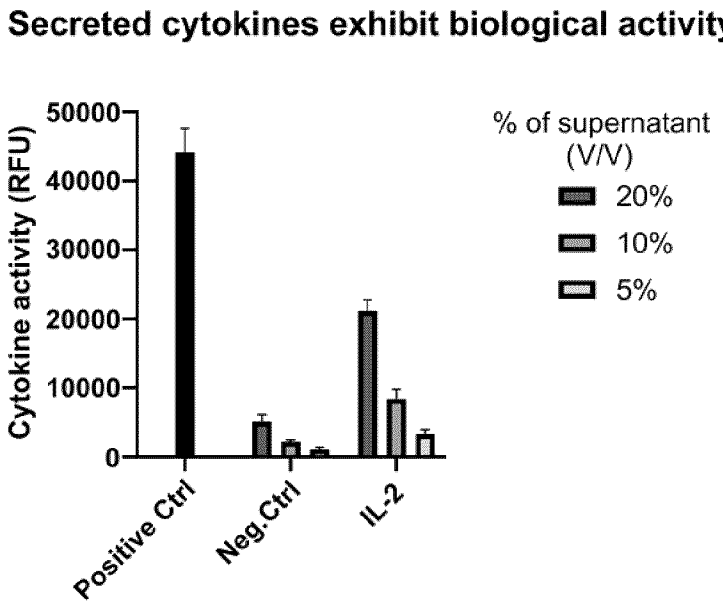

FIGURE 4

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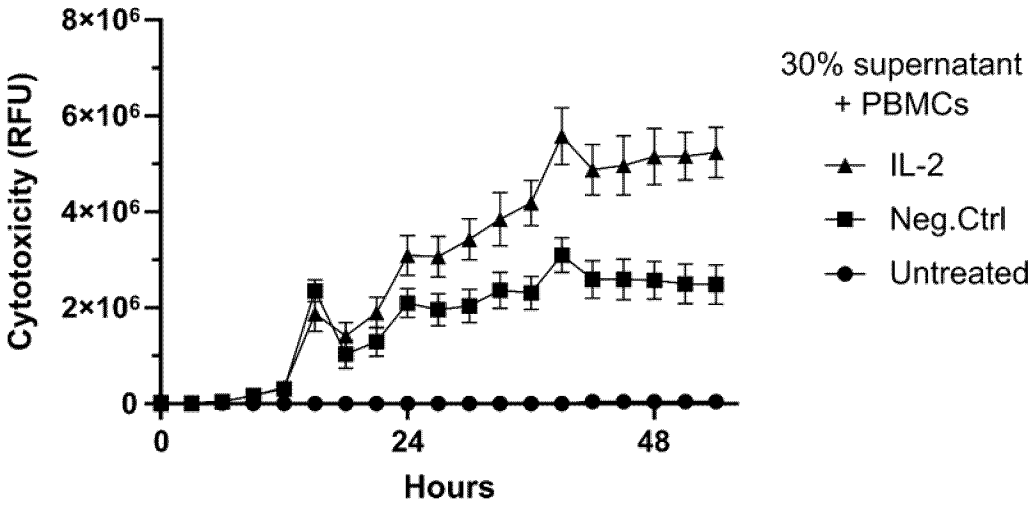

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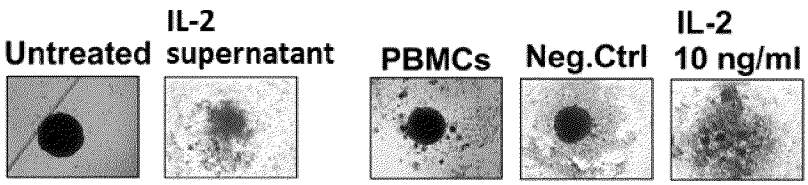

FIGURE 5

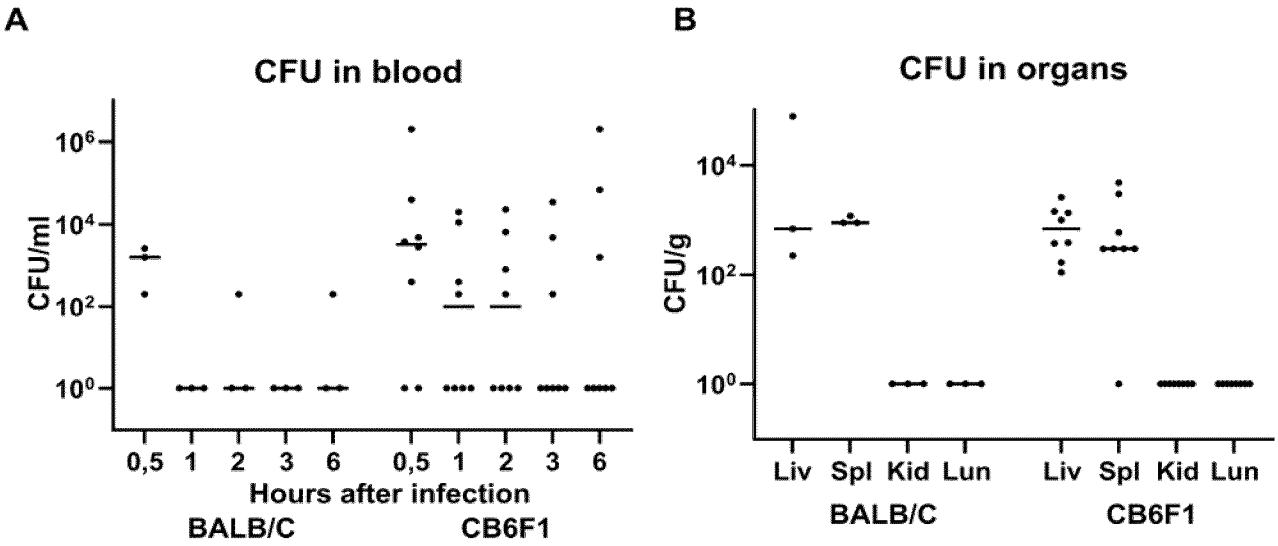

FIGURE 6

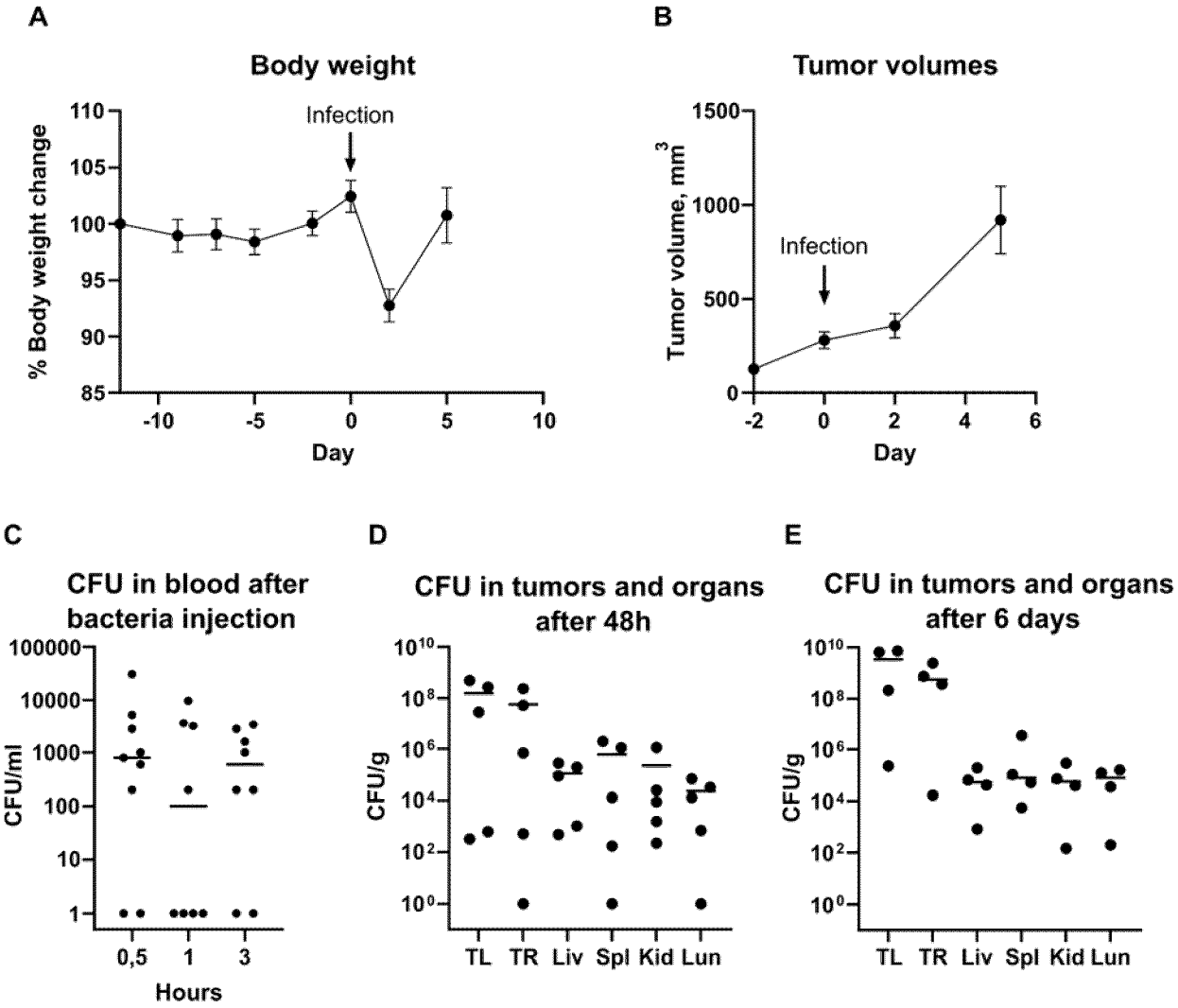

FIGURE 7

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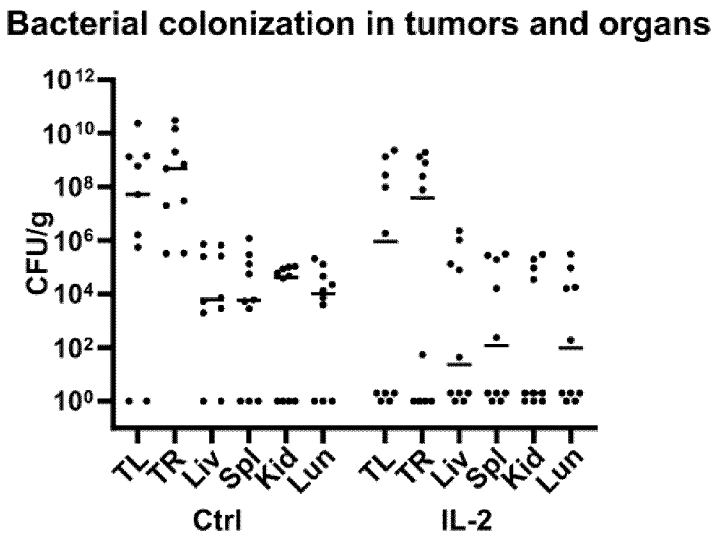

FIGURE 8

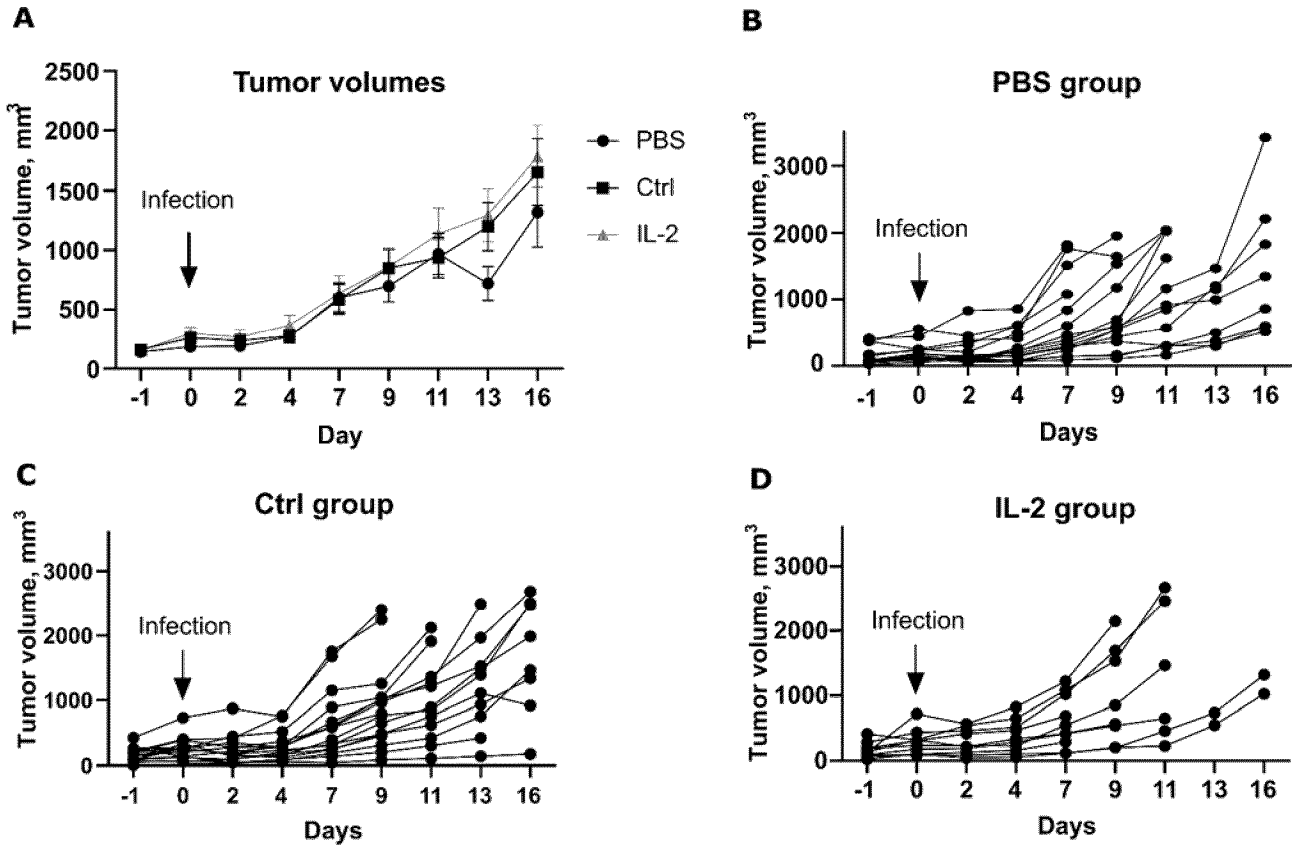

FIGURE 9

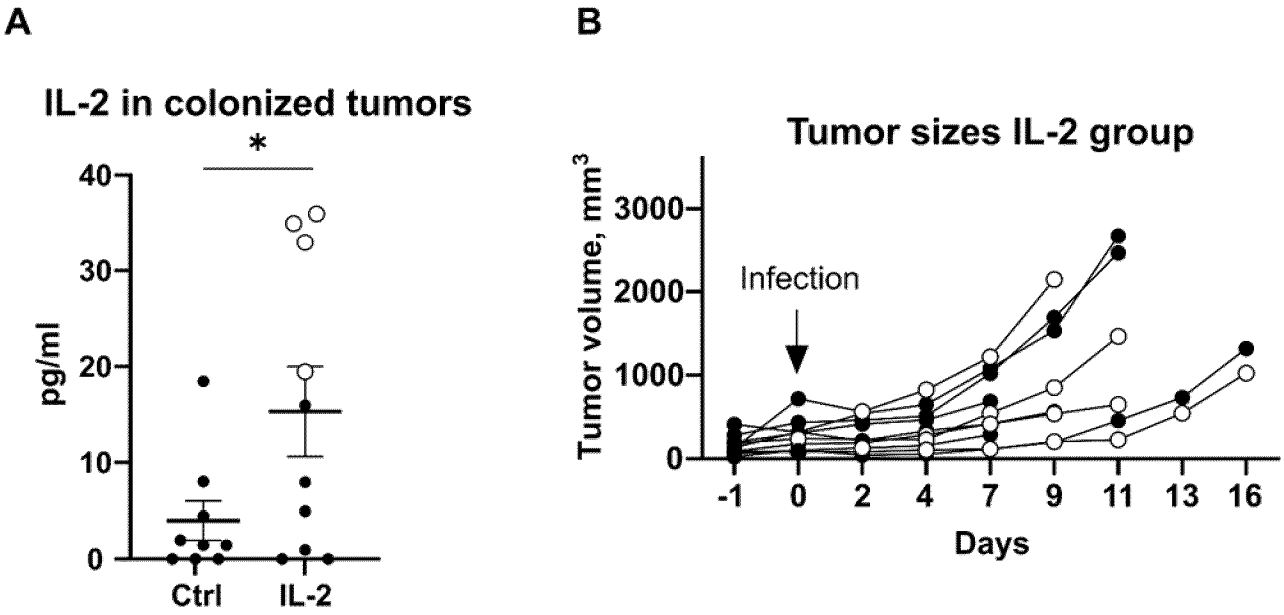

FIGURE 10

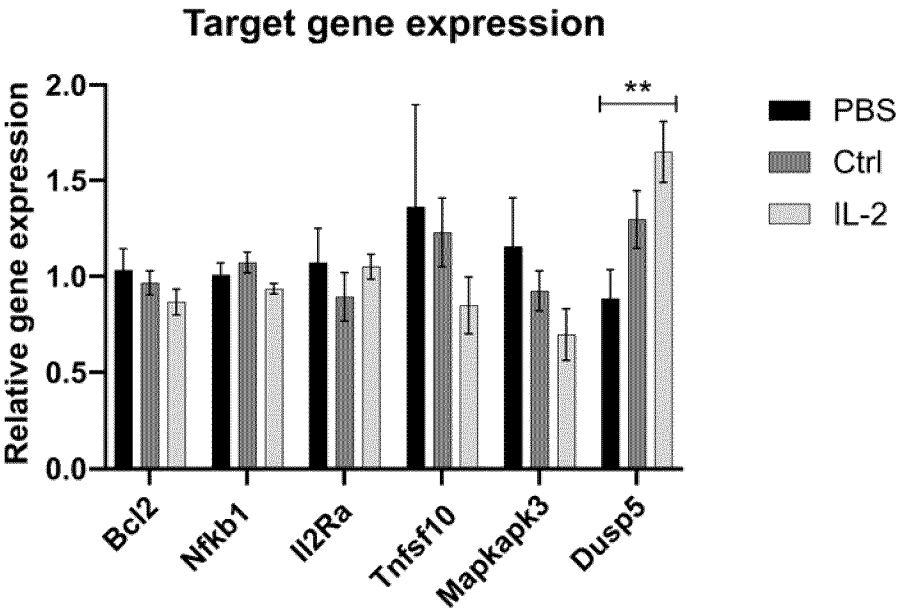

FIGURE 11

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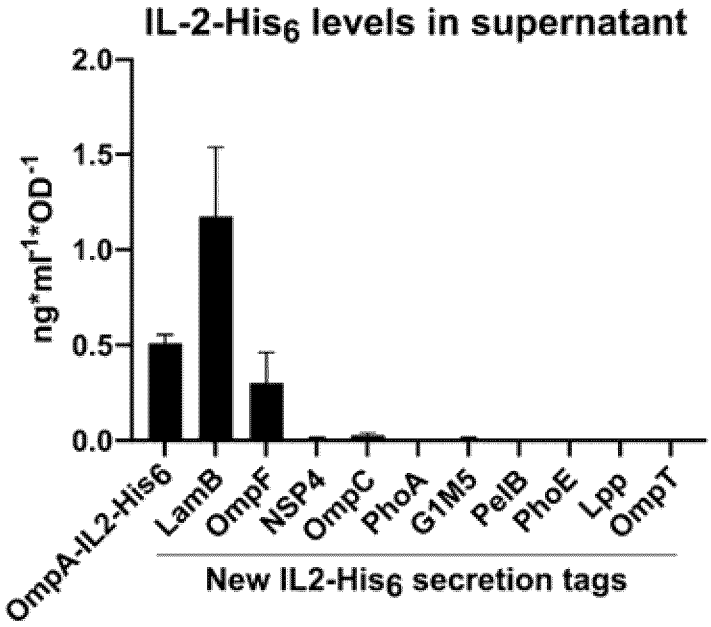

IL-2-His6 levels in supernatant
ng*ml-1*OD-1
2.0
1.5
1.0
0.5
0.0
OmpA-IL2-His6
LamB
OmpF
NSP4
OmpC
PhoA
G1M5
PelB
PhoE
Lpp
OmpT
New IL2-His6 secretion tags

B

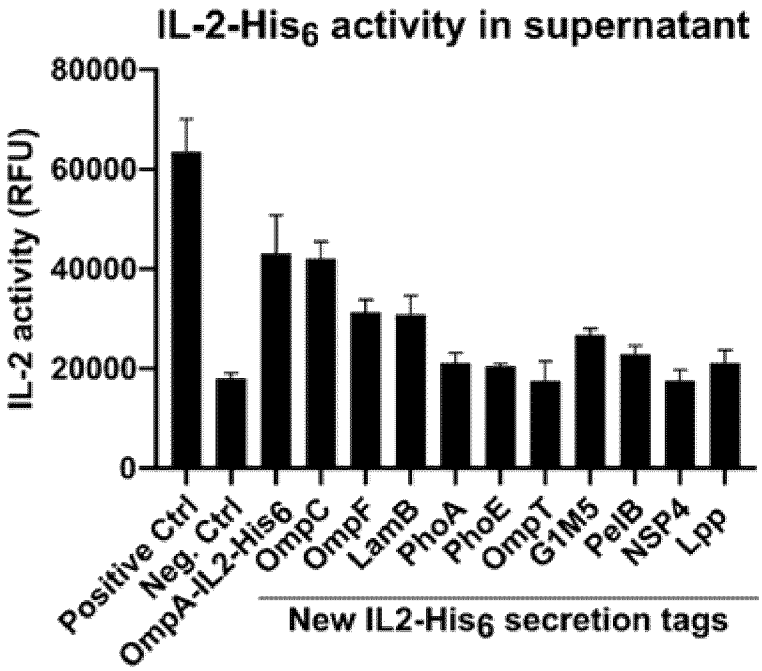
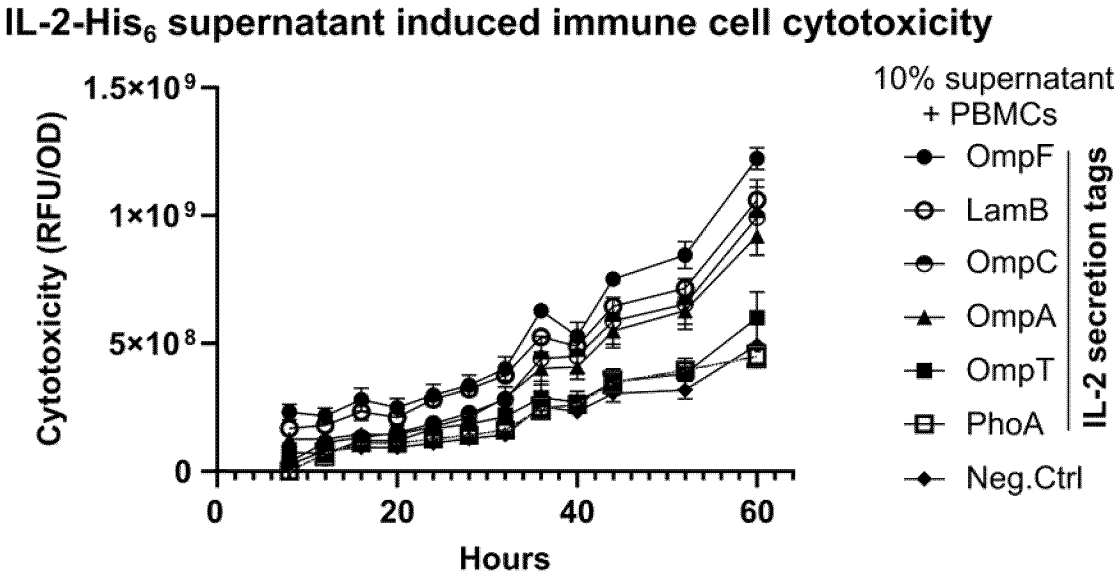
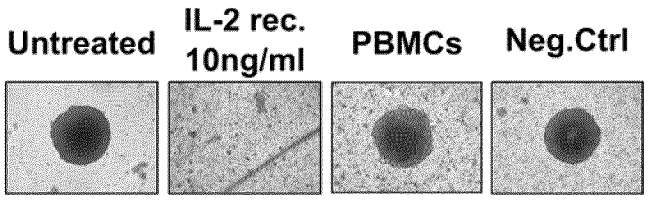

IL-2-His6 activity in supernatant
IL-2 activity (RFU)
80000
60000
40000
20000
0
Positive Ctrl
Neg. Ctrl
OmpA-IL2-His6
OmpC
OmpF
LamB
PhoA
PhoE
OmpT
G1M5
PelB
NSP4
Lpp
New IL2-His6 secretion tags

FIGURE 12

A


IL-2-His6 supernatant induced immune cell cytotoxicity
Cytotoxicity (RFU/OD)
1.5×10⁹
1×10⁹
5×10⁸
0
0
20
40
60
Hours
10% supernatant
+ PBMCs
OmpF
LamB
OmpC
OmpA
OmpT
PhoA
Neg.Ctrl
IL-2 secretion tags

B


Untreated
IL-2 rec.
10ng/ml
PBMCs
Neg.Ctrl

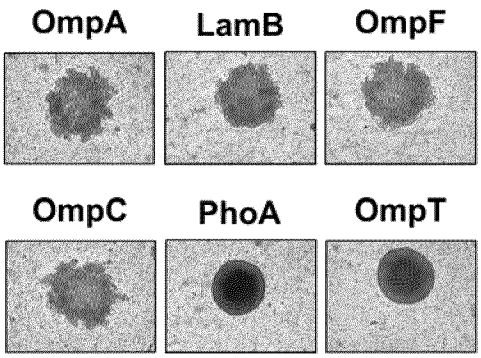
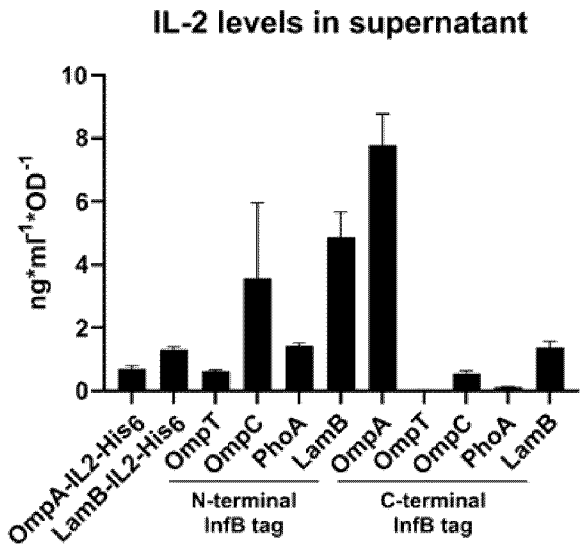
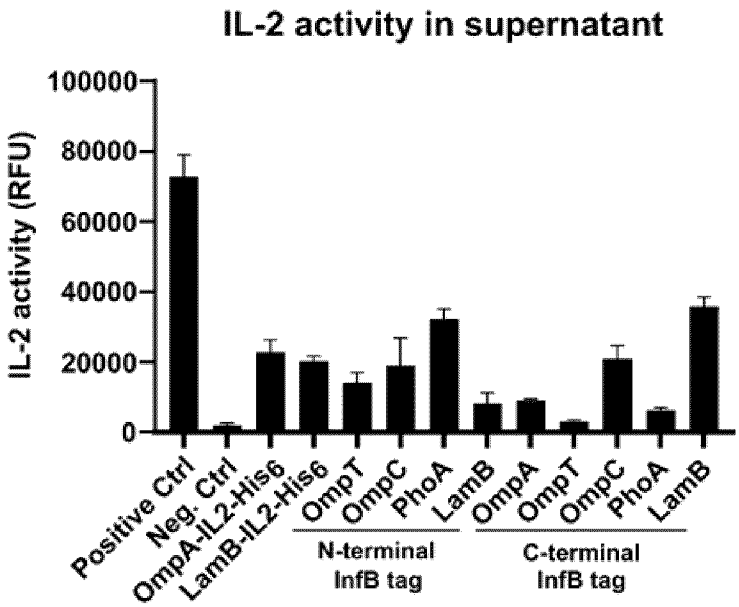

OmpA
LamB
OmpF
OmpC
PhoA
OmpT

FIGURE 13

A



B



C

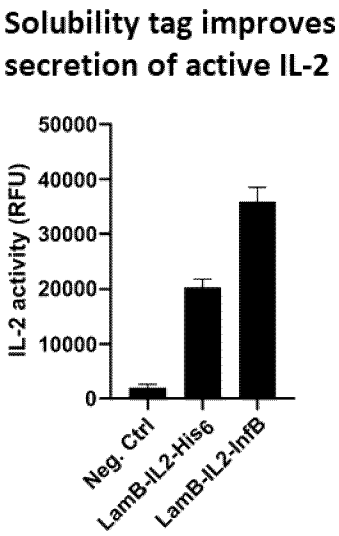
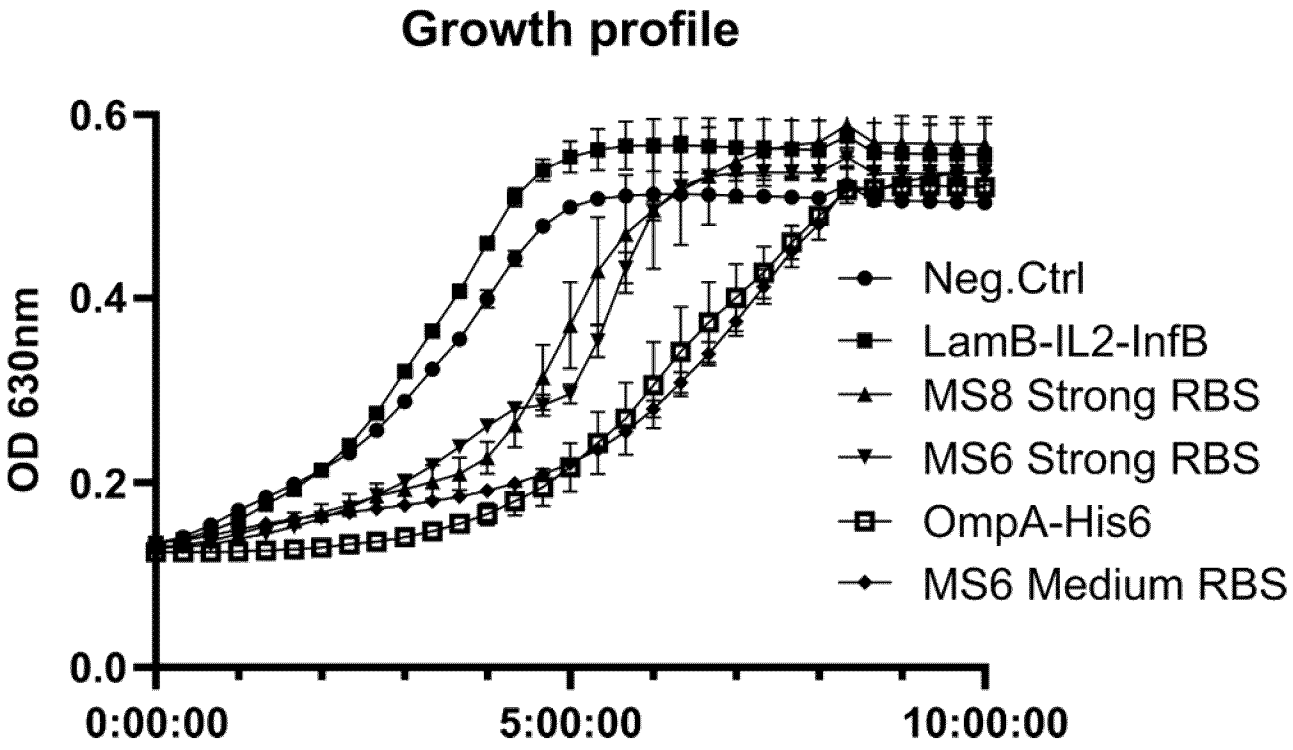
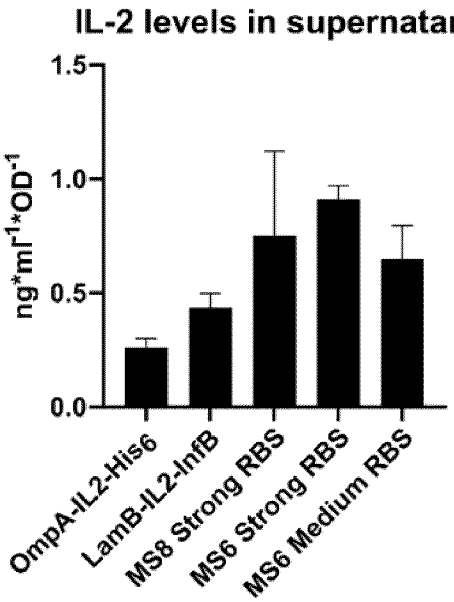


FIGURE 14

A



B



C

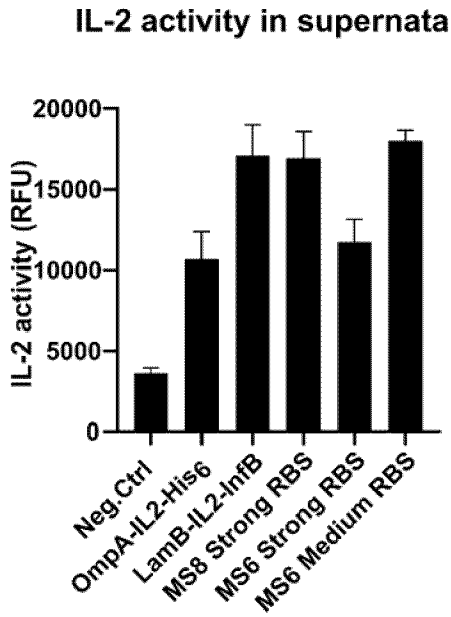
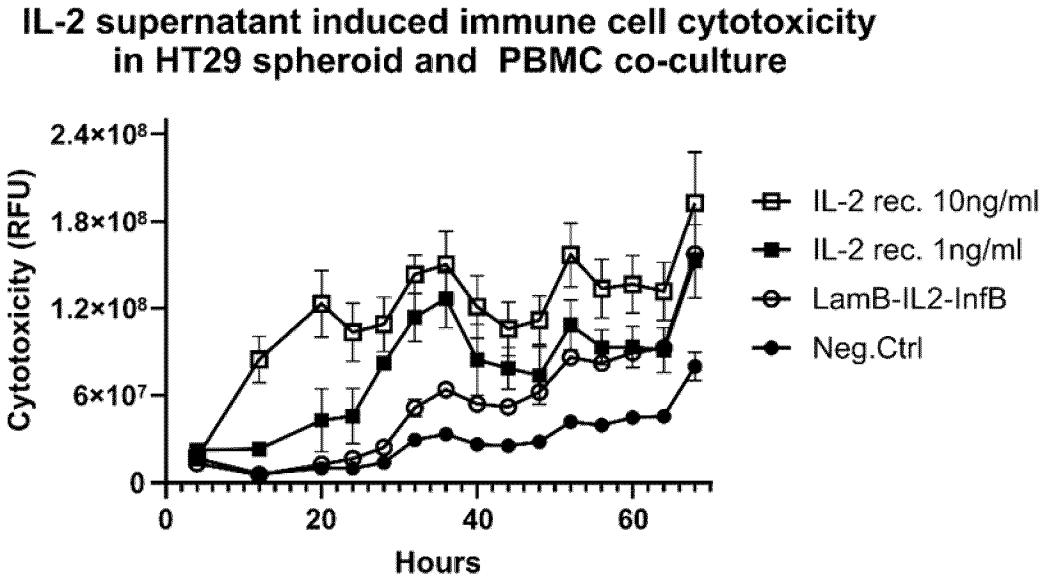


FIGURE 15

A


IL-2 supernatant induced immune cell cytotoxicity
in HT29 spheroid and PBMC co-culture
Cytotoxicity (RFU)
2.4×10⁸
1.8×10⁸
1.2×10⁸
6×10⁷
0
0
20
40
60
Hours
IL-2 rec. 10ng/ml
IL-2 rec. 1ng/ml
LamB-IL2-InfB
Neg.Ctrl

B

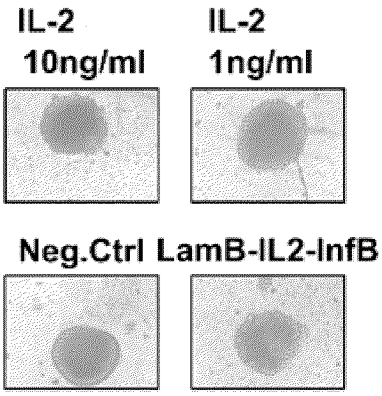
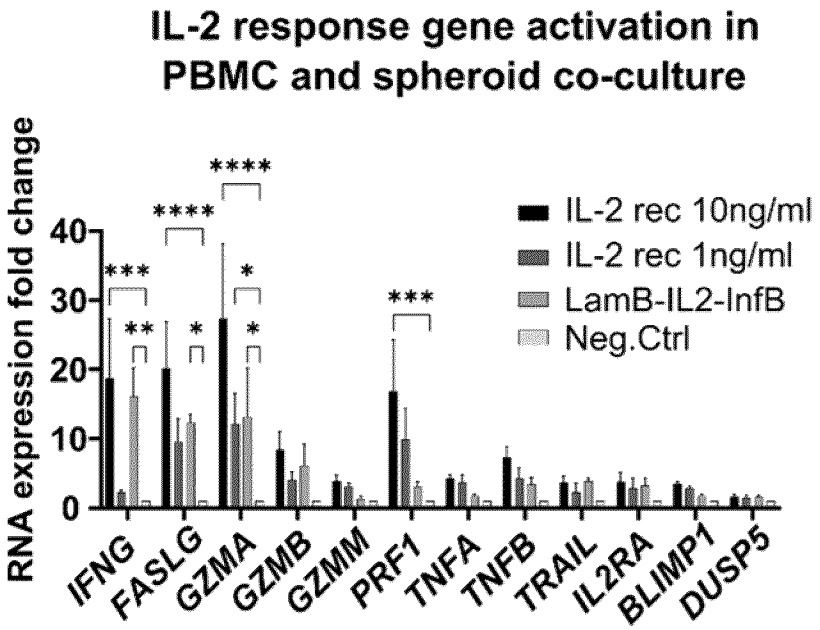

IL-2
10ng/ml
IL-2
1ng/ml
Neg.Ctrl
LamB-IL2-InfB

FIGURE 15 (continued)

C



D

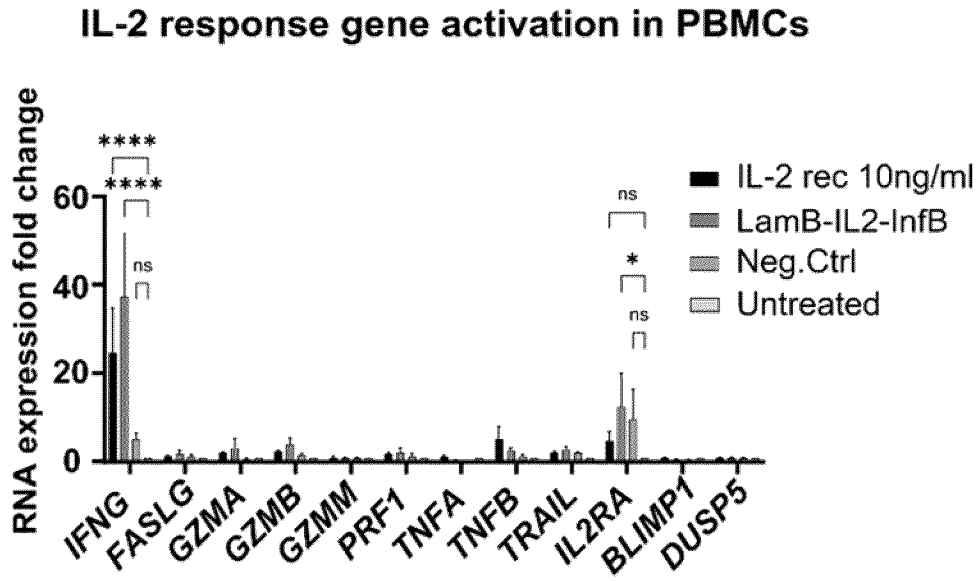
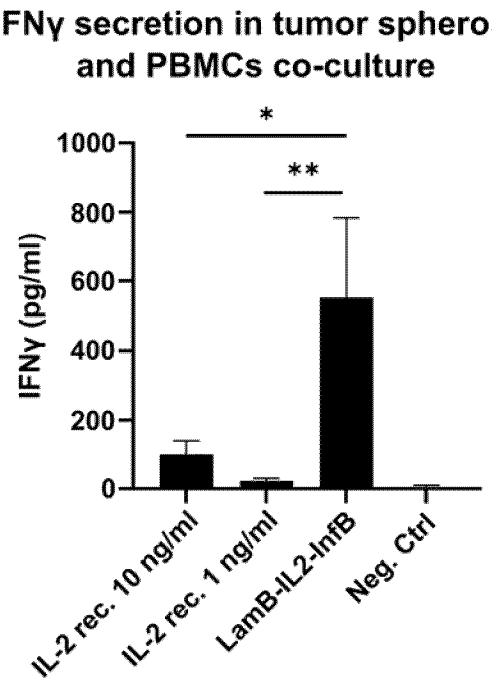


FIGURE 16

A


IFNγ secretion in tumor spheroid and PBMCs co-culture
IFNγ (pg/ml)
1000
800
600
400
200
0
*
**
IL-2 rec. 10 ng/ml
IL-2 rec. 1 ng/ml
LamB-IL2-InfB
Neg. Ctrl

B

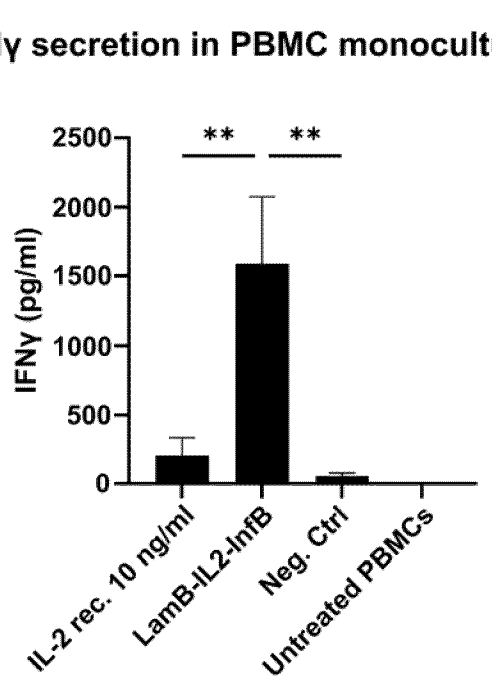

IFNγ secretion in PBMC monoculture
IFNγ (pg/ml)
2500
2000
1500
1000
500
0
**
**
IL-2 rec. 10 ng/ml
LamB-IL2-InfB
Neg. Ctrl
Untreated PBMCs

FIGURE 17

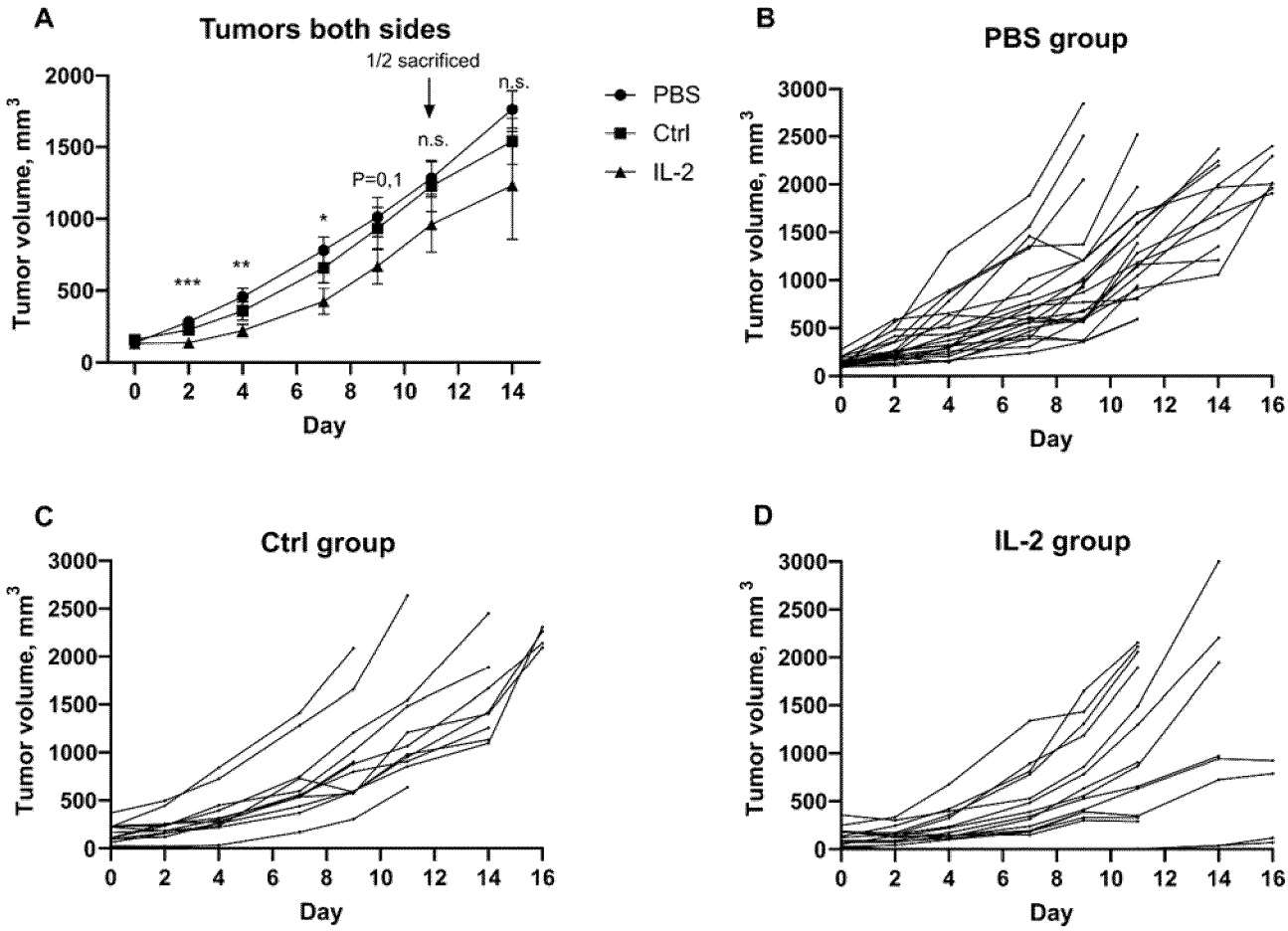


FIGURE 18

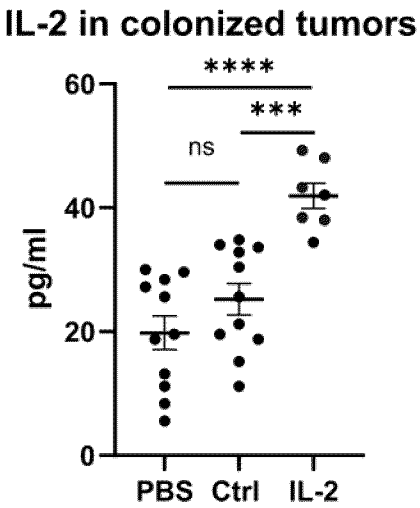
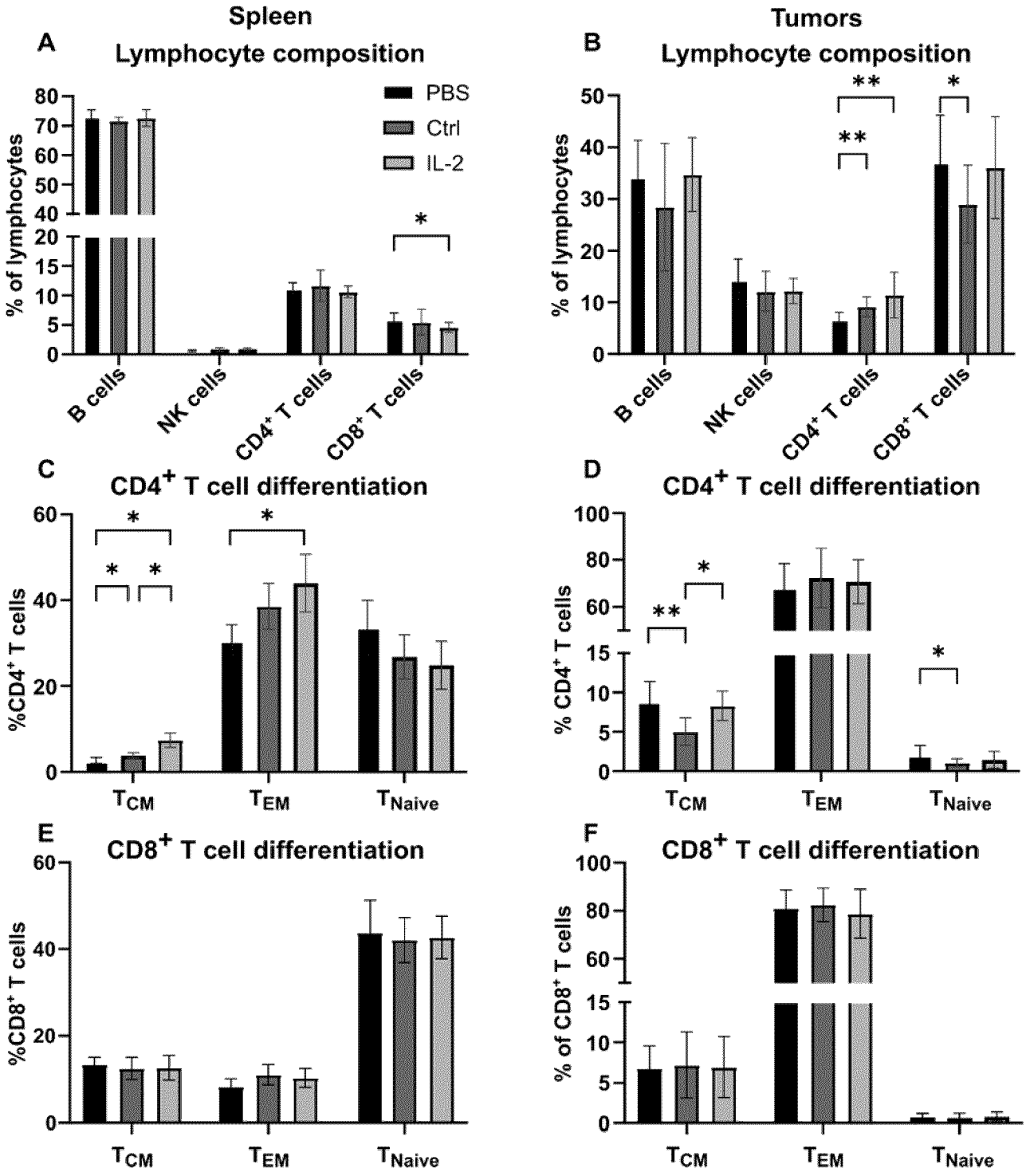


FIGURE 19


Spleen
A
Lymphocyte composition
PBS
Ctrl
IL-2
% of lymphocytes
B cells
NK cells
CD4+ T cells
CD8+ T cells
Tumors
B
Lymphocyte composition
C
CD4+ T cell differentiation
%CD4+ T cells
TCM
TEM
TNaive
D
CD4+ T cell differentiation
% CD4+ T cells
E
CD8+ T cell differentiation
%CD8+ T cells
F
CD8+ T cell differentiation
% of CD8+ T cells

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/054604

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/55 C12N15/62 A61P35/00 A61K38/20
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/336544 A1 (FALB DEAN [US] ET AL) 7 November 2019 (2019-11-07)	1,2,5-11
Y	sequences 156, 1178, 1189, 1194 paragraphs [0104], [0522] paragraph [0667] paragraph [0007] - paragraph [0010] paragraphs [0085], [0092] examples 34-37 paragraph [0222] paragraph [0572] - paragraph [0573] paragraph [0092] - paragraph [0094] tables 7, 44 paragraph [0107]	3

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 2023	06/06/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Solyga-Zurek, A

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/094288 A1 (UNIV AARHUS [DK]; MORTENSEN KIM KUSK [DK] ET AL.) 26 August 2010 (2010-08-26) figure 3 sequences 2, 7, 8 example 3 figure 11 page 2, lines 8-11 page 24, lines 1-6	3
A	WO 2021/245130 A1 (ASCENDIS PHARMA ONCOLOGY DIV A/S [DK]) 9 December 2021 (2021-12-09) page 34, line 31 - page 35, line 9 page 39, line 6 - line 11 page 39, line 20 - line 25 sequences 13, 14, 45 example 26 claims 39, 40	1-15
A	GUPTA KAJAL H. ET AL: "Bacterial-Based Cancer Therapy (BBCT): Recent Advances, Current Challenges, and Future Prospects for Cancer Immunotherapy", VACCINES , vol. 9, no. 12 18 December 2021 (2021-12-18), page 1497, XP055947402, DOI: 10.3390/vaccines9121497 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8707929/pdf/vaccines-09-01497.pdf table 2	1-15
X,P	WO 2022/043493 A1 (ASCENDIS PHARMA ONCOLOGY DIV A/S [DK]) 3 March 2022 (2022-03-03) sequence 237 page 94, line 28 - page 95, line 6 page 16, lines 5-9	1,5-11
X,P	WO 2022/072636 A1 (SYNLOGIC OPERATING CO INC [US]) 7 April 2022 (2022-04-07) example 32 sequences 144, 1129 paragraph [0131]	1,2,5-11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/054604

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/054604

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2019336544 A1	07-11-2019	AU 2018205276 A1	18-07-2019
		BR 112019013863 A2	03-03-2020
		CA 3049579 A1	12-07-2018
		CN 110913875 A	24-03-2020
		EP 3565566 A1	13-11-2019
		IL 267830 A	26-09-2019
		JP 2020505016 A	20-02-2020
		SG 11201906161V A	27-08-2019
		US 2019336544 A1	07-11-2019
		WO 2018129404 A1	12-07-2018
WO 2010094288 A1	26-08-2010	NONE	
WO 2021245130 A1	09-12-2021	AU 2021286177 A1	01-12-2022
		BR 112022022826 A2	13-12-2022
		CA 3178074 A1	09-12-2021
		CN 116133676 A	16-05-2023
		EP 4161956 A1	12-04-2023
		IL 298642 A	01-01-2023
		KR 20230019889 A	09-02-2023
		TW 202210502 A	16-03-2022
		WO 2021245130 A1	09-12-2021
WO 2022043493 A1	03-03-2022	AU 2021335032 A1	09-03-2023
		BR 112023003476 A2	11-04-2023
		CA 3189715 A1	03-03-2022
		KR 20230057447 A	28-04-2023
		TW 202219060 A	16-05-2022
		WO 2022043493 A1	03-03-2022
WO 2022072636 A1	07-04-2022	NONE	