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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 November 2023 (23.11.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/224554 A1

(51) International Patent Classification:
C12N 15/63 (2006.01) *A61P 37/00* (2006.01)
C12N 15/20 (2006.01) *A61P 1/00* (2006.01)
A61K 38/21 (2006.01)

(21) International Application Number:
PCT/SG2023/050337

(22) International Filing Date:
17 May 2023 (17.05.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10202205282W 19 May 2022 (19.05.2022) SG

(71) Applicant: **NATIONAL UNIVERSITY OF SINGAPORE** [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: **CHANG, Matthew Wook**; c/o National University of Singapore,Yong Loo Lin School of Medicine, Synthetic Biology Translational Research Programme, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **CHUA, Koon Jiew**; c/o National University of Singapore, Yong Loo Lin School of Medicine, Synthetic Biology Translational Research Programme, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **LING, Hua**; c/o National University of Singapore, Yong Loo Lin School of Medicine, Synthetic Biology Translational Research Programme, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **HWANG, In Young**; c/o National University of Singapore, Yong Loo Lin School of Medicine, Synthetic Biology Translational Research Programme, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) Agent: **DOWSING, Bruce John**; Marks & Clerk Singapore LLP, Tanjong Pagar Post Office, P.O. Box 636, Singapore 910816 (SG).

(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, MU, 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.

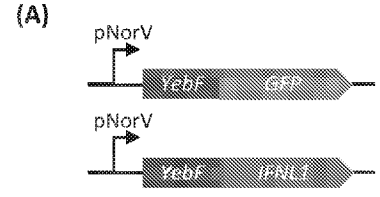
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, 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))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: AN ENGINEERED PROBIOTIC PRODUCES A TYPE III INTERFERON IFNL1 AND REDUCES INFLAMMATIONS IN IN VITRO INFLAMMATORY BOWEL DISEASE MODELS

Figure 1



(57) Abstract: The present invention relates to an expression cassette comprising a Type III interferon gene, and a nitric oxide-inducible promoter operably linked to the Type III interferon gene. In an embodiment, the Type III interferon gene encodes human interferon L1 (IFNL1). The invention also relates to a genetically engineered bacterium comprising such an expression cassette, composition comprising the bacterium, and use of the expression cassette to produce recombinant Type III interferon.

WO 2023/224554 A1

AN ENGINEERED PROBIOTIC PRODUCES A TYPE III INTERFERON IFNL1 AND REDUCES INFLAMMATION IN IN VITRO INFLAMMATORY BOWEL DISEASE MODELS

FIELD OF THE INVENTION

The present invention relates to an expression cassette comprising a Type III interferon gene, and a nitric oxide-inducible promoter operably linked to the Type III interferon gene. The invention also relates to a genetically engineered bacterium comprising such an expression cassette, a pharmaceutical composition comprising the bacterium, and its use for treatment of inflammation, such as IBD or gut inflammation.

BACKGROUND OF THE INVENTION

Inflammatory bowel diseases (IBDs) are a collective term for various pathological subtypes of intestinal epithelium inflammation. Any part of the small and large intestines can be inflamed and inflamed tissue can develop into intestinal cancer if left untreated. Healthy intestines house trillions of commensal microbes that aid in digestion and influence the development and function of the host's mucosal immune system. The intestinal epithelium functions as a physical and biochemical barrier between the host and these microbes. Epithelium inflammation can disrupt the host-microbe barrier, causing intestinal barrier dysfunction where the tight junctions of epithelial cells are disrupted. The tight junction disruptions allow uncontrolled paracellular transport of sodium, potassium and fluid, contributing to diarrhea and increasing the risk of infection to the host along with microbial translocation into the bloodstream. Ulcerative colitis (UC) and Crohn's disease (CD), the most common IBDs, are typically attributed to the imbalance of T helper 2 (Th2)-associated cytokines in UC and Th1-associated cytokines in CD [Nemeth *et al.*, 2017].

Currently available treatments for IBDs are multifaceted, aiming to control the disease by regulating the patient's immune system [Weisshof *et al.*, 2018]. The most common immunotherapy in IBDs involves designing antibodies to target pro-inflammatory cytokines implicated during IBD development, such as TNFα and IL12/23 [Hvas *et al.*, 2018]. While these antibody treatments can remit IBD initially, long-term usage of such therapies may increase the risk of opportunistic infections [Holmer *et al.*, 2019]. Supplementation with the anti-inflammatory cytokine IL-10 is an alternative to reverse the inflammatory environment. However, its use in clinical trials for CD patients has been disappointing [Buruiana *et al.*, 2010].

Type III interferons (IFN-III), including IFNL1 (IL-29) have protein sequences and structures highly similar to IL-10 [Li *et al.*, 2004]. IFN-III are associated with immunomodulatory effects in various diseases, including autoimmune diseases, viral

infections and cancer [Wack *et al.,* 2015]. For instance, IFN-III administration is reported to reduce the expression of Th2-associated cytokines such as IL-13 in mouse models of autoimmune disease. Reduced expression of IFNL1 and IFNL2 was observed in asthma [Li *et al.*, 2014] and autoimmune arthritis [Blazek *et al.,* 2015], respectively. IFN-III signals through a heterodimeric receptor complex consisting of its own unique IFNL1 receptor 1 (IFNLR1) and the shared IL-10 receptor subunit 2 (IL10R2) [Kotenko *et al.*, 2003]. *IFNLR1* displays a restricted cellular expression and is found mostly on epithelial cells, with the highest expression in the intestinal epithelial cells [Sheppard *et al.*, 2003]. The downstream signaling pathways activated by IFN-III are similar to but non-redundant to that of type I IFN (IFN-I). Previous studies using IFN-I as a therapeutic to treat IBD have demonstrated disappointing effects in both animal and clinical trials, mostly due to the unwanted effects [Rauch *et al.*, 2014].

As well as being a member of the IL-10 superfamily, the ability of IFNL1 to signal via various pathways involving IL-10 and type I IFNs, makes it a potential candidate to be investigated as a therapeutic for IBD [Eslam and George, 2016]. Various studies have demonstrated an increase of anti-inflammatory cytokines including IL-10 in the blood serum of IBD patients [Kucharzik *et al.*, 1995; Mitsuyama *et al.*, 2006]. Several immunotherapies developed to target a specific pro-inflammatory cytokine or pathway have been unsuccessful [Raad *et al.*, 2016].

There is a need for alternative methods to ameliorate or treat inflammation, in particular gut inflammation, such as IBD.

SUMMARY OF THE INVENTION

The inventors hypothesized that instead of targeting a specific cytokine or pathway, the inflammatory IBD environment can be more effectively reversed by modulating the expression of Th1- and Th2-associated cytokines. According to the invention, a probiotic strain *Escherichia coli* Nissle 1917 (EcN) was engineered to promote anti-inflammatory effects via recombinant expression and secretion of IFNL1 under the control of a nitric oxide (NO)-inducible promoter. Recombinant IFNL1 was expressed from a plasmid (EcN-IFNL1), or integrated and expressed from a chromosome (EcN-gIFNL1). NO is a biomarker for intestinal inflammation [Kolios *et al.*, 2004]. The anti-inflammatory effect of the engineered EcN strain was validated in two *in vitro* IBD models. EcN-gIFNL1 suppressed various pro-inflammatory cytokine production, enhanced anti-inflammatory cytokine production and promoted an increase in the population of regulatory T cells. Moreover, EcN-gIFNL1 rescued the integrity of the inflamed epithelial cell monolayer. The invention demonstrates a potential for developing live biotherapeutics for IBD immunotherapy.

In a first aspect, the invention provides an expression cassette comprising: i) a Type III interferon gene, and ii) a nitric oxide-inducible promoter operably linked to the Type III interferon gene.

In some embodiments, the Type III interferon gene encodes a Type III interferon having at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% amino acid sequence identity to SEQ ID NO: 2 for human interferon L1 (IFNL1).

In some embodiments, the Type III interferon gene encodes interferon L1 (IFNL1), interferon L2 (IFNL2), interferon L3 (IFNL3), or interferon L4 (IFNL4), or a functional variant thereof.

In some embodiments, IFNL1 comprises the amino acid sequence set forth in SEQ ID NO: 2, IFNL2 comprises the amino acid sequence set forth in SEQ ID NO: 3, IFNL3 comprises the amino acid sequence set forth in SEQ ID NO: 4.

In some embodiments, the Type III interferon gene encodes interferon L1 (IFNL1).

In some embodiments, the type III interferon is encoded by a gene sequence comprising a nucleic acid sequence that has at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleic acid sequence comprising the nucleic acids at and between positions 605 and 1150 of SEQ ID NO: 1.

In some embodiments, IFNL1 is encoded by a gene sequence comprising a nucleic acid sequence that has at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleic acid sequence comprising the nucleic acids at and between positions 605 and 1150 of SEQ ID NO: 1, due to redundancy of the genetic code.

In some embodiments, the nitric oxide-inducible promoter is *pNorV* from *E. coli,* preferably comprising the nucleic acid sequence comprising the nucleic acids at and between positions 1 and 203 of SEQ ID NO: 1, or a functional variant thereof.

In some embodiments, the expression cassette further comprises a bacterial secretion tag.

In some embodiments, the secretion tag comprises *YebF*.

In some embodiments, the cassette further comprises FRT sites flanking an antibiotic resistance gene; and/or the cassette comprises *pNorV-YebF-IFNL1*, preferably having the nucleotide sequence set forth in SEQ ID NO: 1.

In some embodiments, the cassette further comprises regions homologous to a genome site in a host bacterium.

In some embodiments, the regions comprise *lacI*/*lacZ* homologous regions for integration; and/or the cassette is comprised in one or more plasmid vectors.

In some embodiments, the Type III interferon gene has a polynucleotide sequence that is codon-optimised for expression in a probiotic bacterium.

In some embodiments, the probiotic bacterium is selected from the group comprising *E. coli sp., Bacteroides sp.*, *Clostridium sp., Faecalibacterium sp., Lactococcus lactis,* and *Lactocbacillus sp.*

In a second aspect, the invention provides an isolated genetically engineered bacterium comprising an expression cassette.

In some embodiments, the bacterium is selected from the group comprising *E. coli sp., Bacteroides sp.*, *Clostridium sp., Faecalibacterium sp., Lactococcus lactis,* and *Lactocbacillus sp.*

In a third aspect, the invention provides a pharmaceutical composition comprising the genetically engineered bacterium, in combination with one or more of a pharmaceutically acceptable carrier, diluent or excipient.

In some embodiments, the composition is for the treatment of inflammatory bowel disease (IBD) or gut inflammation.

In some embodiments, the gut inflammation is caused by gut viral infection.

In some embodiments, the composition is formulated for oral administration.

In a fourth aspect, the invention provides a use of an expression cassette for the recombinant production of Type III interferon that shares at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or a functional variant thereof.

In a fifth aspect, the invention provides a method of treatment comprising administering to a subject in need of such treatment an efficacious amount of a composition.

In some embodiments, the subject has IBD or gut inflammation.

In a sixth aspect, the invention provides a use of an isolated genetically engineered probiotic bacterium, or a composition for the manufacture of a medicament for the treatment or IBD or gut inflammation.

In some embodiments, the gut inflammation is caused by gut viral infection.

Details of the invention shall be described in the following sections.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a functional characterization of EcN-IFNL1 expressing and secreting IFNL1. (**A**) Schematics of pNorV-controlled expression of secretion tag YebF-fused GFP or IFNL1. (**B**) Fluorescence intensity of GFP secreted by the transformed EcN was determined with (10 mM) and without (0 mM) inducer SNP. (**C**) The amount of IFNL1 produced and secreted by the engineered EcN was determined by ELISA. Inducers were supplemented as indicated. n=3 biological repeats (**D**) Transcription levels of Treg transcription factor, Foxp3, in Jurkat T cells, were normalized to the transcription levels of housekeeping gene *GAPDH* and determined by real-time PCR. FC, fold change. (**E**) Permeability of Caco-2 epithelial layer determined by FITC-dextran assay. (**F**) Western blot showing the differential expressions of the different tight junction proteins of Caco-2 cells with the different indicated treatments for 8 h. n=6 biological repeats, *$p<0.05$.

Figure 2 shows the effect of sodium nitroprusside to cell growth and nitrite generation. (**A**) Sodium nitroprusside (SNP) at various concentrations was supplemented to EcN cells for 24h. Cell density was determined and nitrite concentration in the supernatants were obtained using the Griess method. (**B**) Amount of nitrite was determined after 8 h of SNP supplementation to culture media. n = 5.

Figure 3 shows the expression level of *iNOS* gene in inflamed Caco-2 cells co-cultured with Jurkat T cells. FC, fold change.

Figure 4 shows a multiplex assay of pro-inflammatory cytokine expression in Caco-2 cells co-cultured with Jurkat cells and treated with Mesalazine, wild-type EcN or EcN-IFNL1. (**A**) Concentrations of pro-inflammatory cytokines was confirmed in inflamed co-culture of Caco-2/Jurkat T cells. IL-4 (**B**), IL-13 (**C**), IL-33 (**D**) and IL-12 (**E**) were significantly downregulated by EcN-IFNL1. FC, fold change.

Figure 5 shows the expression level of tight junction proteins E-cadherin, Occludin, Tricellulin and Claudin-2 in inflamed and uninflamed Caco-2 cells co-cultured with wild-type

EcN, rhIFNL1 or EcN-IFNL1. Fold changes of various tight junction proteins without treatment (**A**) and with treatment, including rhINFL1, EcN, and EcN-INFL1 (**B**) by quantifying the western blot results from Fig. 1D are shown.

Figure 6 shows chromosomal expression and secretion of IFNL1 and amelioration of inflammation by EcN-gIFNL1. (**A**) Schematic of IFNL1 production-secretion cassette for genomic integration. *KanR* was used as the selection marker gene for successfully integrated clones. (**B**) The concentration of secreted IFNL1 by EcN-gIFNL1 determined by ELISA. (**C**) Induced nitric oxide synthase (iNOS) expression was confirmed in inflamed primary intestinal epithelial cells (IECs). (**D**) iNOS expression was significantly downregulated by EcN-gIFNL1.

Figure 7 shows a flow cytometric analysis of the effect of EcN-gIFNL1 on enhancing the population of CD4+CD25+Foxp3+ induced Treg (iTreg) cells. The dot plots of flow cytometry are shown.

Figure 8 shows the effect of EcN-gIFNL1 on regulatory T (Treg) cell populations. Flow cytometric analysis showed a synergistic effect of EcN-gIFNL1 on enhancing the population of $CD4^+CD25^+Foxp3^+$ induced Treg (iTreg) cells (**A**). Naïve $CD4^+$ T cells were cultured for 24 h under an inflammatory condition and treated for 10 h with the indicated treatments. (**B**) Naïve $CD4^+$ T cells were co-cultured with primary IECs under inflammatory conditions and subsequently treated for 10 h with the indicated treatments. The concentration of IL-2 in the cell supernatants was analyzed by multiplex assay. Similarly, flow cytometric analysis of the T cells in co-culture model revealed a synergistic effect of EcN-gIFNL1 on the enhancement of Treg cells. (**C**) Plots of iTregs ($CD25^+Foxp3^+$) and effector Tregs ($CD25^{hi}Foxp3^{hi}$) were boxed and labelled accordingly. Bar graphs of iTregs (**D**) and effector Tregs (**E**) were derived accordingly. n=6 biological replicates, *p<0.05, **p<0.01.

Figure 9 shows regulation of Th1-, Th2- and Th17-related pro-inflammatory cytokines by EcN-gIFNL1. Th2-related (**A i-iv**), Th1 (**B**) and Th17 (**C i-ii**) cytokines expression in primary $CD4^+$ T cells were significantly downregulated upon treatment with EcN-gIFNL1. (**D**) IL-10 expression was upregulated. n=6 biological replicates, *p<0.05, **p<0.01.

Figure 10 shows protective effects of EcN-gIFNL1 on epithelial tight junction under inflammation. (**A**) Permeability of intestinal epithelial cells (IECs) layer determined by 10 kDa FITC-dextran assay. Localization of claudin-2 (**B-i**) and E-cadherin (**B-ii**) were determined in transverse sectioning of the IECs layer, by confocal microscopy. White solid arrows indicate

the positions of nucleus, while white dotted arrows indicate the cell membrane (WGA, magenta). (**C**) Fluorescence intensity of claudin-2 (**i**) and E-cadherin (**ii**) in IECs were quantified from at least eight different fields from each sample.

Figure 11 shows that EcN-gIFNL1 influences tight junction protein localization. Localizations of (**A**) Claudin-2 (light grey in the "Claudin-2" column) and (**B**) E-cadherin (light grey in the "E-cadherin" column) were determined in transverse sectioning of the IECs layer. White solid arrows indicate the positions of nuclei, while white dotted arrows indicate the cell membrane (grey in the "WGA" column). Images before overlay are shown.

Figure 12 shows a proposed mechanism of anti-inflammation and immunomodulation by EcN-gIFNL1. EcN-gIFNL1 secretes cytokine IFNL1 in an inflamed environment. Upon binding with its receptor on epithelial cells, a cascade of downstream signaling is activated. One such event includes the induction of transcription factor Foxp3 in naïve $CD4^+$ T cells in the lamina propria, promoting the differentiation into iTregs. The iTregs in turn suppress the different T helper (Th) cells, which were signaled to the epithelial barrier upon inflammation. Our data has shown a significant suppression of pro-inflammatory cytokines expression by these Th cells. Besides immunomodulation, EcN-gIFNL1 can reduce the expression channel protein claudin-2 and restore the expression of tight junction protein E-cadherin. Upregulation (black solid arrows) and downregulation (black dotted arrows) are indicated.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

The terms "amino acid" or "amino acid sequence," as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

The phrases "nucleic acid" or "nucleic acid sequence," as used herein, refer to an oligonucleotide, nucleotide, polynucleotide, or any fragment thereof, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material.

As used herein, the term "oligonucleotide", refers to a nucleic acid sequence of at least about 6 nucleotides to 60 nucleotides, preferably about 15 to 30 nucleotides, and most preferably about 20 to 25 nucleotides, which can be used in PCR amplification or in a hybridization assay or microarray. As used herein, the term "oligonucleotide" is substantially equivalent to the terms "amplimers," "primers," "oligomers," and "probes," as these terms are commonly defined in the art.

The term "variant" as used herein, refers to an amino acid sequence that is altered by one or more amino acids, but retains the ability to function as an IFN III peptide in the present invention. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "non-conservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR® software (DNASTAR, Inc. Madison, Wisconsin, USA). Polynucleotide sequence variants include those which differ from a reference polynucleotide sequence but still encode the same protein as the reference polynucleotide, due to redundancy in the genetic code. It would be understood that to produce IFNL1 protein, a polynucleotide may encode it while having less than 100% identity with a reference sequence set forth in nucleic acids at position 605-1150 of SEQ ID NO: 1.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base, or were common general knowledge in the

field relevant to the present disclosure as it existed before the priority date of each of the appended claims.

Bibliographic references mentioned in the present specification are for convenience listed at the end of the examples. The whole content of such bibliographic references are herein incorporated by reference.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2012).

EXAMPLE 1

Materials and Methods

Plasmids, DNA constructs and oligonucleotides

All plasmids were constructed according to standard restriction cloning procedures. Codon-optimized human *IFNL1* (*IFNL1*) was synthesized by IDT (Singapore). *YebF* was PCR amplified from TOP10 genome, while promoter pNorV was PCR amplified from EcN genome, using Kapa HiFi polymerase (Kapa Biosystems). The promoter pNorV, RBS j23100, *YebF* and *IFNL1* were cloned into pUC18 vector, resulting in recombinant plasmids including pUC18-pNorV-YebF (Fig. 1A). Similarly, pUC18-pNorV-YebF-GFP was also constructed. A gene cassette comprising FRT-flanked kanamycin resistance gene (*kanR*), pNorV-YebF-IFNL1 and lacI/lacZ homologous regions was amplified using Q5 polymerase (New England Biolabs) and cloned into pUC18, resulting in gene cassettes for genomic integration (Fig. 6A).

Integration of IFNL1 expression cassette into EcN genome

The pNorV-YebF-IFNL1 cassette was knocked into the lacI/lacZ domain in EcN genome using phage λ Red recombinase as described by Datsenko et al [Datsenko and Wanner, 2000]. Briefly, an approximately 3kb fragment consisting of FRT-flanked *kanR*, pNorV-YebF-IFNL1 and lacI/lacZ homologous regions was amplified and transformed into EcN expressing λ Red recombinase [Hwang *et al.*, 2021]. Transformants were then grown on LB agar supplemented with IPTG (40 µg/mL), X-Gal (2 µg/mL), kanamycin (50 µg/mL) at 37°C and white colonies were selected, and the knock-in sequence was verified by colony

PCR. Positive colonies carrying pNorV-YebF-IFNL1 were made electro-competent and transformed with pCP20, an ampicillin resistance vector that carries a temperature-sensitive replicon and allows thermal induction of FLP synthesis. Positive transformants were selected on LB agar added with 100 μg/mL ampicillin at 30°C and purified non-selectively at 43°C, before being tested for complete loss of antibiotic resistance. The obtained colonies were confirmed by PCR using genome DNA as a template. The confirmed strain was named EcN-gIFNL1. A control strain chromosomally integrated with pNorV-YebF was constructed using the same method.

Cell lines and culture conditions

Human colon epithelial cells Caco-2 (ATCC HTB-37™) were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, ThermoFisher) supplemented with 15% fetal bovine serum (FBS, Biowest, France) and 1% penicillin/streptomycin (Sigma-Aldrich). T cells Jurkat (ATCC TIB-152™) were maintained in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37°C in a humidified 5% CO_2-containing atmosphere.

For FITC-dextran permeability assays and Western blot analysis, Caco-2 cells were seeded at a density of 7.5 x 10^4 cells per well in 24-well transwells (Millipore) 5 d before subjecting to polarization. Polarization was achieved by culturing the cells in FBS-free DMEM on the apical compartment, while FBS-supplemented DMEM was provided on the basal compartment, for at least 14 d. The medium was changed to both apical and basal sides every 2 d.

To induce inflammation, a cocktail of pro-inflammatory cytokines (interleukin 1-beta (rhIL-1β, 25 ng/ml), tumor necrosis factor alpha (rhTNF-α, 50 ng/ml), interferon gamma (rhIFN-γ, 50 ng/ml) and lipopolysaccharide (LPS, 1 μg/ml) [Zhu *et al.*, 2015]) were supplemented in low glucose (1 mg/mL) DMEM without antibiotics and FBS, and added to the apical compartment. In the basal compartment, 2x10^5 cells/mL of Jurkat T were seeded in RPMI as described above. Inflammation was induced for 24 h before 8 h treatments with 10 mM Mesalazine (Sigma-Aldrich), 100 ng/mL rhIFNL1, 10^7 cells/mL EcN-WT, EcN-YebF, EcN-IFNL1, EcN-gYebF, or EcN-gIFNL1. Caco-2 cells, Jurkat T cells, and culture supernatants were collected and analyzed. Recombinant human IFNL1 (rhIFNL1), IL-1β, TNF-α and IFN-γ were purchased from R&D Systems. LPS was purchased from Sigma-Aldrich.

Primary cells and culture conditions

Primary intestinal epithelial cells (InEpC) and myofibroblasts (InMyoFib) were maintained in Smooth Muscle Growth Basal Medium (SmBM) supplemented with insulin, hFGF-B, hEGF, 5% FBS and 0.1% gentamycin. InMyoFibs (10^5 cells/cm^2) were seeded on the basal side of each transwell one day before seeding of the InEpCs. InEpCs (10^5 cells/cm^2) cultured on the apical compartment pre-coated with 30 µg/mL rat tail type I collagen (Sigma-Aldrich). InEpC, InMyoFib, SmBM and the medium supplements were purchased from Lonza. All cells were incubated at 33°C in a humidified 5% CO_2 atmosphere.

$CD4^+$ T cells were isolated from human peripheral blood mononuclear cells (PBMCs, ATCC PCS-800-011) using EASYSEP™ Human $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies Singapore Pte Ltd), according to manufacturer's protocol. Isolated $CD4^+$ T cells were maintained in IMMUNOCULT™ XF medium supplemented with IMMUNOCULT™ Human CD3/CD28/CD2 T Cell Activator and 600 IU/mL hIL-2 (R&D Biosystems) and incubated at 37 °C in a humidified 5% CO_2 atmosphere until the cell count reached approximately 10^7 cells/mL. The cells were then cryopreserved in CRYOSTOR® CS10 preservation medium until further use. IMMUNOCULT™ XF medium, T cell activator, CRYOSTOR® CS10 were purchased from STEMCELL Technologies.

Four hours before inflammation, the medium was changed to fresh SmBM medium in the apical compartment, while 2 x 10^5 cells/mL of the isolated $CD4^+$ T cells supplemented with hIL-2 were added to the basal compartment. Inflammation was induced for 36 h as above described. Treatments with EcN-gYebF and EcN-gIFNL1 were carried out for 10 h before cells and supernatant in both compartments were collected for further analysis. All cell culture media used in co-culture studies were antibiotics free.

RNA extraction and real-time PCR

Total RNA was extracted from treated Caco-2 cells, Jurkat T cells and IECs using TRIZOL™ (Invitrogen), and 1 µg of total RNA was reverse-transcribed using qScript cDNA Supermix (Quantabio, USA). Real-time PCR was carried out using Luna Universal qPCR Master Mix (New England Biolabs) and performed on CFX Connect Real-time PCR Detection System (Biorad). All procedures were performed according to manufacturers' protocols. The primers used were:

iNOS_F: ACCTCCAGTCCAGTGACACA (SEQ ID NO: 5),

iNOS_R: AATCCCTTTGGCCTTATGGT (SEQ ID NO: 6),

Foxp3_F: AACAGCACATTCCCAGAGTTCCT (SEQ ID NO: 7),

Foxp3_R: CATTGAGTGTCCGCTGCTTCT (SEQ ID NO: 8),

GAPDH_F: GCTCTCTGCTCCTCCTGTTC (SEQ ID NO: 9),

GAPDH_R: AAATGAGCCCCAGCCTTCTC (SEQ ID NO: 10).

Protein extraction and Western Blot

Epithelial cells were carefully removed from the membranes with 100 µL protein extraction buffer (0.1% Triton-X) by repeatedly pipetting up and down. Cell suspension was centrifuged at 10,000 rpm, 4 °C for 10 min and the lysate was removed to a clean new tube. The protein concentration in each sample lysate was measured using a NanoDrop UV-Vis spectrophotometer at 280 nm. The same amount of each sample was removed, mixed with 6x loading buffer and denatured at 100 °C for 5 min before applying to a gradient (10-15%) SDS-PAGE gel. Proteins were then transferred to 0.45 µm pore size nitrocellulose membrane (GE Healthcare) before blotting for different tight junction proteins. SIGNALFIRE™ ECL reagent (Cell Signaling Technology) was used to detect the protein bands, and the bands were visualized with Amersham Imager (GE Life Sciences). Anti-E-cadherin, anti-claudin-2, anti-tricellulin and anti-beta-actin (Cell Signaling Technology) antibodies were used to detect respective proteins. All antibodies were purchased from Life Technologies unless otherwise stated.

Cryopreservation, immunofluorescence and confocal microscopy

Membrane from each transwell was removed using a scalpel and briefly washed with sterile 1xPBS. The membranes were first treated with 4% paraformaldehyde (PFA) for 10 min at 4 °C, washed thrice with 1xPBS before treatment with 30% sucrose for 15 min. The membrane was then incubated overnight at 4 °C in a 30 % sucrose solution. The membranes were then removed and incubated in 30% sucrose:OCT (1:1) for at least an hour at room temperature and finally incubated in 100% OCT for 15 min. Each membrane was then embedded in a vertical position in 100% OCT on a cold metal plate on dry ice. Embedded membranes were stored at -80 °C until sectioning. 6 µm sections were made from each membrane with CRYOSTAR™ NX50 cryostat (Thermo Fisher). The sections were placed on poly-L-lysine coated glass slides and fixed with 4% PFA for 5 min, before being washed with HBSS thrice. Labeling with ALEXA FLUOR™ 644 wheatgerm agglutinin (WGA, 5 µg/mL, ThermoFisher Scientific) was carried out for 10 min at room temperature. The slides were washed thrice with HBSS before being blocked with 0.1% BSA for 30 min at room temperature before co-incubation with anti-E-cadherin and anti-claudin-2 antibodies overnight at 4 °C. Samples were then incubated for 1 h at room temperature with FITC-labelled secondary antibodies (Cell Signaling Technology), stained with and then with

nuclear stain 4',6-diamidino-2-phenylindole (DAPI, NUCBLUE™ Fixed Cell READYPROBES™ Reagent, LifeTech) before a clean cover slip was mounted over each sample. All incubation steps were carried out on a rotator. Samples were gently washed with 0.1% TBST. Prolong Gold anti-fade mountant (LifeTech) was used to preserve the fluorescence in each sample. Confocal microscopy was carried out using Olympus FV3000 confocal microscope and images were analysed using ImageJ software.

FITC-dextran permeability assay

Supernatants from both apical and basal compartments were removed and 1 µg/mL of 10 kDa FITC-dextran (FD10) was diluted in fresh cell culture medium and applied to the apical compartment at the end of each indicated treatment. Sterile 1xPBS was supplemented to the basal compartment, and the culture was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 1 h before 100 µL of supernatant was removed from both apical and basal compartment. Fluorescence intensities were determined at an excitation wavelength of 485 nm and emission wavelength of 525 nm using a Synergy H1 microplate reader (BioTek).

Flow cytometry

Cells in the basal compartments in all co-culture setups were stained with surface markers FITC -labelled anti-CD4 (Cell Signaling Technology), APC-labelled anti-CD25 (Cell Signaling Technology), PE-Cy7-labelled anti-CD127 (eBioScience) and Treg-specific transcription factor PE-labelled anti-FoxP3 (eBioscience) antibodies for identification of Treg cells. The eBioscience Foxp3/Transcription Factor Staining Buffer Set was purchased from ThermoFisher Scientific. Single cell suspensions were resuspended at $1x10^6$ cells/mL and were firstly stained with the surface markers for at least 30 min in 4 °C. Cells were then fixed and permeabilized. Intracellular staining of Foxp3 was carried out according to manufacturer's protocol. Flow cytometry was performed with BD LSR FORTESSA™ and analyzed by FLOWJO™ Software.

ELISA and multiplex assay

ELISA was carried out for the detection of secreted IFNL1 by the engineered cells. Anti-IFNL1 capture antibodies (4 µg/mL) (ThermoFisher) were coated on 96-well MAXISORP™ plates (ThermoFisher) overnight at 4 °C. All wells were then blocked with 0.1% BSA for 1 h at room temperature. Samples or IFNL1 standards prepared using rhIFNL1 (R&D Systems) were loaded in triplicates and incubated at 4 °C for overnight. Anti-IFNL1 detection antibodies (2 µg/mL) (R&D Systems) and subsequently secondary anti-mouse antibodies (Cell Signaling Technology) were added to each well and incubated at

room temperature for 1 h in a stepwise manner. All antibodies were diluted in 0.1% BSA blocking buffer. Wells were washed with 1×PBS three times and blotted by patting the plate against a paper towel between every step. TMB substrate (Pierce) was added to each well, allowed to sit in the dark for 10 min, and the reaction was stopped with sulphuric acid. Quantitative readings of the absorbance at 450nm were carried out using Synergy H1 microplate reader. Multiplex assays of co-culture supernatants were analyzed using customized PROCARTAPLEX™ plates (Thermo Fisher Scientific). All steps were carried out according to manufacturer's protocol.

EXAMPLE 2

Production and secretion of IFNL1 by EcN-IFNL1 in response to nitric oxide

In this study, the inventors selected a probiotic bacterial strain EcN and engineered it to produce and secrete IFNL1 in a controllable manner. To this end, the inventors first constructed a genetic circuit to produce and secrete green fluorescent protein (GFP) in the presence of NO, an inflammation marker observed in the gut. Particularly, *GFP* fused with a Gram-negative bacterial secretion tag *YebF* was cloned under the control of an NO-inducible promoter pNorV in pUC18 (Fig. 1A) [Archer *et al.*, 2012]. The resulting plasmid pUC18-pNorV-YebF-GFP was introduced into EcN, resulting in strain EcN-GFP. To confirm the inducible production and secretion of GFP in EcN-GFP, the inventors inoculated EcN-GFP cells at 10^7 CFU/mL in phenol red-free DMEM medium supplemented with sodium nitroprusside (SNP), a source of NO, and evaluated cell densities, GFP induction and secretion. Figure 2 shows that the cell density of EcN-GFP with ≤ 5 mM SNP was comparable to EcN-GFP without SNP, and the density decreased by 20% (10 mM SNP) and 90% (30 mM SNP). There was relatively low growth inhibition and high nitrite (^{14} μM) produced at 8 h (Fig. 2) at 10 mM SNP. Hence, the inventors chose to use 10 mM SNP to induce IFNL1 expression for subsequent functional assays. Figure 1B shows that the fluorescence intensity of the supernatant collected from EcN-GFP+10 mM SNP was >2-fold higher than EcN-GFP without SNP, suggesting successful induction of GFP production and secretion in the presence of the inducer SNP. These results confirmed the induction of GFP expression by SNP and its secretion mediated by the YebF secretion tag.

Following the confirmation of SNP-inducible GFP production and secretion, the inventors replaced *GFP* with *IFNL1*. To determine the amount of IFNL1 secreted, ELISA was carried out. Briefly, wild-type (WT) EcN or EcN-IFNL1 were inoculated at 10^7 CFU/mL for 8 h. Approximately 17 ng/mL of IFNL1 was detected in EcN-IFNL1 cultures supplemented with 10 mM SNP, while less than 5 ng/mL of IFNL1 was detected in the culture of EcN-IFNL1 in

the absence of SNP (Fig. 1C). As expected, WT-EcN showed no observable IFNL1 expression. This result suggests successful production and secretion of IFNL1 induced by 10 mM SNP.

EXAMPLE 3

Anti-inflammatory effects of EcN-IFNL1 in Caco-2/Jurkat T cell co-culture model

The inventors then proceeded to test the functionality of EcN-IFNL1 using a co-culture model consisting of Caco-2 (apical compartment) and Jurkat T cells (basal compartment). An inflammatory cocktail was added to the co-culture 24 h prior to EcN-IFNL1 treatment. For a clearer comparison, the inventors included controls with the treatments of either EcN-WT or 10 mM Mesalazine, a first line drug for UC. The inventors first sought to confirm NO upregulation in the inflamed co-culture model by determining the expression of induced nitric oxide synthase (iNOS), an enzyme which generates NO and is an indicator of inflammation [Green *et al.,* 1994]. Figure S2 shows that *iNOS* was upregulated by 200-fold in the inflamed Caco-2 cells co-cultured with Jurkat T cells in the basal compartment. This result suggests that our inflammation model produced NO, which served as an inducer for the engineered EcN to express and secrete IFNL1.

Given the role of IFNL in the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) population [Li *et al.*, 2014; Mennechet and Uze, 2006], a question to answer was whether *Foxp3* expression is altered in the presence of the engineered EcN expressing IFNL1 (EcN-IFNL1) and pro-inflammatory cytokines. When inflammation was induced in a co-culture model consisting of Caco-2 cells and Jurkat T cells, the transcription level of *Foxp3* in Jurkat T cells was moderately decreased. When treated with rhIFNL1 protein, EcN-WT or EcN-IFNL1, the transcription level of *Foxp3* increased over 6-folds compared to the untreated group (Fig. 1D). Following the confirmation that *Foxp3* transcription was enhanced, changes in pro-inflammatory cytokines IL-4, IL-13, IL-33 and IL-12 were determined. Figure 4A shows that pro-inflammatory cytokines related to both Th1 (IL-12) and Th2 cells (IL-4, IL-13, IL-33) were increased up to 92-fold when the co-culture models were inflamed. EcN-IFNL1 treatment significantly reduced the production of these pro-inflammatory cytokines compared to the controls (no treatment, Mesalazine, EcN-WT) (Fig. 4B, C, D and E). These results confirmed the upregulation of Foxp3 expression in the presence of EcN-INFL1 and the downregulation of pro-inflammatory cytokines IL-12, IL-4, IL-13 and IL-33 with the EcN-IFNL1 treatment, suggesting inflammation amelioration.

To confirm the physiological effects on the inflamed epithelial cells with or without EcN-IFNL1 treatment, an FITC-dextran assay was performed to analyze the permeability of

the Caco-2 epithelial layer. There was an increase of FD10 concentration (FC 2.2) in the basal compartment of the inflamed and untreated model, compared to the uninflamed control (Fig. 1E). No significant reduction in the concentration of FD10 was detected in the basal compartment of co-cultures treated with either rhIFNL1 or EcN-WT, compared to the uninflamed model. EcN-IFNL1 treatment gave a significant reduction of FD10 in the basal compartment, indicating a tighter epithelial layer or less permeability (Fig. 1E). Next, Western blot was performed to determine whether EcN-IFNL1 treatment influenced tight junction protein expression. Figures 1F and 5 show that inflammation of the co-cultures reduced the expression of tight junction proteins (i.e., E-cadherin, occludin and tricellulin) while increasing the expression of claudin-2, an ion-channel protein. EcN-IFNL1 treatment increased the tight junction proteins and reduced claudin-2, consistent with the results of FD10 measurements. Therefore, the results suggest EcN-IFNL1 improves the integrity of tight junctions of the inflamed Caco-2 cells.

EXAMPLE 4

Anti-inflammatory effects of EcN-gIFNL1 in primary epithelial cells

After demonstrating the anti-inflammatory effects of EcN-IFNL1 with the plasmid-based *IFNL1* expression, the inventors sought to develop EcN as a chassis that stably delivers IFNL1. To this end, the inventors integrated the IFNL1 production-secretion cassette into EcN genome using a lambda red recombinase-based method described by Datsenko, K. A.et al,, 2000 (Fig. 6A). Particularly, the inventors chose the lacI-lacZ region which is non-essential to be the integration site, and used *KanR* as a marker gene for selecting the colonies that carried the IFNL1 production-secretion cassette. The colonies were confirmed by genomic PCR and the obtained strain was named EcN-gIFNL1 (data not shown). Next, IFNL1 production was induced by adding 10 mM SNP and measured IFNL1 concentration in the supernatant. Figure 6B shows that IFNL1 at 6 ng/mL in the presence of inducer SNP was detected in the supernatant of EcN-IFNL1, 3-fold higher than uninduced EcN-IFNL1. The results suggest that the engineered EcN carrying a genomic copy of the IFNL1 production-secretion cassette (EcN-gIFNL1) successfully produced IFNL1 upon SNP induction and secreted IFNL1 into supernatant mediated by the YebF secretion tag.

Given upregulation of *iNOS* expression in inflamed Caco-2 cells co-cultured with Jurkat T cells (Fig. 3), the inventors were interested in the effect of EcN-gIFNL1 treatment on *iNOS* expression level in the inflamed primary intestinal epithelial cells (IECs). To mimic a colorectal environment, a scaffold-based 3D co-culture model was set up, where IECs were seeded onto the membranes of transwells (“apical/epithelial”), and cultured myofibroblasts and T cells in the basal compartment (“basal/lamina propria”) followed by RT-PCR analysis

of *iNOS* expression. Figure 6C shows that the transcription level of *iNOS* was increased by 32.4-fold in the inflamed IECs over the uninflamed IECs. Figure 6D shows that the *iNOS* transcription level was reduced by 84.1% in the inflamed IECs treated with EcN-gIFNL1, significantly higher than the EcN-gYebF treatment (46.5%). The reduction of *iNOS* expression level in the inflamed primary IECs with EcN-gIFNL1 treatment suggests EcN-gIFNL1 mediated anti-inflammatory effects.

EXAMPLE 5

Effects of EcN-gIFNL1 on induced and effector Treg cell populations

Given the increase of *Foxp3* transcription in the inflamed Jurkat T cells treated with EcN-IFNL1 (Fig. 1D), the inventors hypothesized that EcN-gIFNL1 treatment could promote the differentiation of induced Treg cell (iTregs) populations under inflammation conditions. As the name suggests, iTregs arise from peripherally circulating naïve $CD4^+$ T cells upon certain stimulations, different from naturally occurring Treg cells (nTregs) that develop from progenitor cells in the bone marrow [Workman *et al.*, 2009]. The inventors induced inflammation in $CD4^+$ T cells isolated from human peripheral blood mononuclear cells (PBMCs), and treated the $CD4^+$ T cells with rhIFNL1, EcN-gYebF or EcN-gIFNL1 by applying the bacteria culture on a transwell. Next, the $CD4^+$ T cells were collected and analyzed with anti-CD4, anti-CD25, anti-CD127 and anti-Foxp3 by flow cytometry. The results (Fig. 10 and A) show that under inflammation, EcN-gIFNL1 treatment resulted in an iTregs cell population of 14.6%, which is 6% higher than with the rhIFNL1 treatment (8.6%), 2.8% higher than with the EcN-gYebF treatment (11.8%), and 3.5% higher than the negative control without inflammation. These changes are statistically significant and support our hypothesis of EcN-gIFNL1 driving the iTregs population to increase.

The population of $CD4^+CD25^+Foxp3^+$ Tregs cells were then analyzed in the scaffold-based 3D co-culturing model, where Naïve $CD4^+$ T cells were co-cultured with primary IECs under inflammation and subsequently treated with EcN-gIFNL1. IL-2 is required for the proliferation and survival of $CD4^+CD25^+Foxp3^+$ Treg cells, and low-dose exposure to IL-2 selectively promotes the expansion of $CD4^+CD25^+Foxp3^+$ Treg cells in several different clinical studies [Matsuoka *et al.*, 2013; Rosenzwajg *et al.*, 2015]. Therefore, the inventors first measured and compared IL-2 concentrations in the supernatant under inflammation and various treatments. Figure 8B shows that the IL-2 concentrations from EcN-gIFNL1 or EcN-gYebF treatment was 40% lower than following no treatment. Lower IL-2 concentration in the supernatant from EcN treatments suggests higher iTreg cell and effector Treg cell populations. Our flow cytometry results (Fig. 8C-E) show a significant increase in the population of both iTreg ($CD4^+CD25^+Foxp3^+$) and effector Treg cells ($CD4^+CD25^{hi}Foxp3^{hi}$)

from EcN-gIFNL1 treatment compared to no treatment but with inflammation. The reduction in IL-2 concentration correlates with the increase in both iTreg and effector Treg cell populations in the inflamed co-cultures with EcN-gIFNL1 treatment, where low dose IL-2 was observed to enhance and maintain Treg populations. Effector Tregs are further differentiated from iTregs depending on the environmental cues and produce a high amount of immunosuppressive molecules such as IL-10 [Zhou *et al.*, 2019; Zheng *et al.*, 2007]. Furthermore, these observations also correlate with previous reports that Foxp3 negatively regulates the expression of IL-2 [Hench and Su, 2011].

EXAMPLE 6

EcN-gIFNL1 suppresses the expression of pro-inflammatory cytokines

Following the confirmation of upregulation of $CD4^+CD25^+Foxp3^+$ Tregs (Fig. 8) and the reduction of pro-inflammatory cytokines (Fig. 10), the inventors sought to confirm the concentrations of the various cytokines in the scaffold-based 3D co-culture model. EcN-gIFNL1 treatment significantly reduced the concentration of the Th2 pro-inflammatory cytokines IL-4, IL-5, IL-13, and IL-33, among which IL-33 was reduced to 33% compared to the control without EcN-gIFNL1 treatment (Table 1). IL-33 has been recently discovered as a pro-inflammatory cytokine highly produced by epithelial cells and implicated in the pathology of UC via the IL-4 pathway *in vivo* [Pushparaj *et al.*, 2013]. Furthermore, IL-33 was found to drive the differentiation of naïve helper T cells to Th2 cells, as well as type II innate lymphoid cells (ILCs), and is also implicated in the development of UC [Seidelin *et al.*, 2015]. The inventors note that IL-33 was upregulated by 3-fold in the inflamed co-culture model without treatment and upon EcN-gIFNL1 treatment, it was significantly reduced to a level similar to the uninflamed control. The inventors did not observe significant reduction of IL-33 upon EcN-gYebF treatment (Table 1).

Table 1. Concentration of cytokines (pg/mL) from scaffold-based 3D co-culture model

Cytokines	Uninflamed	Inflamed		
		Untreated	EcN-gYebF	EcN-gIFNL1
IL-4	281.86 ± 35.49	310.46 ± 35.95	203.07 ± 14.45	224.85 ± 27.02
IL-5	353.42 ± 13.45	434.49 ± 30.11	387.84 ± 74.01	286.115 ± 6.78
IL-10	7.26 ± 0.38	13.26 ± 3.11	26.907 ± 1.96	17.985 ± 1.35
IL-12p70	0.11 ± 0.01	0.415 ± 0.06	0.145 ± 0.05	0.03 ± 0.01
IL-13	1580.115 ± 93.5	1924.1 ± 60.8	1484.31 ± 174.63	1151.175 ± 135.12
IL-17AF	1977.015 ± 39.72	2028.9725 ± 174.1	2271.235 ± 231.33	1778.1925 ± 92.68
IL-22	1.025 ± 0.46	3.25 ± 0.67	1.875 ± 0.32	0.9275 ± 0.29
IL-33	6892 ± 767.79	15961.53 ± 2293.93	12895.035 ± 2042.75	4966.03 ± 1503.01

Although IFNL1 was previously found to modulate Th1/Th2 cytokine expression by promoting Th1 cytokine production and suppressing Th2 cytokine expression [Jordan *et al.*, 2007; Dai *et al.,* 2009], the inventors have observed a reduction in Th1 cytokine IL-12p70 upon EcN-gIFNL1 treatment (Fig. 9B). The upregulation of IL-12p70 in the inflammation and untreated model is likely due to the presence of IFNγ while inducing inflammation. Although IL-12 is often implicated in the pathology of CD, it was also found to be upregulated in the serum and intestinal samples of UC patients [Lee *et al.*, 2016]. In addition to the downregulation of Th1 and Th2 pro-inflammatory cytokines, the inventors also observed significant downregulation of Th17-related cytokines (IL-17AF and IL-22) upon EcN-gIFNL1 treatment (Fig. 9C). Treg and Th17 cells develop from naïve $CD4^+$ T cells and can be induced by the same cytokine, TGFβ, although the environment determines which subtypes naïve $CD4^+$ T cells develop into [Hatton and Weaver, 2009]. Th17 cells are heavily implicated in various autoimmune diseases, and Treg cells are often dysregulated in autoimmune diseases [Noack and Miossec, 2014]. IL-17AF and IL-22 are the two most common IL-17 cytokines, which are highly produced by Th17 cells and pro-inflammatory. While the IL-17AF level did not increase under inflammation conditions, EcN-gIFNL1 treatment caused a slight but significant decrease of IL-17AF. IL-22 is a pleiotropic cytokine that can be either pro-inflammatory or anti-inflammatory, depending on environment [Rutz *et al.,* 2013; Wei *et al.*, 2020]. The inventors observed a significant increase in the levels of IL-22 in the inflamed co-culture model. When the inflamed model was treated with EcN-gIFNL1, the IL-22 level was significantly reduced and comparable to the uninflamed control cells. This result suggests a positive role of EcN-gIFNL1 in anti-inflammation partially through reducing IL-22 levels, which has been observed at a higher concentration in both murine colitis models and clinical IBD samples, compared to healthy controls [Li *et al.*, 2014].

Furthermore, the inventors observed a slight increase in the IL-10 level in inflamed models treated with EcN-gIFNL1 (Fig. 9D) despite significant increases of iTreg and effector Treg populations (Fig. 8). The slight increase of IL-10 is likely attributed to the possible reduction of IL-10-producing T-helper cells [Moore *et al.*, 2001], or IL-10 production in Treg cells in the presence of IFNL1. Nevertheless, our results indicate that the EcN-gIFNL1 reduced pro-inflammatory cytokine production and suppressed expression of Th2, Th1, Th17 pro-inflammatory cytokines. Taken together, these results support the activity of the EcN-gIFNL1 towards ameliorating inflammation that drives IBD development.

EXAMPLE 7

Protective effect of EcN-gIFNL1 on the colorectal epithelial layer

Given the above anti-inflammatory effects of EcN-gIFNL1, the inventors hypothesized that EcN-gIFNL1 treatment might promote the integrity of the inflamed epithelial cell layer. Permeability assays were performed to confirm the effects of the EcN-IFNL1 on inflamed epithelial cells. Figure 10A shows that the EcN-gIFNL1 treatment cultures had 41% less FD10 in the basal compartment than the inflamed control without EcN treatment or with EcN-gYebF treatment, suggesting lower permeability of the inflamed epithelial cell layer and improved tight junctions with EcN-gIFNL1 treatment. The FITC-Dextran concentration with inflammation and EcN-gIFNL1 treatment was comparable to the uninflamed control. EcN-gYebF treatment did not improve epithelial layer integrity, similar to the EcN-WT treatment (Fig. 1E), which confirms that the protective effect on the inflamed epithelial cell tight junctions was attributed to EcN-gIFNL1 rather than EcN-WT.

To further confirm the protective effects of EcN-gIFNL1 on the inflamed epithelial tight junctions, the localization and expression of two representative tight junction proteins, claudin-2 and E-cadherin in IECs were examined by confocal microscopy. Figure 10B shows that more claudin-2 (white dotted) localized to the cellular membrane (white solid arrows) in the inflamed IECs compared to the uninflamed control without EcN treatment. The inventors also observed that more claudin-2 localized to the nucleus (white arrows) in the inflamed IECs with EcN-gIFNL1 treatment compared to the controls without or with EcN-gYebF treatment. Figure 10B also shows that more E-cadherin (white) was localized to the cellular membrane in the inflamed cells with EcN-gIFNL1 treatment than the controls without or with EcN-gYebF treatment (Fig. 11). The fluorescence intensity of claudin-2 with EcN-gIFNL1 treatment was 40% lower than the inflamed control, suggesting downregulation of claudin-2 production by EcN-gIFNL1 treatment (Fig. 10C-i). The fluorescence intensity of E-cadherin with EcN-gIFNL1 treatment was slightly higher than the inflamed control, suggesting a rescue of E-cadherin expression by EcN-gIFNL1 treatment (Fig. 10C-ii). Upregulation of claudin-2 expression may lead to increased paracellular transport of water and cations [Heller *et al.*, 2005]. Furthermore, claudin-2 expression is dependent on the presence of other pro-inflammatory cytokines such as IL-13, IL-17, IL-22 and TNF-α and thus, claudin-2 upregulation is heavily implicated in various diseases of the small and large intestines [Luettig *et al.*, 2015; Wang *et al.*, 2017]. In contrast, downregulation or complete loss of E-cadherin is a requirement of metagenesis, which can destabilize epithelial monolayers [Smyth *et al.*, 2012] or cause cell deaths [Schneider *et al.*, 2010; Grill *et al.*, 2015]. In the inflamed IECs with EcN-gIFNL1 treatment, the reduced expression of claudin-2 (Fig. 10C-i) and increased expression of E-cadherin (Fig. 10C-ii) may contribute to maintaining the proper localization of other tight junction proteins including E-cadherin (Fig. 10B-ii) and consequently a lower permeability (Fig. 10A). These results are consistent to the

upregulation of tight junction proteins (E-cadherin, occludin and tricellulin) in inflamed Caco-2 cells with EcN-IFNL1 treatment (Figs. 1F and 5), which contributed to protecting epithelial barrier integrity. Overall, the results support our hypothesis that EcN expressing IFNL1 protect epithelial tight junctions through modulating localization and expression of tight junction proteins.

EXAMPLE 8

Protective effect of EcN-gIFNL1 on the colorectal epithelial layer in vivo

Briefly, lab animals are given dextran sulfate sodium (DSS) in drinking water, as a way to induce colitis (Chassaing et al, 2014; Taghipour et al, 2016). The exact period and dosage of DSS-treatment needs to be confirmed, as colitogenic potential of DSS is known to exist between vendors and manufacturing batches. Also, acute and chronic DSS-treatment are quite different. Briefly, an initial concentration between 1.5-3.0% w/v DSS in autoclaved water is given to C57BL/6 mice for up to 7 days. The animals are weighed and monitored daily. Animals with weight loss of >25% are euthanized as guidelines. The concentration of DSS and length of administration is determined by 100% survival rate and clinical assessment of inflammation such as body weight loss and stool consistency. Acute colitis should be established after one cycle of DSS treatment, while chronic colitis development should occur by subjecting the animals to repeated cycles of DSS followed by drinking water without DSS. After colitis is confirmed in the animals, they are given the engineered cells as a treatment. Again, the period of engineered probiotics treatment needs to be confirmed. The animals are then sacrificed to study the pathological changes with or without the engineered probiotics treatment. Organs are cryopreserved for subsequent disease scoring and fluorescence microscopy studies. The blood is collected and subjected to multiplex assays as well as flow cytometry of immune cells population studies.

Summary

In this study, the inventors engineered EcN strains that express and secrete IFNL1 upon NO induction. In an active inflammatory environment, treatment with the engineered strain EcN-gIFNL1 reduced pro-inflammatory cytokines (related to Th1, Th2 and Th17 cells), increased anti-inflammatory cytokine IL-10 as well as iTreg and effector Treg cell populations. It also improved the expression of tight junction proteins (e.g. E-cadherin, occludin, tricellulin), reduced claudin-2 expression, maintained tight junction protein localization and thereby ameliorated intestinal permeability dysfunction in the inflamed epithelial layer in *in vitro* cell line and 3D scaffold co-culture models. Figure 12 is a schematic of the inventors proposed mode of action of EcN-gIFNL1 in ameliorating

inflammation in the IBD models (Fig. 12). To the best of the inventors' knowledge, this work represents the first study on engineering probiotic bacteria to express and secrete IFNL1 in a controllable manner and demonstrating its anti-inflammatory effects in the *in vitro* IBD models. Our study shows a potential to provide a new route for delivering IFNL1 as a therapeutic agent in a probiotic chassis, an alternative to *IFNL1* gene transfer via recombinant adenovirus [Li *et al.*, 2014; Hasegawa *et al.*, 2016]. As a GRAS substance [Reister *et al.*, 2014], the development of EcN as a chassis to deliver IFNL1 to the inflamed colorectal epithelium is more applicable than delivery by other means, including adenovirus delivery which is restricted to gene transfer and unstable.

References

Archer, E. J.; Robinson, A. B.; Suel, G. M., Engineered E. coli that detect and respond to gut inflammation through nitric oxide sensing. *ACS Synth Biol* **2012,** *1* (10), 451-7. DOI: 10.1021/sb3000595.

Blazek, K.; Eames, H. L.; Weiss, M.; Byrne, A. J.; Perocheau, D.; Pease, J. E.; Doyle, S.; McCann, F.; Williams, R. O.; Udalova, I. A., IFN-lambda resolves inflammation via suppression of neutrophil infiltration and IL-1beta production. *J Exp Med* **2015,** *212* (6), 845-53. DOI: 10.1084/jem.20140995.

Buruiana, F. E.; Solà, I.; Alonso-Coello, P., Recombinant human interleukin 10 for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* **2010,** (11), Cd005109. DOI: 10.1002/14651858.CD005109.pub3.

Commins, S.; Steinke, J. W.; Borish, L., The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol* **2008,** *121* (5), 1108-11. DOI: 10.1016/j.jaci.2008.02.026.

Dai, J.; Megjugorac, N. J.; Gallagher, G. E.; Yu, R. Y.; Gallagher, G., IFN-lambda1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. *Blood* **2009,** *113* (23), 5829-38. DOI: 10.1182/blood-2008-09-179507.

Datsenko, K. A.; Wanner, B. L., One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **2000,** *97* (12), 6640-5. DOI: 10.1073/pnas.120163297.

Eslam, M.; George, J., Targeting IFN-lambda: therapeutic implications. *Expert Opin Ther Targets* **2016,** *20* (12), 1425-1432. DOI: 10.1080/14728222.2016.1241242.

Green, S. J.; Scheller, L. F.; Marletta, M. A.; Seguin, M. C.; Klotz, F. W.; Slayter, M.; Nelson, B. J.; Nacy, C. A., Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunol Lett* **1994,** *43* (1-2), 87-94. DOI: 10.1016/0165-2478(94)00158-8.

Grill, J. I.; Neumann, J.; Hiltwein, F.; Kolligs, F. T.; Schneider, M. R., Intestinal E-cadherin Deficiency Aggravates Dextran Sodium Sulfate-Induced Colitis. *Dig Dis Sci* **2015,** *60* (4), 895-902. DOI: 10.1007/s10620-015-3551-x.

Hasegawa, K.; Tagawa, M.; Takagi, K.; Tsukamoto, H.; Tomioka, Y.; Suzuki, T.; Nishioka, Y.; Ohrui, T.; Numasaki, M., Anti-tumor immunity elicited by direct intratumoral administration of a recombinant adenovirus expressing either IL-28A/IFN-lambda2 or IL-29/IFN-lambda1. *Cancer Gene Ther* **2016,** *23* (8), 266-77. DOI: 10.1038/cgt.2016.29.

Hatton, R. D.; Weaver, C. T., Duality in the Th17-Treg developmental decision. *F1000 Biol Rep* **2009,** *1*, 5. DOI: 10.3410/b1-5.

Heller, F.; Florian, P.; Bojarski, C.; Richter, J.; Christ, M.; Hillenbrand, B.; Mankertz, J.; Gitter, A. H.; Burgel, N.; Fromm, M.; Zeitz, M.; Fuss, I.; Strober, W.; Schulzke, J. D., Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* **2005,** *129* (2), 550-64. DOI: 10.1016/j.gastro.2005.05.002.

Hench, V. K.; Su, L., Regulation of IL-2 gene expression by Siva and FOXP3 in human T cells. *BMC Immunol* **2011,** *12*, 54. DOI: 10.1186/1471-2172-12-54.

Holmer, A.; Singh, S., Overall and comparative safety of biologic and immunosuppressive therapy in inflammatory bowel diseases. *Expert Rev Clin Immunol* **2019,** *15* (9), 969-979. DOI: 10.1080/1744666x.2019.1646127.

Hvas, C. L.; Bendix, M.; Dige, A.; Dahlerup, J. F.; Agnholt, J., Current, experimental, and future treatments in inflammatory bowel disease: a clinical review. *Immunopharmacol Immunotoxicol* **2018,** *40* (6), 446-460. DOI: 10.1080/08923973.2018.1469144.

Hwang, I. Y.; Kim, H. R.; De Sotto, R.; Chang, M. W., Engineered probiotics modulate the endocannabinoid system. *Biotechnology Notes* **2021,** *2*, 33-38.

Jordan, W. J.; Eskdale, J.; Srinivas, S.; Pekarek, V.; Kelner, D.; Rodia, M.; Gallagher, G., Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. *Genes Immun* **2007,** *8* (3), 254-61. DOI: 10.1038/sj.gene.6364382.

Kolios, G.; Valatas, V.; Ward, S. G., Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* **2004,** *113* (4), 427-37. DOI: 10.1111/j.1365-2567.2004.01984.x.

Kotenko, S. V.; Gallagher, G.; Baurin, V. V.; Lewis-Antes, A.; Shen, M.; Shah, N. K.; Langer, J. A.; Sheikh, F.; Dickensheets, H.; Donnelly, R. P., IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* **2003,** *4* (1), 69-77. DOI: 10.1038/ni875.

Kucharzik, T.; Stoll, R.; Lügering, N.; Domschke, W., Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol* **1995,** *100* (3), 452-6. DOI: 10.1111/j.1365-2249.1995.tb03721.x.

Lee, H. W.; Chung, S. H.; Moon, C. M.; Che, X.; Kim, S. W.; Park, S. J.; Hong, S. P.; Kim, T. I.; Kim, W. H.; Cheon, J. H., The Correlation of Serum IL-12B Expression With Disease Activity in Patients With Inflammatory Bowel Disease. *Medicine* **2016,** *95* (23), e3772. DOI: 10.1097/md.0000000000003772.

Li, L. J.; Gong, C.; Zhao, M. H.; Feng, B. S., Role of interleukin-22 in inflammatory bowel disease. *World J Gastroenterol* **2014,** *20* (48), 18177-88. DOI: 10.3748/wjg.v20.i48.18177.

M. Li; He, S., IL-10 and its related cytokines for treatment of inflammatory bowel disease. *World J Gastroenterol* **2004,** *10* (5), 6. DOI: 10.3748/wjg.v10.i5.620.

Li, Y.; Gao, Q.; Yuan, X.; Zhou, M.; Peng, X.; Liu, X.; Zheng, X.; Xu, D.; Li, M., Adenovirus expressing IFN-lambda1 (IL-29) attenuates allergic airway inflammation and airway hyperreactivity in experimental asthma. *Int Immunopharmacol* **2014,** *21* (1), 156-62. DOI: 10.1016/j.intimp.2014.04.022.

Luettig, J.; Rosenthal, R.; Barmeyer, C.; Schulzke, J. D., Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. *Tissue Barriers* **2015,** *3* (1-2), e977176. DOI: 10.4161/21688370.2014.977176.

Matsuoka, K.; Koreth, J.; Kim, H. T.; Bascug, G.; McDonough, S.; Kawano, Y.; Murase, K.; Cutler, C.; Ho, V. T.; Alyea, E. P.; Armand, P.; Blazar, B. R.; Antin, J. H.; Soiffer, R. J.; Ritz, J., Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med* **2013,** *5* (179), 179ra43. DOI: 10.1126/scitranslmed.3005265.

Mennechet, F. J.; Uzé, G., Interferon-lambda-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells. *Blood* **2006,** *107* (11), 4417-23. DOI: 10.1182/blood-2005-10-4129.

Mitsuyama, K.; Tomiyasu, N.; Takaki, K.; Masuda, J.; Yamasaki, H.; Kuwaki, K.; Takeda, T.; Kitazaki, S.; Tsuruta, O.; Sata, M., Interleukin-10 in the pathophysiology of inflammatory bowel disease: increased serum concentrations during the recovery phase. *Mediators Inflamm* **2006,** *2006* (6), 26875. DOI: 10.1155/mi/2006/26875.

Moore, K. W.; de Waal Malefyt, R.; Coffman, R. L.; O'Garra, A., Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **2001,** *19*, 683-765. DOI: 10.1146/annurev.immunol.19.1.683.

Nemeth, Z. H.; Bogdanovski, D. A.; Barratt-Stopper, P.; Paglinco, S. R.; Antonioli, L.; Rolandelli, R. H., Crohn's Disease and Ulcerative Colitis Show Unique Cytokine Profiles. *Cureus* **2017,** *9* (4), e1177. DOI: 10.7759/cureus.1177.

Noack, M.; Miossec, P., Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* **2014,** *13* (6), 668-77. DOI: 10.1016/j.autrev.2013.12.004.

Pushparaj, P. N.; Li, D.; Komai-Koma, M.; Guabiraba, R.; Alexander, J.; McSharry, C.; Xu, D., Interleukin-33 exacerbates acute colitis via interleukin-4 in mice. *Immunology* **2013,** *140* (1), 70-7. DOI: 10.1111/imm.12111.

Raad, M. A.; Chams, N. H.; Sharara, A. I., New and Evolving Immunotherapy in Inflammatory Bowel Disease. *Inflamm Intest Dis* **2016,** *1* (2), 85-95. DOI: 10.1159/000445986.

Rauch, I.; Hainzl, E.; Rosebrock, F.; Heider, S.; Schwab, C.; Berry, D.; Stoiber, D.; Wagner, M.; Schleper, C.; Loy, A.; Urich, T.; Müller, M.; Strobl, B.; Kenner, L.; Decker, T., Type I interferons have opposing effects during the emergence and recovery phases of colitis. *Eur J Immunol* **2014,** *44* (9), 2749-60. DOI: 10.1002/eji.201344401.

Rosenzwajg, M.; Churlaud, G.; Mallone, R.; Six, A.; Dérian, N.; Chaara, W.; Lorenzon, R.; Long, S. A.; Buckner, J. H.; Afonso, G.; Pham, H. P.; Hartemann, A.; Yu, A.; Pugliese, A.; Malek, T. R.; Klatzmann, D., Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J Autoimmun* **2015,** *58*, 48-58. DOI: 10.1016/j.jaut.2015.01.001.

Rutz, S.; Eidenschenk, C.; Ouyang, W., IL-22, not simply a Th17 cytokine. *Immunol Rev* **2013,** *252* (1), 116-32. DOI: 10.1111/imr.12027.

Schneider, M. R.; Dahlhoff, M.; Horst, D.; Hirschi, B.; Trülzsch, K.; Müller-Höcker, J.; ogelmann, R.; Allgäuer, M.; Gerhard, M.; Steininger, S.; Wolf, E.; Kolligs, F. T., A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. *PLoS One* **2010,** *5* (12), e14325. DOI: 10.1371/journal.pone.0014325.

Seidelin, J. B.; Coskun, M.; Kvist, P. H.; Holm, T. L.; Holgersen, K.; Nielsen, O. H., IL-33 promotes GATA-3 polarization of gut-derived T cells in experimental and ulcerative colitis. *J Gastroenterol* **2015,** *50* (2), 180-90. DOI: 10.1007/s00535-014-0982-7.

Sheppard, P.; Kindsvogel, W.; Xu, W.; Henderson, K.; Schlutsmeyer, S.; Whitmore, T. E.; Kuestner, R.; Garrigues, U.; Birks, C.; Roraback, J.; Ostrander, C.; Dong, D.; Shin, J.; Presnell, S.; Fox, B.; Haldeman, B.; Cooper, E.; Taft, D.; Gilbert, T.; Grant, F. J.; Tackett, M.; Krivan, W.; McKnight, G.; Clegg, C.; Foster, D.; Klucher, K. M., IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* **2003,** *4* (1), 63-8. DOI: 10.1038/ni873.

Wack, A.; Terczynska-Dyla, E.; Hartmann, R., Guarding the frontiers: the biology of type III interferons. *Nat Immunol* **2015,** *16* (8), 802-9. DOI: 10.1038/ni.3212.

Wang, Y.; Mumm, J. B.; Herbst, R.; Kolbeck, R.; Wang, Y., IL-22 Increases Permeability of Intestinal Epithelial Tight Junctions by Enhancing Claudin-2 Expression. *J Immunol* **2017,** *199* (9), 3316-3325. DOI: 10.4049/jimmunol.1700152.

Wei, H. X.; Wang, B.; Li, B., IL-10 and IL-22 in Mucosal Immunity: Driving Protection and Pathology. *Front Immunol* **2020,** *11*, 1315. DOI: 10.3389/fimmu.2020.01315.

Weisshof, R.; El Jurdi, K.; Zmeter, N.; Rubin, D. T., Emerging Therapies for Inflammatory Bowel Disease. *Adv Ther* **2018,** *35* (11), 1746-1762. DOI: 10.1007/s12325-018-0795-9.

Workman, C. J.; Szymczak-Workman, A. L.; Collison, L. W.; Pillai, M. R.; Vignali, D. A., The development and function of regulatory T cells. *Cell Mol Life Sci* **2009,** *66* (16), 2603-22. DOI: 10.1007/s00018-009-0026-2.

Zheng, S. G.; Wang, J.; Wang, P.; Gray, J. D.; Horwitz, D. A., IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J Immunol* **2007,** *178* (4), 2018-27. DOI: 10.4049/jimmunol.178.4.2018.

Zhou, L.; Chu, C.; Teng, F.; Bessman, N. J.; Goc, J.; Santosa, E. K.; Putzel, G. G.; Kabata, H.; Kelsen, J. R.; Baldassano, R. N.; Shah, M. A.; Sockolow, R. E.; Vivier, E.; Eberl, G.; Smith, K. A.; Sonnenberg, G. F., Innate lymphoid cells support regulatory T cells in the intestine through interleukin-2. *Nature* **2019,** *568* (7752), 405-409. DOI: 10.1038/s41586-019-1082-x.

Zhu, H.; Wan, X.; Li, J.; Han, L.; Bo, X.; Chen, W.; Lu, C.; Shen, Z.; Xu, C.; Chen, L.; Yu, C.; Xu, G., Computational Prediction and Validation of BAHD1 as a Novel Molecule for Ulcerative Colitis. *Sci Rep* **2015,** *5*, 12227. DOI: 10.1038/srep12227.

Claims

1. An expression cassette comprising;

i. a Type III interferon gene, and

ii. a nitric oxide-inducible promoter operably linked to the Type III interferon gene.

2. The expression cassette according to claim 1, wherein the Type III interferon gene encodes a Type III interferon having at least 70% amino acid sequence identity to the sequence set forth in SEQ ID NO: 2 for human interferon L1 (IFNL1).

3. The expression cassette according to claim 1 or 2, wherein the Type III interferon gene encodes interferon L1 (IFNL1), interferon L2 (IFNL2), interferon L3 (IFNL3), or interferon L4 (IFNL4), or a functional variant thereof.

4. The expression cassette according to claim 2 or 3, wherein IFNL1 comprises the amino acid sequence set forth in SEQ ID NO: 2, IFNL2 comprises the amino acid sequence set forth in SEQ ID NO: 3, IFNL3 comprises the amino acid sequence set forth in SEQ ID NO: 4.

5. The expression cassette according to any one of claims 1 to 4, wherein the type III interferon is encoded by a gene sequence comprising a nucleic acid sequence that has at least 80% sequence identity to the nucleic acid sequence comprising the nucleic acids at and between positions 605 and 1150 of SEQ ID NO: 1.

6. The expression cassette according to any one of claims 1 to 5, wherein the nitric oxide-inducible promoter is *pNorV* from *E. coli,* preferably comprising the nucleic acid sequence comprising the nucleic acids at and between positions 1 and 203 of SEQ ID NO: 1, or a functional variant thereof.

7. The expression cassette according to any one of the preceding claims, further comprising a bacterial secretion tag.

8. The expression cassette according to claim 7, wherein the secretion tag comprises *YebF*.

9. The expression cassette according to any one of claims 1 to 8, wherein the cassette further comprises FRT sites flanking an antibiotic resistance gene; and/or

wherein the cassette comprises *pNorV-YebF-IFNL1*, preferably having the nucleotide sequence set forth in SEQ ID NO: 1.

10. The expression cassette according to any one of claims 1 to 9, wherein the cassette further comprises regions homologous to a genome site in a host bacterium.

11. The expression cassette according to claim 10, wherein the regions comprise *lacI*/*lacZ* homologous regions for integration; and/or

wherein the cassette is comprised in one or more plasmid vectors.

12. The expression cassette according to any one of claims 2 to 11, wherein the Type III interferon gene has a polynucleotide sequence that is codon-optimised for expression in a probiotic bacterium.

13. The expression cassette according to claim 12, wherein the probiotic bacterium is selected from the group comprising *E. coli sp., Bacteroides sp.*, *Clostridium sp., Faecalibacterium sp., Lactococcus lactis,* and *Lactocbacillus sp*.

14. An isolated genetically engineered bacterium comprising an expression cassette of any one of claims 1 to 13.

15. The isolated genetically engineered bacterium of claim 14, wherein the bacterium is selected from the group comprising *E. coli sp., Bacteroides sp.*, *Clostridium sp., Faecalibacterium sp., Lactococcus lactis,* and *Lactocbacillus sp*.

16. A pharmaceutical composition comprising the genetically engineered bacterium of claim 14 or 15, in combination with one or more of a pharmaceutically acceptable carrier, diluent or excipient.

17. The composition of claim 16 for the treatment of inflammatory bowel disease (IBD) or gut inflammation.

18. The composition of claim 17, wherein the gut inflammation is caused by gut viral infection.

19. The composition of any one of claims 16 to 18, wherein the composition is formulated for oral administration.

20. Use of an expression cassette according to any one of claims 1 to 13 for the recombinant production of Type III interferon that shares at least 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or a functional variant thereof.

21. A method of treatment comprising administering to a subject in need of such treatment an efficacious amount of a composition defined in any one of claims 16 to 19.

22. The method of claim 21, wherein the subject has IBD or gut inflammation.

23. Use of an isolated genetically engineered probiotic bacterium of claim 14 or 15, or a composition of any one of claims 16 to 19 for the manufacture of a medicament for the treatment or IBD or gut inflammation.

24. The use according to claim 23, wherein the gut inflammation is caused by gut viral infection.

Figure 1

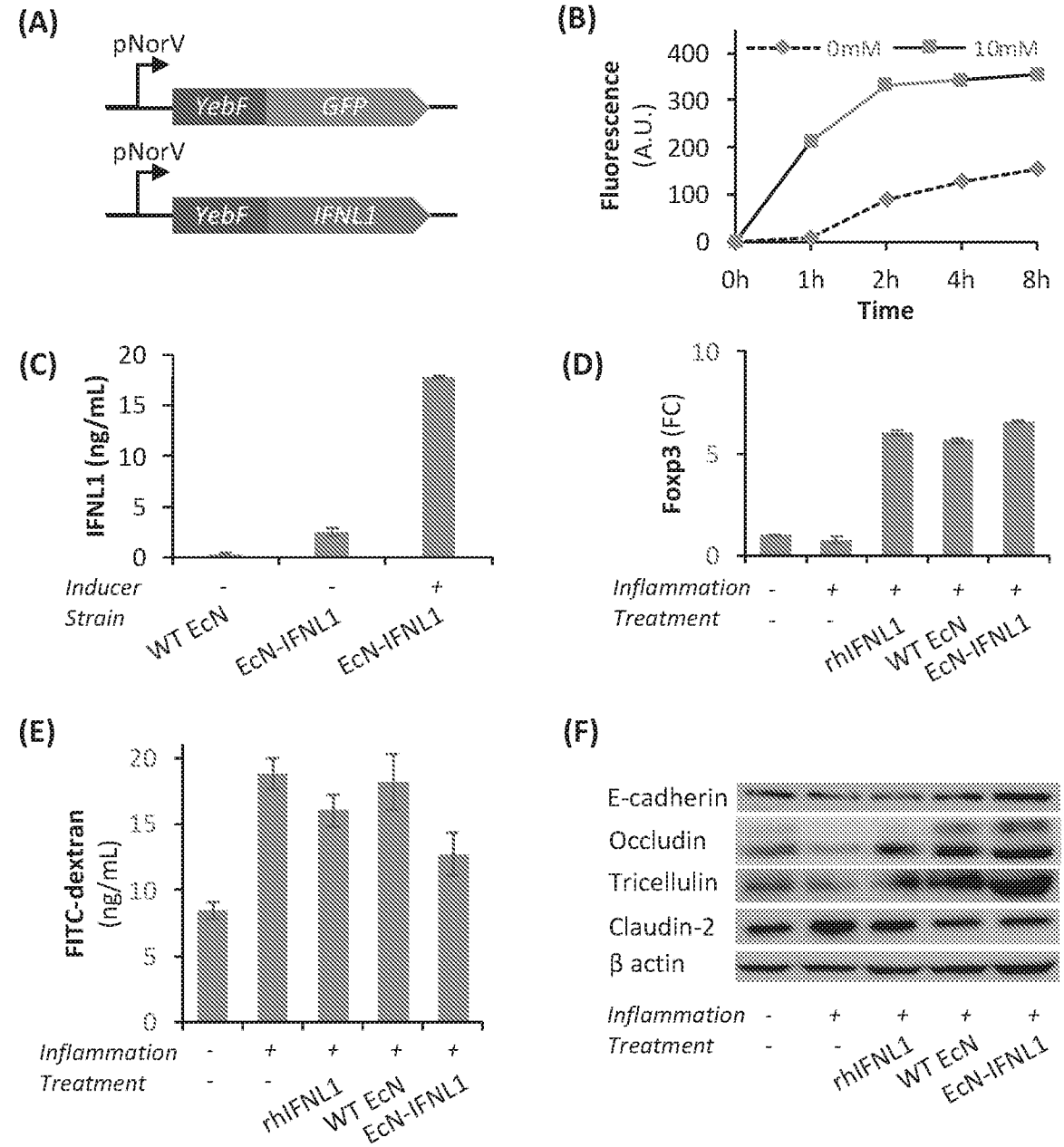


Figure 2

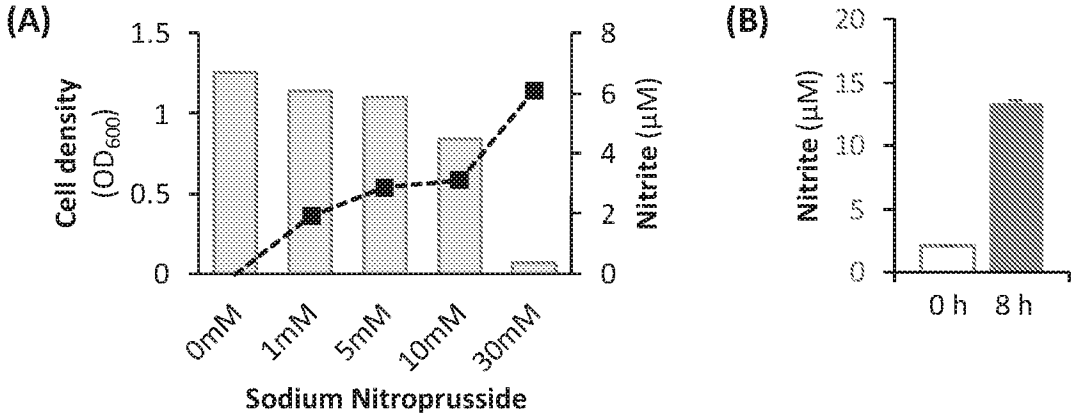


Figure 3

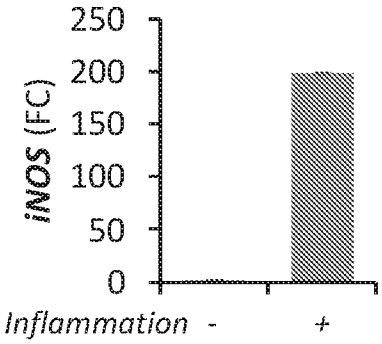
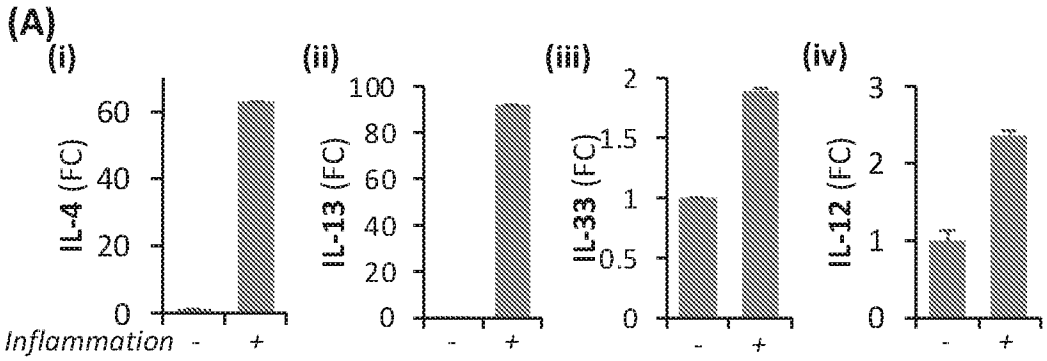
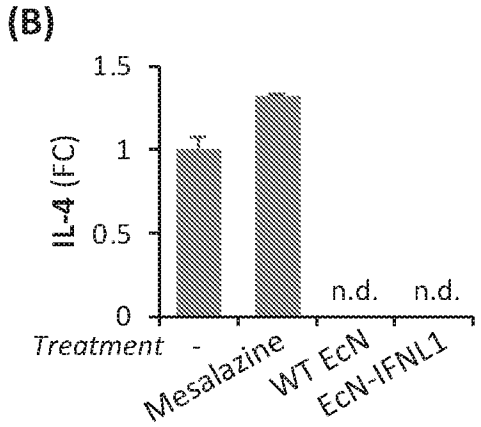
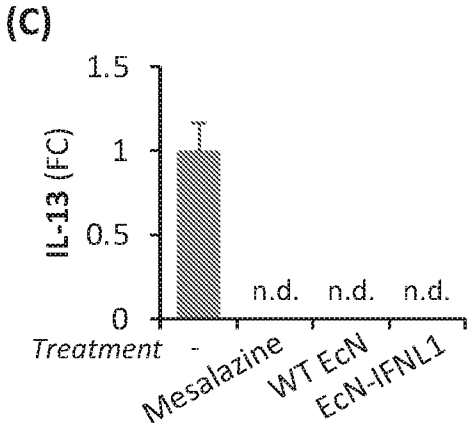
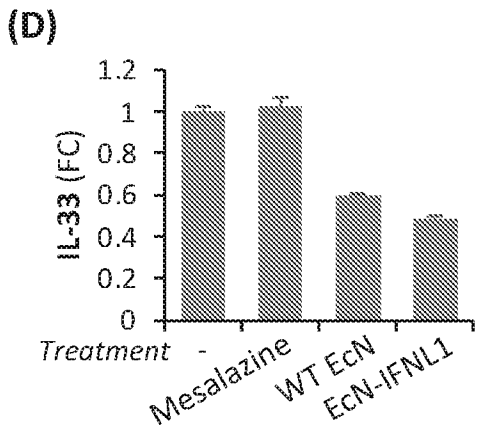


Figure 4









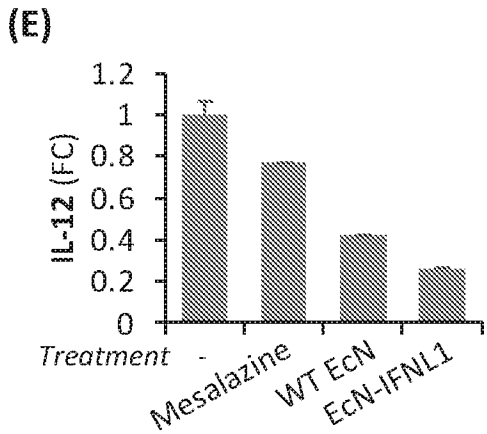


Figure 5

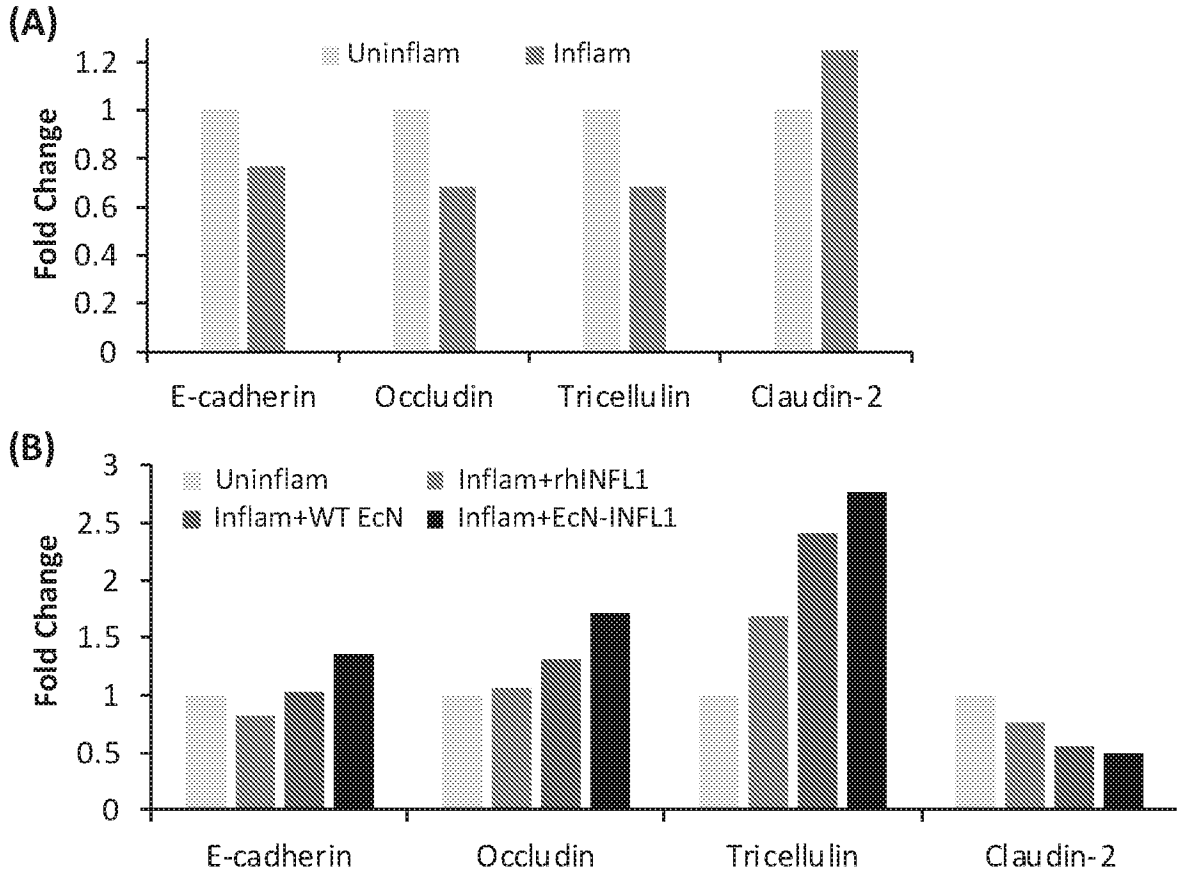
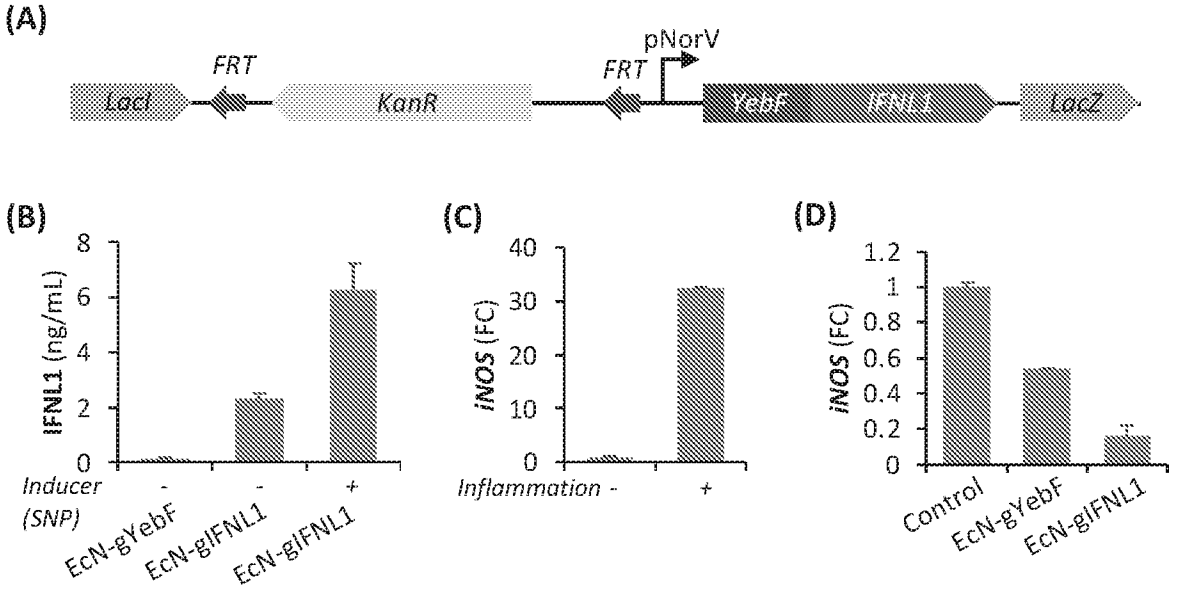


Figure 6



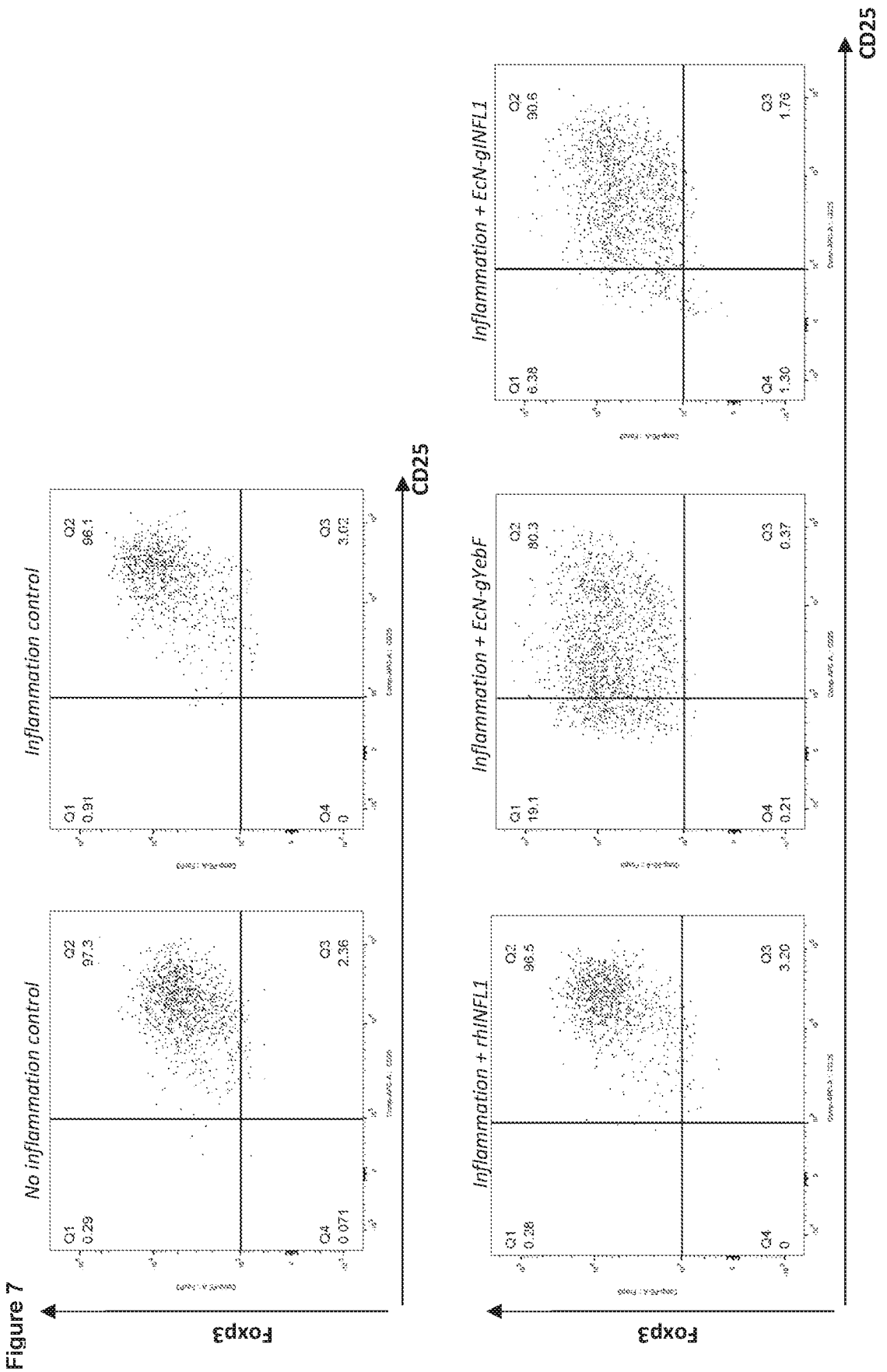
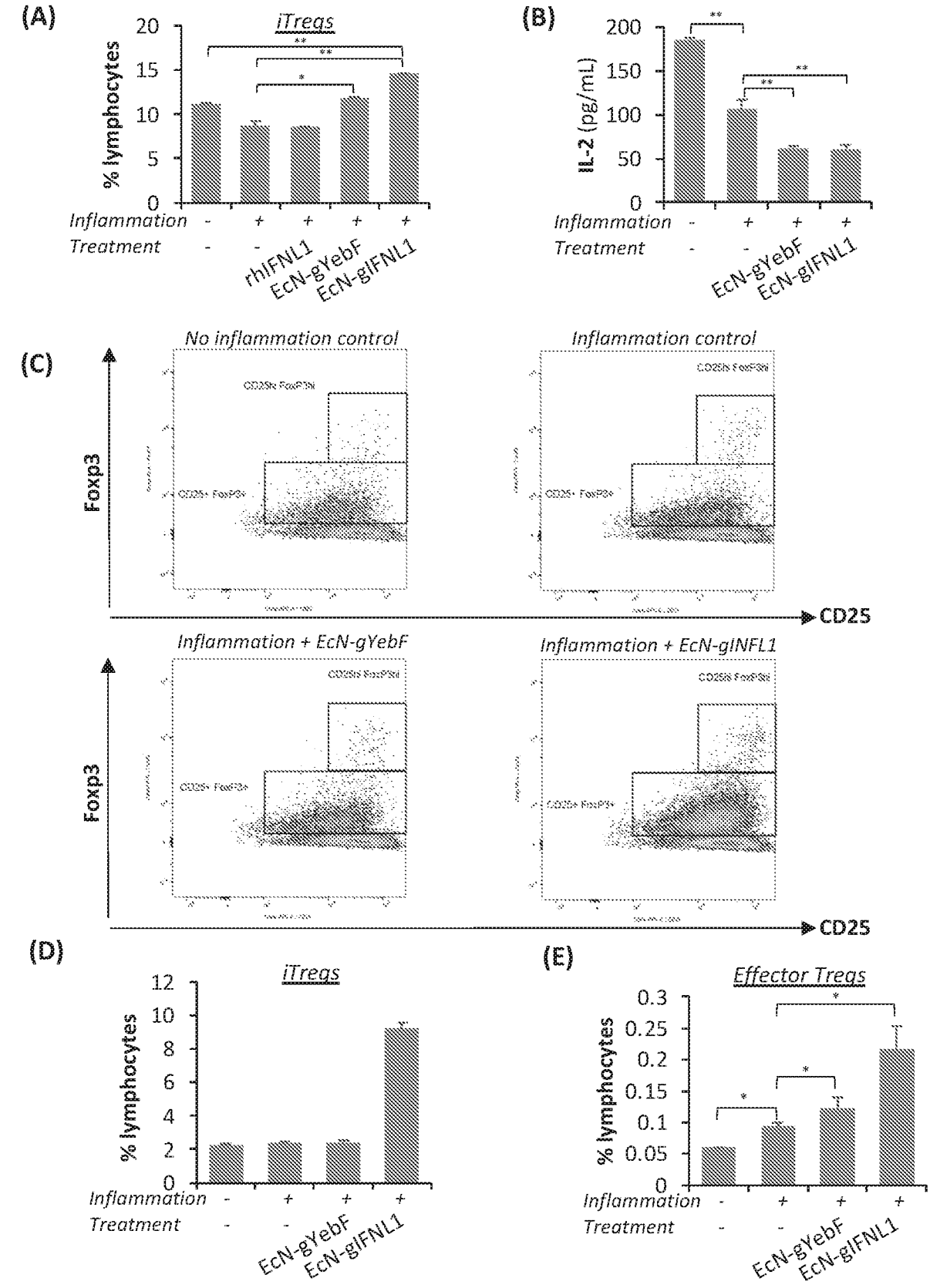

No inflammation control
Q1 0.29
Q2 97.3
Q3 2.36
Q4 0.071
Inflammation control
Q1 0.91
Q2 96.1
Q3 3.02
Q4 0
Inflammation + rhINFL1
Q1 0.28
Q2 96.5
Q3 3.20
Q4 0
Inflammation + EcN-gYebF
Q1 19.1
Q2 80.3
Q3 0.37
Q4 0.21
Inflammation + EcN-gINFL1
Q1 6.38
Q2 90.6
Q3 1.76
Q4 1.30
Foxp3
CD25

Figure 7

Figure 8


(A)
iTregs
% lymphocytes
20
15
10
5
0
Inflammation - + + + +
Treatment - - rhIFNL1 EcN-gYebF EcN-gIFNL1
(B)
IL-2 (pg/mL)
200
150
100
50
0
Inflammation - + + +
Treatment - - EcN-gYebF EcN-gIFNL1
(C)
No inflammation control
Inflammation control
Inflammation + EcN-gYebF
Inflammation + EcN-gINFL1
Foxp3
CD25
(D)
iTregs
% lymphocytes
12
10
8
6
4
2
0
Inflammation - + + +
Treatment - - EcN-gYebF EcN-gIFNL1
(E)
Effector Tregs
% lymphocytes
0.3
0.25
0.2
0.15
0.1
0.05
0
Inflammation - + + +
Treatment - - EcN-gYebF EcN-gIFNL1

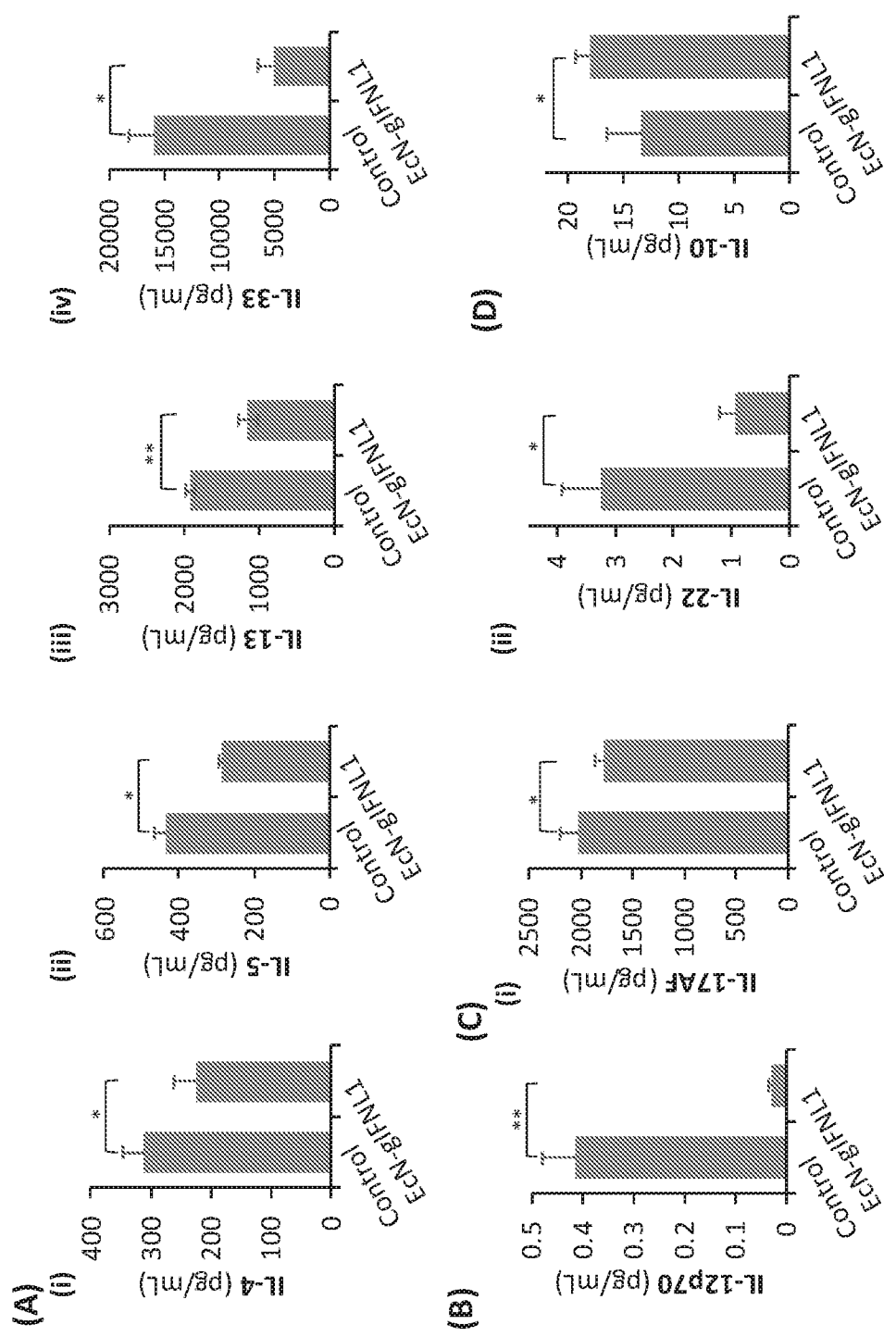

(A)
(i)
IL-4 (pg/mL)
400
300
200
100
0
Control
ECN-gIFNL1
(ii)
IL-5 (pg/mL)
600
400
200
0
(iii)
IL-13 (pg/mL)
3000
2000
1000
(iv)
IL-33 (pg/mL)
20000
15000
10000
5000
(B)
IL-12p70 (pg/mL)
0.5
0.4
0.3
0.2
0.1
(C)
(i)
IL-17AF (pg/mL)
2500
2000
1500
1000
500
(ii)
IL-22 (pg/mL)
4
3
2
1
(D)
IL-10 (pg/mL)
20
15
10
5

Figure 9

Figure 10

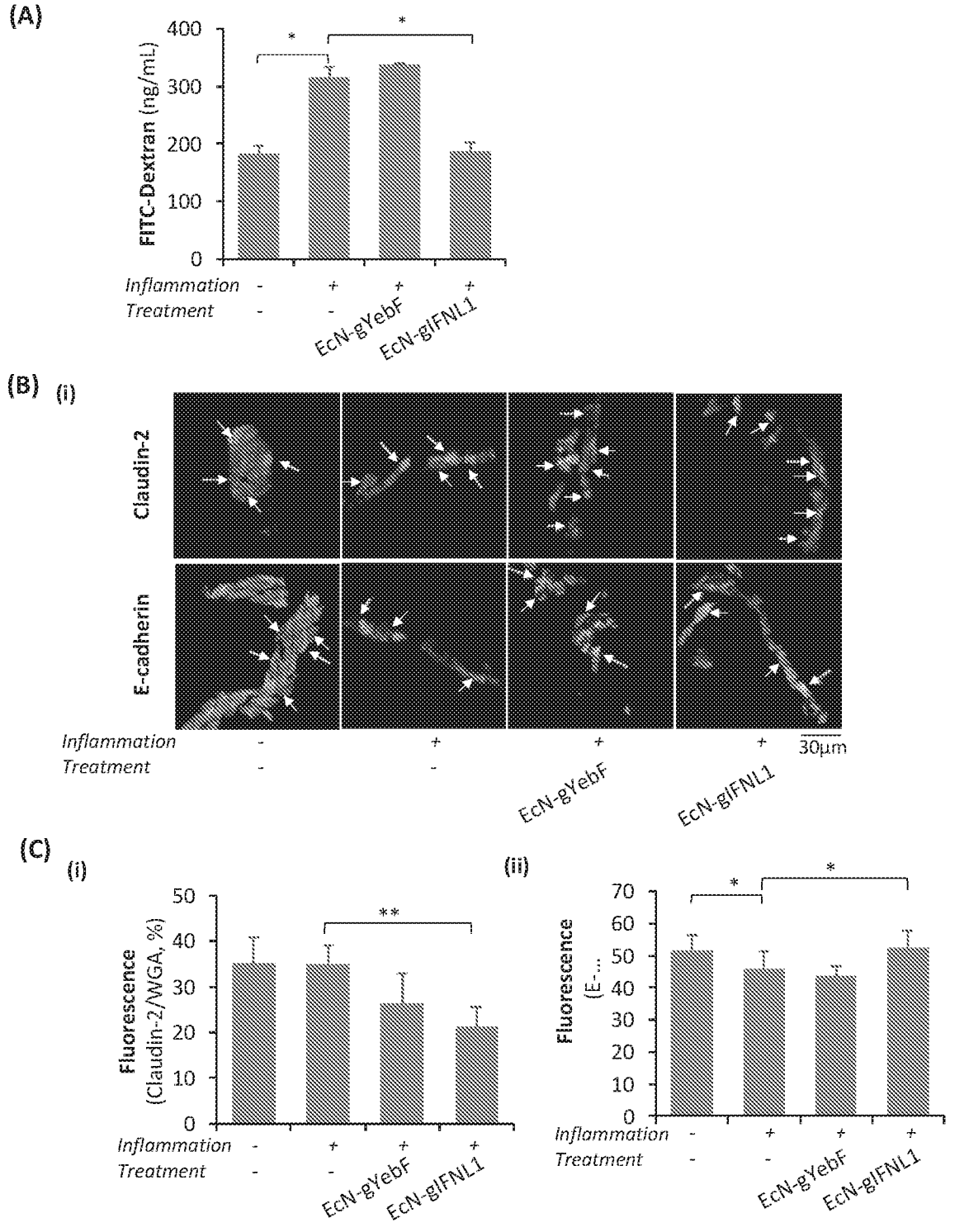

(A)
FITC-Dextran (ng/mL)
400
300
200
100
0
*
*
Inflammation - + + +
Treatment - - EcN-gYebF EcN-glFNL1
(B) (i)
Claudin-2
E-cadherin
Inflammation - + + +
Treatment - -
EcN-gYebF
EcN-glFNL1
30µm
(C)
(i)
Fluorescence (Claudin-2/WGA, %)
50
40
30
20
10
0
**
Inflammation - + + +
Treatment - - EcN-gYebF EcN-glFNL1
(ii)
Fluorescence (E-...
70
60
50
40
30
20
10
0
*
*
Inflammation - + + +
Treatment - - EcN-gYebF EcN-glFNL1

Figure 11

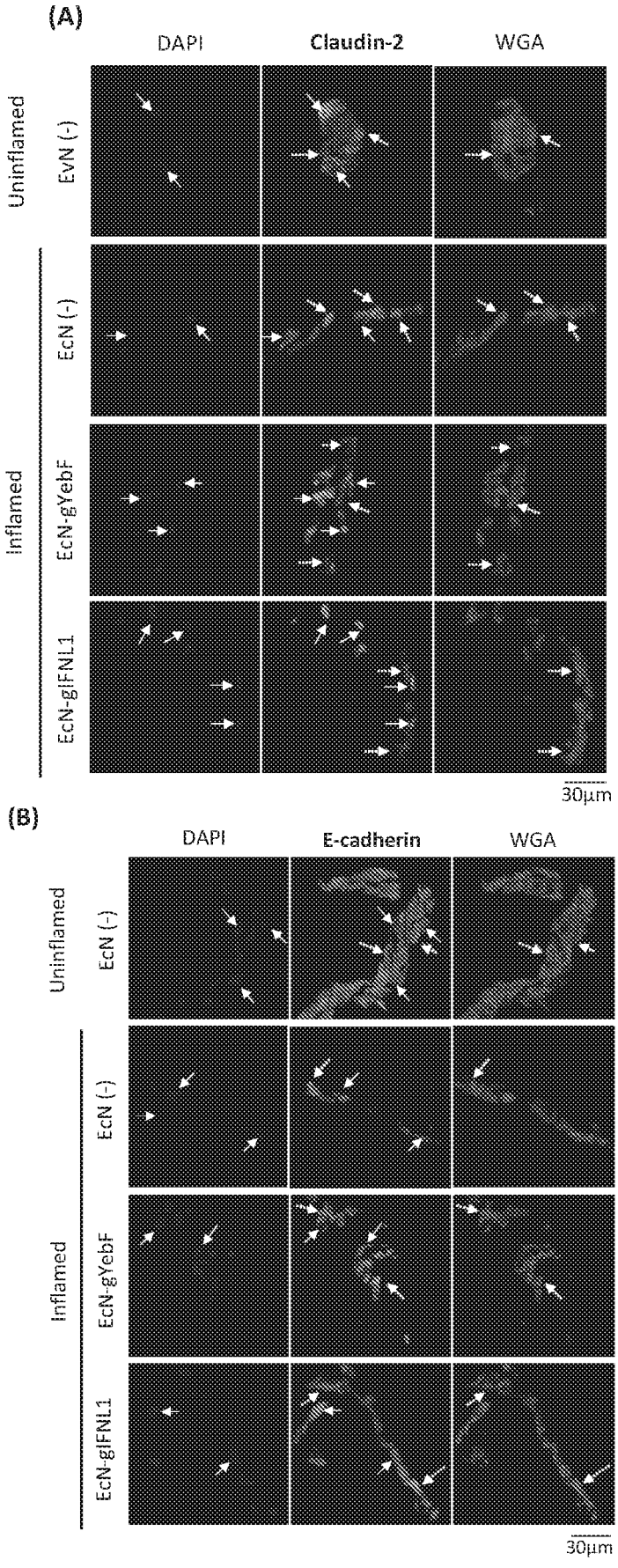
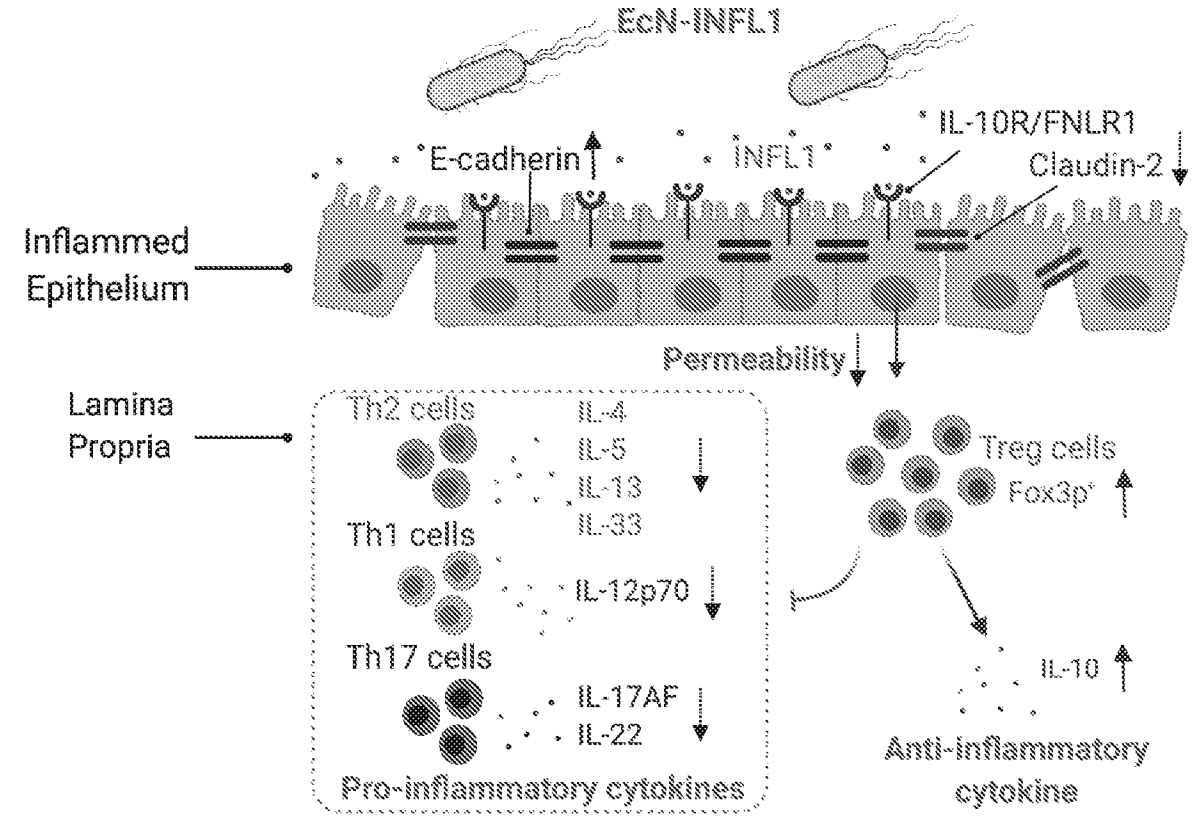

(A)
DAPI
Claudin-2
WGA
Uninflamed
EvN (-)
Inflamed
EcN (-)
EcN-gYebF
EcN-gIFNL1
30µm
(B)
DAPI
E-cadherin
WGA
Uninflamed
EcN (-)
Inflamed
EcN (-)
EcN-gYebF
EcN-gIFNL1
30µm

Figure 12


EcN-INFL1
IL-10R/FNLR1
E-cadherin
INFL1
Claudin-2
Inflammed Epithelium
Permeability
Lamina Propria
Th2 cells
IL-4
IL-5
IL-13
IL-33
Th1 cells
IL-12p70
Th17 cells
IL-17AF
IL-22
Pro-inflammatory cytokines
Treg cells
Fox3p+
IL-10
Anti-inflammatory cytokine

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050337

A. CLASSIFICATION OF SUBJECT MATTER

See Supplemental Box

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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)

FAMPAT, EMBASE, BIOSIS, and MEDLINE: Type III interferon, IFNL1, IFNL2, IFNL3, IFNL4, IL-28, IL-29, nitric oxide, inducible, responsive, promoter, pNorV, YebF, inflammation, inflammatory bowel disease, IBD, and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Team NEU China A, Design submission to the International Genetically Engineered Machine 2018 (iGEM 2018). 28 October 2018 [Retrieved on 2023-10-16 from https://2018.igem.org/Team:NEU_China_A/Design] Overview, Section 4, and Figure 3	1-24
Y	MANIVASAGAM S. & KLEIN R. S., Type III Interferons: Emerging Roles in Autoimmunity. *Front Immunol*, 26 November 2021, Vol. 12, No. 764062, pages 1-8 [Retrieved on 2023-10-16] <DOI: 10.3389/FIMMU.2021.764062> Page 5	1-24
A	MCKAY R. ET AL., A platform of genetically engineered bacteria as vehicles for localized delivery of therapeutics: Toward applications for Crohn's disease. *Bioeng Transl Med*, 28 August 2018, Vol. 3, No. 3, pages 209-221 [Retrieved on 2023-10-16] <DOI: 10.1002/BTM2.10113> Abstract and Figures 1 and 7	-

☒ Further documents are listed in the continuation of Box C. ☐ 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

"D" document cited by the applicant in the international application

"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
16/10/2023 *(day/month/year)*	18/10/2023 *(day/month/year)*

Name and mailing address of the ISA/SG	Authorized officer
Intellectual Property Office of Singapore 1 Paya Lebar Link, #11-03 PLQ 1, Paya Lebar Quarter Singapore 408533 Email: pct@ipos.gov.sg	Kenneth Tan (Dr) IPOS Customer Service Tel. No.: (+65) 6339 8616

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG X. ET AL., Targeting IL-10 Family Cytokines for the Treatment of Human Diseases. *Cold Spring Harb Perspect Biol*, 28 February 2019, Vol. 11, No. a028548, pages 1-30 [Retrieved on 2023-10-16] <DOI: 10.1101/CSHPERSPECT.A028548> Page 19 and Figure 1	-
A	BARRA M. ET AL., Engineered Probiotics for Detection and Treatment of Inflammatory Intestinal Diseases. *Front Bioeng Biotechnol*, 31 March 2020, Vol. 8, No. 265, pages 1-10 [Retrieved on 2023-10-16] <DOI: 10.3389/FBIOE.2020.00265> Figure 1D	-
P, X	CHUA K. J. ET AL., An Engineered Probiotic Produces a Type III Interferon IFNL1 and Reduces Inflammations in in vitro Inflammatory Bowel Disease Models. *ACS Biomater Sci Eng*, 18 November 2022, Vol. 9, No. 9, pages 5123-5135 [Retrieved on 2023-10-16] <DOI: 10.1021/ACSBIOMATERIALS.2C00202> Abstract and Figures 1, 2, and 6	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050337

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. ☐ 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:

   Although a sequence listing was not used for the purposes of search, the sequences mentioned in the claims have been taken into consideration for the purposes of this opinion.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050337

Supplemental Box
(Classification of Subject Matter)

Int. Cl.
C12N 15/63 (2006.01)
C12N 15/20 (2006.01)
A61K 38/21 (2006.01)
A61P 37/00 (2006.01)
A61P 1/00 (2006.01)