Activation of LANCL2 by BT-11 Ameliorates IBD by Supporting Regulatory T Cell Stability Through Immunometabolic Mechanisms

Andrew Leber, Ph.D,* Raquel Hontecillas, Ph.D, DVM,*

Victoria Zoccoli-Rodriguez, B.S.,* and Josep Bassaganya-Riera, Ph.D, DVM*

Background: Inflammatory bowel disease (IBD) afflicts 5 million people and is increasing in prevalence. There is an unmet clinical need for safer and effective treatments for IBD. The BT-11 is a small molecule oral therapeutic that ameliorates IBD by targeting lanthionine synthetase C-like 2 (LANCL2) and has a benign safety profile in rats.

Methods: Mdr1a-/-, dextran sodium sulphate, and adoptive transfer mouse models of colitis were employed to validate therapeutic efficacy and characterize the mechanisms of therapeutic efficacy of BT-11. In vitro cultures of CD4+ T cell differentiation and human peripheral blood mononuclear cells from Crohn's disease patients were used to determine its potential for human translation.

Results: BT-11 reduces inflammation in multiple mouse models of IBD. Oral treatment with BT-11 increases the numbers of lamina propria regulatory T cells (Tregs) in a LANCL2-dependent manner. In vitro, BT-11 increases the differentiation in Treg phenotypes, the upregulation of genes implicated in Treg cell stability, and conditions Treg cells to elicit greater suppressive actions. These immunoregulatory effects are intertwined with the ability of BT-11 to regulate late stage glycolysis and tricarboxylic acid cycle. Immunometabolic mechanistic findings translate into human peripheral blood mononuclear cells from healthy individuals and Crohn's disease patients.

Conclusions: BT-11 is a safe, efficacious oral therapeutic for IBD with a human translatable mechanism of action that involves activation of LANCL2, immunometabolic modulation of CD4+ T cell subsets leading to stable regulatory phenotypes in the colonic LP.

Key Words: inflammatory bowel disease, immunometabolism, regulatory T cells, LANCL2

INTRODUCTION

Inflammatory bowel diseases (IBD), encompassing both Crohn's disease (CD) and ulcerative colitis (UC), afflict 1.6 million North Americans and 5 million worldwide, with nearly 15% growth over the last 5 years.^{1,2} Because of the complex and multifactorial nature of IBD, with symptoms, risk factors, and severity varying along a wide spectrum, the development of efficacious, safer treatments has been a slow and arduous process.³ Currently, dominant market therapeutics benefit only small portions of the overall population,⁴ have high rates of loss of response,⁵ or induce high rates of side effects,

including cancer, infection, and death.^{6,7} Therefore, there is an unmet clinical need for safer and more efficacious therapeutics for IBD.

Among the array of factors linked to the development of IBD is an imbalance and expansion of effector, proinflammatory CD4+ T cells.⁸ Their spontaneous high-level production of IFN γ , TNF α , and IL17 is the major driver of the random flares of inflammation.⁹ In contrast, the main role of regulatory CD4+ T (Treg) cells is to prevent this expansion, activation, and inflammatory cytokine production. Despite these clear benefits in maintaining mucosal homeostasis, Treg cells experience an expected compensatory increase in the gastrointestinal (GI) of IBD patients¹⁰ and display normal suppressive function in vitro¹¹ compared to healthy controls, raising controversy over their tangible impact in disease. Recent evidence suggests that whereas Tregs characterized by traditional markers do expand in number, these cells might be coproducers of inflammatory cytokines or cells that unstably express FOXP3 in situ.¹²⁻¹⁴ This coproduction creates an intermediate phenotype that more closely associates with an effector/inflammatory CD4+ T cell rather than a typical anti-inflammatory Treg cell. Additionally, expansion of Tregs has been linked to the responsiveness to current IBD treatments.¹⁵ Thus, therapeutically targeting the stability of the fully committed Treg profile might restore efficacy to natural and induced Treg cell function within the GI mucosa.

Received for publications January 10, 2018; Editorial Decision March 8, 2018.

*Landos Biopharma Inc, Blacksburg, VA, 24060.

Conflicts of Interest: Andrew Leber, Raquel Hontecillas, and Victoria Zoccoli-Rodriguez have no conflicts of interest. Josep Bassaganya-Riera is the founder, director, officer, and shareholder of Landos Biopharma.

Supported by: This work was supported by funds from NIH SBIR/STTR programs (1R43DK097940 and 1R41DK099027), the Virginia Biosciences Health Research Corporation, and Landos Biopharma.

Correspondence address: Dr Josep Bassaganya-Riera Landos Biopharma Inc, 1800 Kraft Drive, Suite 216 Blacksburg VA 24060. E-mail: jbr@landosbiopharma.com

© 2018 Crohn's & Colitis Foundation. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

doi: 10.1093/ibd/izy167

Published online 28 April 2018

The entwined and complex nature of cellular metabolism and immunity has become more prominent within the study of human disease and the development of immune targeted therapeutics.¹⁶ In particular, inflammatory and autoimmune diseases, such as IBD, have been identified as prime examples of the potency and efficacy of immunometabolic regulators.¹⁷ Effector and regulatory CD4⁺ T cells have pronounced differences in metabolic function with effector cells favoring lactate production and glucose utilization¹⁸, whereas regulatory cell types retain a balanced metabolic profile between fatty acid and glucose oxidation.¹⁹ The interface between immunity and metabolism, known as immunometabolism, gains increasing importance therapeutically as new metabolic enzymes and substrates are identified to exert moonlighting functions impacting immunological behavior from hexokinase²⁰ and GAPDH²¹ to enolase²² and phosphoenolpyruvate.²³ With effects on IL-1 β and IFN γ production, FOXP3 expression, and calcium signaling, these elements, once thought to only produce energy, have a multitude of potent effects on immune function that can be exploited therapeutically. By controlling the metabolism of immune cells, immunometabolic therapeutics can effectively prevent differentiation and polarization into inflammatory subsets.^{24–27}

The previously identified lanthionine synthetase C-like 2 (LANCL2)-mediated regulation of metabolism in enteroendocrine and muscle cells²⁸ suggests that an important aspect of LANCL2 and BT-11 therapeutic efficacy may lay in a potential immunometabolic mechanism of action of BT-11 in the GI tract. Namely, the activation of LANCL2, the critical first step behind the mode of action of BT-11, was first shown to exert metabolic effects in nonimmune cells, as a receptor for the natural and dietary compound, abscisic acid (ABA),²⁸ and a signal transducer for the production of metabolic hormones, before the discovery of its role in inflammation.²⁹ However, the intimate linkages between the metabolic and immunological actions of the LANCL2 pathway have yet to be fully elucidated. As such, LANCL2, and other immunometabolic targets,¹⁸ merit mechanistic evaluation as innovative immunomodulatory methodologies spanning inflammatory and autoimmune disease.

The ability of BT-11 to therapeutically target LANCL2, selectively, has been validated biochemically in vitro and in vivo in mice with colitis.^{30,31} BT-11 actions are highly localized to the GI mucosa with a low systemic bioavailability of <10% and plasma half-life of 3.1 hours. Furthermore, ample safety studies in rats indicate a very benign safety profile up to the limit dose of 1000 mg/kg po.³² Early evaluations of efficacy define an improvement in disease activity, gut pathology, and decrease in inflammatory markers in the colons of mice challenged with dextran sodium sulphate (DSS). Beyond the activation of LANCL2, the underlying mechanisms of therapeutic action by which BT-11 reduces disease severity in IBD remains to be elucidated. Herein, we propose that activation of LANCL2 by BT-11 induces Treg cell stability via immunometabolic

mechanisms that suppress excessive inflammation in the GI mucosa and ameliorate IBD.

MATERIALS AND METHODS

Mice

Mdr1a^{-/-} on a FVB background were obtained from Taconic Biosciences. Lnc2^{-/-} and LANCL2^{fl/fl} were used on C57Bl/6 backgrounds as previously described.²⁵ Lnc2^{fl/fl} mice were bred to CD4-cre transgenic mice to generate T cell-specific Lnc2 knockout (KO) animals (Lnc2^{ΔT}). The tissue specific knockout of LANCL2 was previously characterized³³ with additional characterization of the T cell deletion provided in [Supplemental Figure 1](#). Killing was conducted by CO₂ narcosis followed by secondary cervical dislocation. Experimental animals were age-, sex-, and body weight-matched upon entry into experiments. Mice were housed in ventilated racks with ad libitum access to Teklad 2918 rodent chow. Mice were on a standard 12 hour on-off light cycle with treatments administered during the on cycle. Male and female littermates were used within each study. All studies were performed with the approval of the BioTherapeutics, Inc., Institutional Animal Care and Use Committee (#16-008).

Induction of Experimental IBD

Mdr1a^{-/-} model

Mdr1a^{-/-} mice develop spontaneous colitis. At 4 weeks of age, Mdr1a^{-/-} mice began receiving daily treatment with BT-11 (8 mg/kg) via orogastric gavage. Mice were weighed and scored weekly to monitor the development of colitis. At 10 weeks of age, Mdr1a^{-/-} were killed for the collection of tissue for downstream assay.

DSS model

Lnc2^{ΔT} and Lnc2-expressing controls were given DSS (8% wt/vol) in drinking water for 7 days to induce severe disease. Following 7 days, standard drinking water was returned. Mice were weighed and scored daily. Mice were killed for tissue collection at 7 and 10 days of experimental timeline. *Adoptive transfer model*

Wild-type (WT) and Lnc2^{-/-} donor spleens were enriched for CD4⁺ fraction by magnetic sorting. CD4⁺CD45RB^{hi}CD25⁻ (T_{eff}) and CD4⁺CD45RB^{lo}CD25⁺ (T_{reg}) cells were sorted by a FACS Aria cell sorter. Based on indicated experimental group, Rag2^{-/-} recipient mice received 4 × 10⁵ T_{eff} and 1 × 10⁵ T_{reg} cells from WT or Lnc2^{-/-} origin by intraperitoneal injection. After transfer, mice received daily treatment of BT-11. Mice were weighed and scored weekly until killing at 8 weeks posttransfer.

Flow Cytometry

Colons and mesenteric lymph nodes (MLN) were collected into RPMI/FBS buffer containing collagenase (300U/mL)

and DNase (50U/mL) for digestion. Tissues were digested for 60 minutes under stirring at 37°C. Resultant cellular suspensions were filtered through 100 μ m strainers, centrifuged (300 $\times$ g, 8 min), and washed in fresh RPMI. Following filtration of the resulting single cell suspensions, immune cells were purified by Percoll gradient of cell containing 40% Percoll overlaid onto 70% Percoll solution. After centrifugation, interphase was collected and washed to obtain enriched colonic lamina propria (LP) and MLN cell fractions, respectively. Cells were labeled with mixtures of extracellular (CD45, CD3, CD4, CD8, CD19, NK1.1, CD25, F4/80, CD11b, CX3CR1, CD64) and intracellular (Tbet, ROR γ T, FOXP3, IFN γ , IL17, IL10) antibodies in a sequential live staining in 96-well plates. Data were acquired using a FACS Celesta flow cytometer with FACSDiva software. Representative cytokine responses are shown in [Supplemental Figure 2](#).

Gene Expression

Total RNA from colon and cells was generated using the Qiagen RNeasy mini kit. cDNA was generated using the BioRad iScript cDNA synthesis kit. Standard curves were generated by serial dilution of purified product from a standard polymerase chain reaction (PCR) with Taq DNA polymerase followed by purification using the Qiagen MinElute PCR purification kit. Expression levels were obtained from quantitative real-time PCR with SybrGreen supermix on a BioRad CFX96 Thermal cycler followed by normalization to expression of β -actin as described previously.³⁴

Histopathology

Hematoxylin and eosin (H&E) stained colonic sections were prepared from portions of colons collected into 10% buffered formalin and embedded in paraffin. Slides were examined by a board-certified veterinary pathologist via an Olympus microscope and images were collected with Image-Pro software. Samples were scored (0–4) for leukocytic infiltration, epithelial erosion, and mucosal thickening.

Metabolic Analysis

Colons and cells were suspended within assay-specific buffer and homogenized for 10 seconds. Homogenate was centrifuged for 10 minutes at 10,000 $\times$ g. For enzyme activity assays, supernatant was collected and plated. Samples were mixed with enzyme developer and substrate. Colorimetric detection of nicotinamide adenine dinucleotide (NADH) production was measured using a BioTek μ Quant plate reader in combination with Gen5 software. For phosphoenolpyruvate (PEP) assay, supernatant was deproteinized using perchloric acid mediated purification. Samples were plated and mixed with a probe, converter, and developer mix. PEP concentration was measured by absorbance quantification on plate reader.

In vitro CD4+ T-cell Culture

Spleens from WT and Lnc12 $^{-/-}$ mice were excised and crushed to generate a single cell suspension. The CD4 $^{+}$ T cell fraction was enriched by negative selection via magnetic sorting with the BD IMag system. Naive CD4 $^{+}$ T cells were obtained by incubation with a biotinylated CD62L antibody followed by conjugation to streptavidin-coated magnetic beads. Naive cells were incubated for 48 hours within anti-CD3-coated tissue culture plates in complete IMDM media containing all-trans retinoic acid and purified TGF- β to stimulate differentiation into Tregs.¹⁸ Cells were plated in media containing indicated concentration of BT-11 from 0 to 48 hours. Metabolic modulators, PS-48 (5 μ M), DASA-58 (10 μ M), and thapsigargin (10 nM), were added at 0 hour. For coassay, CD4 $^{+}$ fraction and Tregs were generated as described and plated within the same well at a 1:1 ratio of 2×10^5 cells each without BT-11 and incubated together for 24 hours. Six hours before assay, cells were stimulated with phorbol myristate acetate (PMA) and ionomycin. Cells were collected from plate for downstream analysis by flow cytometry, gene expression, and metabolic assay.

Isolation and Culture of Human PBMCs

Fresh deidentified whole blood was obtained from commercial vendor. Blood was diluted and purified for peripheral blood mononuclear cell (PBMC) fraction by LeukoSep tube. Remaining red blood cells were lysed by hypotonic lysis. Cells were plated in anti-CD3-coated wells in complete Roswell Park Memorial Institute media and incubated with BT-11 for 24 hours. For siRNA experiments, cells were first incubated with an OriGene 3-mer LANCL2 siRNA^{28,35} or scrambled control suspended within Viromer-based transfection reagent^{36,37} for 6 hours. siRNA was adjusted to 11 μ M with Buffer Green reagent and further diluted 1:10 in ViromerGreen transfection reagent. Sixty microliters of resulting siRNA suspension were added to each well containing 200 μ L serum-free cell suspension. Protocol was based on manufacturer instructions and optimized in human PBMCs to provide stable knockdown of LANCL2 with no differences in cell viability or morphology between LANCL2 and scrambled siRNA treatments. After 6 hours, cells were washed and resuspended in fresh media containing BT-11. Cells were stimulated with PMA and ionomycin 6 hours before assay. After 48 hours of BT-11 treatment, cells were collected for flow cytometry ([Supplemental Fig. 3](#)), gene expression, and metabolic assay.

Statistical Analysis

Data are expressed as mean and SEM. Parametric data were analyzed using ANOVA, followed by the Scheffé' multiple-comparisons test. ANOVA was performed using the general linear model procedure of SAS (SAS Institute, Cary, NC). A 2 $\times$ 2 factorial arrangement comparing genotype and treatment was used. Statistical significance was determined at $P < 0.05$.

RESULTS

BT-11 Reduces Histopathological Lesions, Cellular, and Molecular Markers of Inflammation in an *Mdr1a*^{-/-} Model

Mdr1a^{-/-} mice were treated by oral gavage with 8 mg/kg BT-11 or vehicle daily over a 6-week period beginning at 4 weeks of age. Oral treatment with BT-11 ameliorated disease throughout the 6-week period (Fig. 1A) and reduced colonic leukocytic infiltration, epithelial erosion, and mucosal thickening at 10 weeks (Fig. 1B, C). By flow cytometry, significant ($P < 0.05$) reductions in Th1 (CD4⁺Tbet+IFN γ +) and Th17 (CD4⁺ROR γ T+IL17+) cells occurred within the colonic LP and MLN with oral BT-11 treatment. In contrast, BT-11 increased percentages of IL-10-producing cellular subsets including CX3CR1⁺ macrophages (CX3CR1⁺F4/80^{hi}CD11b⁺CD64⁺) in the colonic LP and induced Treg cells (CD4⁺FOXP3⁺IL10⁺) both in the colonic LP and MLN. Downregulation of inflammatory cytokines, IFN γ , IL17A, IL6, TNF α , and MCP1, was validated at mRNA and protein levels by colonic gene expression and cytometric bead array. Furthermore, oral treatment with BT-11 upregulated whole colon LANCL2. Oral treatment with BT-11 (8 mg/kg) demonstrates efficacy when given therapeutically and prophylactically (Supplemental Fig. 4) and outperforms current Investigational New Drugs (INDs)

and established IBD therapeutics in the *Mdr1a*^{-/-} model (Supplemental Fig. 5).

Expression of LANCL2 in CD4⁺ T cells is Required for Therapeutic Efficacy of BT-11

Based on consistent observations within the CD4⁺ T cell compartment with BT-11, we aimed to investigate the T cell intrinsic effects of BT-11. Mice with a T cell-specific deletion of LANCL2 (*Lanc2*^{fl/fl}/CD4^{cre+}; *Lanc2*^{ΔT}; Supplemental Information) were generated using cre-lox technology.³³ *Lanc2*^{ΔT} and *Lanc2*-expressing controls were exposed to a 7-day period of DSS and treated with oral BT-11 or vehicle control. The loss of LANCL2 in CD4⁺ T cells abrogated the efficacy of BT-11 (Fig. 2). *Lanc2*^{ΔT} displayed increased disease activity, weight loss, and increased severity of colonic inflammatory lesions (Fig. 2A–C). Untreated *Lanc2*-expressing controls and both untreated and BT-11-treated *Lanc2*^{ΔT} displayed increased leukocytic infiltration into the LP and submucosal areas and loss of tissue structure. Additionally, *Lanc2*^{ΔT} had an increased incidence of lymphoid aggregates suggesting the presence of LANCL2 in CD4⁺ T cells is important in overall immune cell homeostasis. At the cellular level, *Lanc2*^{ΔT} treated orally with BT-11 failed to induce characteristic reductions in Th1 and Th17 effector cells in the colonic LP and failed to expand IL-10-producing Treg cells (Fig. 2E). BT-11-treated and

FIGURE 1. BT-11 suppresses disease development in *Mdr1a*^{-/-} model of colitis. BT-11 decreases disease activity index (A). Representative photomicrographs of H&E stained colonic sections at 10 weeks of age in vehicle (B) and BT-11-treated (C) animals. Immunophenotyping of Th1 (CD3⁺ CD4⁺ CD8⁻ NK1.1⁻ Tbet⁺ IFN γ ⁺), Th17 (CD3⁺ CD4⁺ CD8⁻ NK1.1⁻ ROR γ T⁺ IL17⁺), and Treg (CD3⁺ CD4⁺ CD25⁺ FOXP3⁺ IL10⁺) cells, respectively in the colonic lamina propria (D) and mesenteric lymph nodes (E) at 10 weeks of age. qRT-PCR of whole colon (F) of *ifn γ* , *il17a*, *mcp1*, *tnfa*, *lancl2*, and *il6* normalized to β -actin. Statistical significance by treatment group ($n = 10$) marked by $*$ ($P < 0.05$).

FIGURE 2. Loss of *Lancl2* in CD4⁺ T cells abrogates BT-11 effects in DSS model. Disease activity (A) and weight change (B) over a 7-day challenge with DSS followed by 3 days of standard water. Representative photomicrographs of H&E stained colonic sections (C) at day 7 of DSS challenge in wild-type mice treated with vehicle and BT-11 and in *Lancl2*^{ΔT} mice treated with vehicle and BT-11. Summarized histopathological scores (D) in epithelial erosion, leukocytic infiltration, and mucosal thickening. Immunophenotyping of Th1 (CD3⁺ CD4⁺ CD8⁻ NK1.1⁻ Tbet⁺ IFN γ ⁺), Th17 (CD3⁺ CD4⁺ CD8⁻ NK1.1⁻ ROR γ T⁺ IL17⁺), and Treg (CD3⁺ CD4⁺ CD25⁺ FOXP3⁺ IL10⁺) cells, respectively in the colonic lamina propria (E) at day 7 of DSS challenge. qRT-PCR (F) of whole colon of *Il10*, *Il17a*, and *Ifng* normalized to β -actin. Statistical significance by treatment group (n = 10) marked by _a (P < 0.05) and _b (P < 0.01) and genotype group (n = 10) marked by _c (P < 0.05).

untreated *Lancl2^{AT}* mice expressed greater levels of inflammatory cytokines within the colon (Fig. 2F). Whereas the DSS model has an epithelial cell-driven pathogenesis, the early indication from this study suggests a dominant effect of BT-11 in CD4⁺ T cells. With proper LANCL2 function in epithelial cells, BT-11 fails to protect against disease in the absence of T cell LANCL2 indicating that BT-11 prevents the amplification of intestinal epithelial cell-mediated inflammatory signaling by CD4⁺ T cells.

Oral Treatment with BT-11 Suppresses Inflammation via a Treg-dependent Mechanism

To further characterize the cellular mechanism of action of BT-11 and the T cell-dependency of its therapeutic

efficacy, we employed a cotransfer of effector (Teff) and regulatory (Treg) CD4⁺ T cells from either WT or *Lancl2^{-/-}* (KO) donors into *Rag2^{-/-}* mice (Fig. 3). Among experimental groups transferred WT Teff, only the group cotransferred WT Treg experienced consistent decreases in inflammation from oral BT-11 treatment across all measures including disease activity (Fig. 3A, B), histopathological evaluation (Fig. 3C), Th1, and neutrophil (Fig. 3D) populations in the colonic LP and inflammatory cytokines (Fig. 3E). Notably, the WT Teff / KO Treg group exhibited no benefit of BT-11 treatment in cellular or histopathological characterizations of gut inflammation. Similarly, oral BT-11 treatment presented efficacy in recipients of WT Treg cells even with the cotransfer of KO Teff. These mice had disease activity, leukocytic infiltration, Th1, and

FIGURE 3. BT-11 exerts primary effects through regulatory CD4⁺ T cells. Disease activity index from *Rag2^{-/-}* mice transferred WT (A) and *Lancl2^{-/-}* (B) effector CD4⁺ T cells in combination with none, WT, or *Lancl2^{-/-}* regulatory CD4⁺ T cells from date of transfer to 6 weeks posttransfer. Summarized scores of leukocytic infiltration within colonic section at 6 weeks posttransfer by histopathological examination (C). Immunophenotyping of Th1 cells (CD3⁺ CD4⁺ CD8⁻ NK1.1⁻ Tbet⁺ IFN γ ⁺) and neutrophils (Gr1^{hi} CD11b⁺), respectively in the colonic lamina propria (D). E, Gene expression analysis of *Il10*, *Ifng*, and *Tnfa*. Statistical significance by treatment group (n = 10) marked by * (P < 0.05).

neutrophil measures on par with the WT Teff / WT Treg group. These findings combined with the loss of efficacy in *Lancl2*^{ΔT} mice challenged with DSS further validate that the BT-11 mechanism of action is through LANCL2 activation within the Treg cell subset.

Treatment of Tregs with BT-11 Increases Suppressive Capability and Regulatory Phenotype Stability

To directly validate the findings from the adoptive transfer model, a coassay of CD4⁺ and Treg cells was conducted. Treg cells from WT and KO donors were isolated for culture within Treg differentiating media (ATRA, TGFβ) and treatment with BT-11 for a 2-day period. After 2 days, cells were collected and plated with freshly collected carboxyfluorescein succinimidyl ester-labeled CD4⁺ T cells. WT Treg treated with BT-11 lowered the proliferative index of both WT and KO cells (Fig. 4A) in addition to the expression of TNFα and IFNγ as measured by cytometric bead array (Fig. 4B). In contrast, the pretreatment of KO Treg with BT-11 did not induce changes in either measure. WT Treg pretreated with BT-11 also displayed

a greater retention of Treg (FOXP3+IL10+) phenotype after the 24-hour coassay period. To examine the phenomenon, we assayed the expression of a panel of genes that define stability of the Treg phenotype,³⁸ including *Socs2*, *Capn3*, *Irf7*, *Lag3*, *Ikzf2*, and *P2rx7* (Fig. 4C). *Socs2*, *Capn3*, *Irf7*, and *Lag3* displayed a dose-dependent increase in WT Tregs after 48 hours of BT-11 treatment resulting in a significant upregulation of expression at a dosage of 0.625 μM BT-11. *P2rx7* showed no effect at lower doses but was significantly upregulated ($P < 0.05$) at a dose of 0.625 μM BT-11. Whereas, *Ikzf2* was the only gene to display significant genotype differences. Combined with the observation of Treg cell phenotype retention, these expression results suggest that activation of LANCL2 by BT-11 induces stable expression of Treg-associated genes.

BT-11 Induces Treg Cell Stability Through Immunometabolic Mechanisms of Glucose Flux Control

Due to the previous implication of LANCL2 in glucose metabolism^{35,39} and the importance of metabolic profile on the differentiation of CD4⁺ T cells, the effect of BT-11 on glycolysis

FIGURE 4. BT-11 induces increased stability and suppressive function in in vitro differentiated Tregs. Measurement of 24-hour cellular proliferation by (A) carboxyfluorescein succinimidyl ester staining and (B) TNFα and IFNγ production by cytometric bead array of CD4⁺ T cells cocultured with in vitro differentiated Tregs treated with BT-11 (0, 0.00975, 0.039, 0.625 μM). C, Gene expression of *Socs2*, *Capn3*, *Irf7*, *Lag3*, *P2rx7* within Tregs after 48-hours of treatment with BT-11. Statistical significance by treatment group ($n = 9$) marked by * ($P < 0.05$).

was examined. Expression of 2 glycolytic enzymes, Pkm2 and Eno1, were significantly altered by the loss of LANCL2 in Treg cells in vitro (Fig. 5A, B). Functional enzyme activity provided a profound change with BT-11 treatment. When assayed for enzyme activity, WT Tregs treated with 0.625 μ M BT-11 displayed an increase in pyruvate kinase activity (Fig. 5C). The substrate, PEP, and concentration of pyruvate kinase were significantly lower in BT-11-treated Tregs compared to vehicle-treated controls (Fig. 5D). In addition, entrance into the tricarboxylic acid (TCA) cycle, as evidenced by the activity of

pyruvate dehydrogenase (PDH), was stimulated by BT-11 treatment, particularly at doses of 0.156 μ M and 0.625 μ M (Fig. 5E). The BT-11 induced reduction in PEP and PDH activity was abrogated by treatment with PS-48, a small molecule inhibitor of PDH (Fig. 5D, E). This suggests that BT-11-mediated effects on the glycolytic pathway are dependent on the efficient progression of substrate towards the TCA cycle. Treatment with PS-48 abrogates the increased differentiation of Tregs generated by BT-11 (Fig. 5F). Additionally, inhibition of SERCA signaling by thapsigargin diminishes the BT-11-induced increase in

FIGURE 5. BT-11 influences late-stage glycolysis to affect Treg differentiation. Gene expression of Pkm2 (A) and Eno1 (B) in WT and Lancl2^{-/-} Tregs treated with BT-11 (0, 0.00975, 0.039, 0.625 μ M). Enzyme activity of pyruvate kinase (C) in WT Tregs treated with BT-11 (0, 0.00975, 0.039, 0.156, 0.625 μ M). Intracellular PEP concentration (D) in WT Tregs treated with BT-11 (0, 0.00975, 0.039, 0.156, 0.625 μ M) without and with PS-48 treatment. Pyruvate dehydrogenase activity (E) in WT Tregs treated with BT-11 without and with PS-48 treatment. Differentiation of WT and Lancl2^{-/-} CD4⁺ T cells into Tregs (F) in the presence of PS-48, thapsigargin, or DASA-58 and BT-11 (0, 0.00975, 0.039, 0.156, 0.625 μ M). Statistical significance by treatment group (n = 9) marked by α ($P < 0.05$).

Treg cell differentiation whereas stimulation of pyruvate kinase activity with DASA-58 promotes high level differentiation in both treated and untreated cells, demonstrating the importance of end-stage glycolysis processes on CD4⁺ T cell differentiation.

Inhibition of Metabolic Effects of BT-11 Abrogates Efficacy in *Mdr1a*^{-/-} Mice with IBD

To validate the *in vitro* immunometabolic findings, *Mdr1a*^{-/-} mice were given weekly intraperitoneal injections of PS-48 or vehicle control. Mice treated orally with BT-11 and given vehicle injections displayed expected trends in disease activity, histopathology (Fig. 6A–C), and CD4⁺ T cell populations (Fig. 6D) as previously observed. Metabolic changes *in vitro* were validated within the colon at 10 weeks of age with decreased PEP concentration and increased PDH activity with BT-11 treatment (Fig. 6E). Additionally, expression of Treg cell-stability-associated genes were upregulated with BT-11 treatment (Fig. 6F). Across the clinical, cellular, and metabolic measures, PS-48 effectively blocked the BT-11-induced changes. With the PS-48 injection, the non-BT-11 and BT-11-treated mice had no observable differences in colonic histology, CD4⁺ T cell populations, and metabolic measures. Similar patterns were observed within the DSS model (Supplemental Fig. 6).

BT-11 Induces Anti-inflammatory Cytokines and Suppresses Inflammatory Cytokines in PBMCs Isolated from CD Donors

Whole blood was obtained from CD patients, clinically categorized as mild to moderate disease. When treated with BT-11, isolated PBMCs had a higher percentage of IL-10⁺ and FOXP3⁺ cells and lower percentages of TNF α ⁺ and IFN γ ⁺ cells (Fig. 7A). To demonstrate the LANCL2 specificity of BT-11 in human cells, PBMCs were transfected with *Lancl2* siRNA or scrambled control. After siRNA transfection, BT-11 effects on IL10⁺ and IFN γ ⁺ cells were lost (Fig. 7B). From the PBMC fraction, naive CD4⁺ T cells were obtained and differentiated into Tregs in the presence of BT-11. As observed within mouse cells, BT-11 induced an upregulation of stability-associated Treg markers in a dose-dependent fraction (Fig. 7C). Additionally, metabolic patterns in pyruvate kinase and PDH activity were observed within human Tregs with an increase in the activity of both enzymes after BT-11 treatment (Fig. 7D). Thapsigargin prevented the increased induction of FOXP3⁺ cells in PBMCs treated with BT-11 (Fig. 7E). Whereas, BT-11 prevented cells from an increased commitment to effector phenotypes in the presence of augmented PEP concentration. These results provide an initial translatability of BT-11 efficacy to humans.

DISCUSSION

BT-11 is a novel orally active, locallyacting first-in-class therapeutic for IBD. Through this study, we demonstrate

therapeutic efficacy in 3 separate mouse models of IBD and define a novel immunometabolic mechanism of action of BT-11 through LANCL2. BT-11 is a small molecule therapeutic with physicochemical properties designed and optimized for low bioavailability, localized action within the GI, minimizing the risk for systemic side effects. A benign safety profile in rats and dogs up to the limit dose of 1000 mg BT-11/kg³² further indicates the low risk for potential side effects. BT-11 targets the LANCL2 pathway, which has not been noted to possess any genetic mutations that would render its activation futile. Therefore, LANCL2 offers a stable, yet potent anti-inflammatory target for IBD in the GI tract. Furthermore, we demonstrate that BT-11 upregulates and rescues low-level expression of LANCL2 in the GI tract, restoring any functional abnormalities induced by inflammation in IBD. BT-11 currently is undergoing IND-enabling studies, has robust merits for further development as a clinical drug candidate for CD and UC, and is anticipated to be in the clinic by 2018.

In this report, we validated the therapeutic efficacy of BT-11 using 3 well-established models encompassing chemical, cellular, and genetic methods of IBD induction. During the preclinical development program, BT-11 has showed efficacy in multiple mouse models of IBD spanning diverse means of pathogenesis. BT-11 inhibits exuberant inflammation even with the loss of epithelial cell barrier function and translocation of bacteria in the DSS model, suggesting an ability to maintain mucosal homeostasis in the presence of transient epithelial injury. Whereas, in the adoptive transfer model, BT-11 displays a capacity for blocking Tcell-driven inflammation in an unrecognized environment. With the loss of recognition to nonharmful gut microbiota antigens, a commonly proposed element to IBD pathogenesis, the ability to promote regulatory responses is critical to a successful treatment.

The *Mdr1a*^{-/-} model specifically is a promising model of human translatability. Unlike other genetic models of disease that generate immunocompromised mice, *Mdr1a*^{-/-} mice are immunocompetent,²³ with the deletion instead impacting the cellular ability to efflux molecules and prevent cellular stress. The accumulation of waste and cellular by-products leads to a dysregulation of the epithelial cell lifecycle and increased secretion of inflammatory cytokines and chemokines. Thus, it provides a chronic and spontaneous onset of disease with primary initiating events occurring within the epithelium. Additionally, the *MDR1* gene is an emerging risk allele for IBD and affect the responsiveness to glucocorticoid-based treatments.^{40,41} A particular polymorphism within the *MDR1* gene, 1236T, has also been associated with an increased risk for prospective surgery in CD patients.⁴² The ability of BT-11 to provide therapeutic efficacy in the absence of this gene is an important indication of robustness in the presence of genetic abnormalities and suggest high potential for human translation in a surgery-sparing context.

FIGURE 6. In vivo validation of immunometabolic effect of BT-11. Representative photomicrographs of H&E stained colonic sections at 10 weeks of age in vehicle (left) and BT-11-treated mice (right) without (A) and with (B) PS-48 administration, respectively. Histological scores of colon at 10 weeks of age (C). Immunophenotyping of total CD4+, Treg (CD3+ CD4+ CD25+ FOXP3+ IL10+), Th17 (CD3+ CD4+ CD8- NK1.1- RORγT+ IL17+) cells, respectively in the colonic lamina propria (D) at 10 weeks of age. PEP concentration and pyruvate dehydrogenase activity (E) within whole colon at 10 weeks of age. (F) qRT-PCR of whole colon of *lfn*, *Tnfa*, *Foxp3-E2*, *Foxp3*, *Socs2*, and *Capn3* normalized to β -actin. Statistical significance by treatment group ($n = 10$) marked by * ($P < 0.05$).

Across all 3 models, BT-11 provides consistent down-regulation of Th1 and neutrophil cellular responses and expression of inflammatory cytokines IFN γ , TNF α , MCP1, and IL-6 combined with an increase in Treg populations and IL-10 expression. In this study, we have used a singular dosage strength (8 mg/kg) despite differences in severity of disease onset across models. Our evidence supports that BT-11 provides significant therapeutic efficacy across multiple levels of disease and severity. Comparative efficacy data demonstrate that BT-11 outperforms current IBD drugs and INDs. With a NOAEL exceeding 1000 mg/kg and strong translational data at sub-micromolar concentrations, BT-11 offers a wide therapeutic window on entry into human clinical trials. Additionally, within the animal models, mRNA expression was measured within whole colon tissue as opposed to within sorted CD4+ T cells. Measurement within the CD4+ T cell fraction may further enhance differences in expression between vehicle- and BT-11-treated groups supported by the studies within mouse and human cells *in vitro*. Whereas this manuscript focuses on the dominant effect of BT-11 in CD4+ T cells, the expression of LANCL2 in epithelial cells and myeloid cells suggests the potential for contributing effects from other cell types.

The advances in the emerging field of immunometabolism are increasingly linked to understanding development of autoimmune disease, digestive disorders, and cancer and offer new unexplored opportunities for therapeutic development. The involvement of the mechanism of action of BT-11 in latestage glycolysis has particular, 3-fold importance in the differentiation and stability of Treg cells. Firstly, increased transcription of *Eno1* decreases expression of the FOXP3-E2, the isoform of FOXP3 associated with greater suppressive capacity.²² Although free enolase itself may be linked to the altered FOXP3 expression, MBP1, a transcriptional repressor of FOXP3 promoters, is an alternative isoform transcribed from the *Eno1* gene.⁴³ Decreased overall *Eno1* transcription and occupation of the enzymatic isoform within glycolysis thereby reduce MBP1 expression and prevent inhibition of FOXP3 expression. Secondly, the balance between lactate production and entrance into the TCA cycle is a critical divide between T_{eff} and Treg CD4+ T cell subsets, with multiple factors controlling this divide linked to the ability to generate and maintain a Treg population. Thirdly, the ability to efficiently process PEP to pyruvate is a requirement for unimpeded SERCA signaling.^{19,23} Proper signaling through the SERCA pathway is a checkpoint for the activity of the transcriptional activators of FOXP3, influencing the ability of STAT3 to bind to the CNS2

region, a silencer site, through histone acetylation.⁴⁴ These histone modifications are essential for the continued expression of FOXP3 in dividing Treg cells and their loss results in a failure to suppress intestinal inflammation.^{45,46} With an altered methylation pattern, Tregs are more susceptible to the coproduction of inflammatory cytokines and the intermediate phenotype associated with IBD.⁴⁷ The buildup of intracellular PEP can promote multiple inflammatory pathways from the production of lactate to the generation of endoplasmic reticulum stress,^{48,49} contributing to the generation of inflammatory immune cells and survival of epithelial cells. Although the impact of SERCA signaling in CD4+ T cells is currently unexplored in IBD, inhibited SERCA function has previously been linked to decreased contractility of the colon and small bowel leading to altered mechanosensory behavior.⁵⁰ Additional studies are needed to explore the effect of BT-11 on this signaling pathway within colonic smooth muscle.

The increased expression of an entire panel of Treg cell stability-associated genes suggests an effect of BT-11 directly on FOXP3 activity or upstream control of the transcription factor. Whereas the genes together indicate greater suppressive capacity and stability, these downstream targets have important individual impact on disease. *Irf7* can increase FOXP3 expression and its absence worsens the severity of colitis.⁵¹ *Lag3* is a critical surface molecule for suppressive ability and its expression greatly enhances the efficacy of Treg-based treatments in IBD.⁵² *Socs2* can downregulate IFN γ expression and the activation of antigen presenting cells.⁵³ Overall, BT-11 provides an increase in regulatory and anti-inflammatory behavior that promotes the establishment of more stable and anti-inflammatory Treg cell subsets.

We provide evidence that BT-11 treatment benefits the behavior of Treg cells in addition to their differentiation through increased suppression of CD4+ proliferation in coassays. Although results within this manuscript focus largely on the actions of iTregs, the evaluation of BT-11 on populations of naturally occurring Tregs could provide additional insights for the therapeutic. Notably, *Helios*, encoded by the *Ikzf2* gene and a candidate marker for the discrimination of nTregs and iTregs,^{54,55} is impacted by the expression of LANCL2 suggesting a potential role for LANCL2 activation in the production of nTreg cells. The onset of IBD and flare-ups in disease severity have been linked to small changes in dietary intake and the commensal gut microbiome with spontaneous development of reactivity to traditionally nonharmful antigens. With current knowledge, oral treatment with BT-11 is expected to induce and restore local gut tolerance in these situations. With further

FIGURE 7. Human PBMC validation of immunometabolic effects of BT-11. A, Percentage of IL10+, FOXP3+, TNF α +, and IFN γ + cells within PBMCs from Crohn's disease donors after 24-hour culture with BT-11 (0, 0.00975, 0.0195, 0.039, 0.156, 0.625 μ M). B, Percentage of IL10+ and IFN γ + cells within silencing of *Lancl2* after 24-hour culture with BT-11 (0, 0.00975, 0.0195, 0.039, 0.156, 0.625 μ M). C, Gene expression of *Lag3*, *Socs2*, *Irf7*, *P2rx7*, *Capn3*, *Ikzf2* within naive CD4+ T cells isolated from human PBMCs differentiated into Tregs in presence of BT-11. D, Pyruvate kinase activity of *in vitro* differentiated human Tregs. E, Percentage of FOXP3+ and IFN γ + cells after 24-hour culture with BT-11 (0, 0.00975, 0.0195, 0.039, 0.156, 0.625 μ M) and presence of thapsigargin or external PEP. Statistical significance by treatment group (n = 9) marked by _a (*P* < 0.05) and _b (*P* < 0.01). Statistical significance by siRNA group (n = 9) marked by _c (*P* < 0.05).

study into nTregs, we may uncover that BT-11 promotes lasting tolerance through induction of regulatory memory.

In addition to therapeutic efficacy in multiple validated mouse models of IBD, and in support of its potential for human translation, BT-11 promotes potent effects in human PBMCs from CD patients. BT-11 reduces production of 2 prominent inflammatory cytokines, TNF α and IFN γ , in cells obtained from CD patients with moderate to severe disease. The ability to induce a native decrease in TNF α expression suggests the potential to occupy a similar therapeutic space as anti-TNF α biologics within moderate to severe CD patients. Aside from drastic shifts in cytokine production and cellular differentiation, we demonstrate that identified mechanisms of action translate from murine to human cells in terms of LANCL2 specificity, regulation of cytokines, involvement of Tregs, and immunometabolic pathways. The shared mechanism of action establishes feasibility for the translation of therapeutic efficacy from preclinical models of disease and pathology toward clinical trials. Through this study and previous efforts,^{31,32} BT-11 has emerged as a promising therapeutic for addressing the unmet clinical need for safer, more efficacious oral therapeutics for the long-term management of CD and UC. Utilizing novel immunometabolic mechanisms through LANCL2, BT-11 occupies a unique space in the IBD therapeutic market and merits further preclinical and clinical development.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

REFERENCES

- Colombel JF, Mahadevan U. Inflammatory bowel disease 2017: innovations and changing paradigms. *Gastroenterology*. 2017;152:309–12.
- Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol*. 2015;12:720–7.
- Danese S, Focichi C, Panés J. Drug development in IBD: from novel target identification to early clinical trials. *Gut*. 2016;65:1233–9.
- Yajnik V, Khan N, Dubinsky M, et al. Efficacy and safety of vedolizumab in ulcerative colitis and Crohn's disease patients stratified by age. *Adv Ther*. 2017;34:542–59.
- Vande Casteele N, Ferrante M, Van Assche G, et al. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. *Gastroenterology*. 2015;148:1320–9.e3.
- Keane J, Gershon S, Wise RP, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med*. 2001;345:1098–104.
- Tillack C, Ehmman LM, Friedrich M, et al. Anti-TNF antibody-induced psoriasisiform skin lesions in patients with inflammatory bowel disease are characterised by interferon- γ -expressing th1 cells and IL-17A/IL-22-expressing th17 cells and respond to anti-IL-12/IL-23 antibody treatment. *Gut*. 2014;63:567–77.
- Zenewicz LA, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med*. 2009;15:199–207.
- Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology*. 2011;140:1756–67.
- Maul J, Loddenkemper C, Mundt P, et al. Peripheral and intestinal regulatory CD4+ CD25(hi) T cells in inflammatory bowel disease. *Gastroenterology*. 2005;128:1868–78.
- Holmén N, Lundgren A, Lundin S, et al. Functional CD4+CD25HIGH regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis*. 2006;12:447–56.
- Eastaff-Leung N, Mabarrack N, Barbour A, et al. Foxp3+ regulatory T cells, th1 effector cells, and cytokine environment in inflammatory bowel disease. *J Clin Immunol*. 2010;30:80–9.
- Shevach EM, Davidson TS, Huter EN, et al. Role of TGF- β in the induction of foxp3 expression and T regulatory cell function. *J Clin Immunol*. 2008;28:640–6.
- Uhlig HH, Coombes J, Mottet C, et al. Characterization of foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol*. 2006;177:5852–60.
- Li Z, Arijis I, De Hertogh G, et al. Reciprocal changes of foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. *Inflamm Bowel Dis*. 2010;16:1299–310.
- Leber A, Hontecillas R, Tubau-Juni N, et al. Translating nutritional immunology into drug development for inflammatory bowel disease. *Curr Opin Gastroenterol*. 2016;32:443–9.
- Mathis D, Shoelson SE. Immunometabolism: an emerging frontier. *Nat Rev Immunol*. 2011;11:81.
- Leber A, Hontecillas R, Tubau-Juni N, et al. NLRX1 regulates effector and metabolic functions of CD4+T cells. *J Immunol*. 2017;198:2260–8.
- Newton R, Priyadharshini B, Turk LA. Immunometabolism of regulatory T cells. *Nat Immunol*. 2016;17:618–25.
- Wolf AJ, Reyes CN, Liang W, et al. Hexokinase is an innate immune receptor for the detection of bacterial peptidoglycan. *Cell*. 2016;166:624–36.
- Chang CH, Curtis JD, Maggi LB Jr, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell*. 2013;153:1239–51.
- De Rosa V, Galgani M, Porcellini A, et al. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nat Immunol*. 2015;16:1174–84.
- Haarberg KM, Wymore Brand MJ, Overstreet AM, et al. Orally administered extract from *prunella vulgaris* attenuates spontaneous colitis in mdr1a(-/-) mice. *World J Gastrointest Pharmacol Ther*. 2015;6:223–37.
- Cantarini L, Pucino V, Vitale A, et al. Immunometabolic biomarkers of inflammation in Behçet's disease: relationship with epidemiological profile, disease activity and therapeutic regimens. *Clin Exp Immunol*. 2016;184:197–207.
- Carbo A, Gandour RD, Hontecillas R, et al. An N,N-bis(benzimidazolylpiperidinyl) piperazine (BT-11): a novel lanthionine synthetase C-like 2-based therapeutic for inflammatory bowel disease. *J Med Chem*. 2016;59:10113–26.
- Fullerton MD, Steinberg GR, Schertzer JD. Immunometabolism of AMPK in insulin resistance and atherosclerosis. *Mol Cell Endocrinol*. 2013;366:224–34.
- Souza CO, Teixeira AA, Lima EA, et al. Palmitoleic acid (n-7) attenuates the immunometabolic disturbances caused by a high-fat diet independently of ppara. *Mediators Inflamm*. 2014;2014:582197.
- Sturla L, Fresia C, Guida L, et al. LANCL2 is necessary for abscisic acid binding and signaling in human granulocytes and in rat insulinoma cells. *J Biol Chem*. 2009;284:28045–57.
- Lu P, Hontecillas R, Horne WT, et al. Computational modeling-based discovery of novel classes of anti-inflammatory drugs that target lanthionine synthetase C-like protein 2. *Plos One*. 2012;7:e34643.
- Bassaganya-Riera J, Carbo A, Gandour RD, et al. Novel LANCL2-based Therapeutics. U.S. patent 9,556,146. 2016.
- Carbo A, Gandour RD, Hontecillas R, et al. An N,N-bis(benzimidazolylpiperidinyl) piperazine (BT-11): A novel lanthionine synthetase C-like 2-based therapeutic for inflammatory bowel disease. *J Med Chem*. 2016;59:10113–26.
- Bissel P, Boes K, Hinckley J, et al. Exploratory studies with BT-11: a proposed orally active therapeutic for Crohn's disease. *Int J Toxicol*. 2016;35:521–9.
- Leber A, Bassaganya-Riera J, Tubau-Juni N, et al. Modeling the role of lanthionine synthetase C-like 2 (LANCL2) in the modulation of immune responses to *helicobacter pylori* infection. *Plos One*. 2016;11:e0167440.
- Bassaganya-Riera J, Reynolds K, Martino-Catt S, et al. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology*. 2004;127:777–91.
- Bassaganya-Riera J, Guri AJ, Lu P, et al. Abscisic acid regulates inflammation via ligand-binding domain-independent activation of peroxisome proliferator-activated receptor gamma. *J Biol Chem*. 2011;286:2504–16.
- Hadadi E, Zhang B, Baidzajevs K, et al. Differential IL-1 β secretion by monocyte subsets is regulated by hsp27 through modulating mrna stability. *Sci Rep*. 2016;6:39035.
- Pichulik T, Khatamzas E, Liu X, et al. Pattern recognition receptor mediated downregulation of microrna-650 fine-tunes mxa expression in dendritic cells infected with influenza A virus. *Eur J Immunol*. 2016;46:167–77.
- Delgoffe GM, Woo SR, Turnis ME, et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature*. 2013;501:252–6.
- Zocchi E, Hontecillas R, Leber A, et al. Abscisic acid: a novel nutraceutical for glycemic control. *Front Nutr*. 2017;4:24.
- Schwab M, Schaeffeler E, Marx C, et al. Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. *Gastroenterology*. 2003;124:26–33.
- Yang QF, Chen BL, Zhang QS, et al. Contribution of MDR1 gene polymorphisms on IBD predisposition and response to glucocorticoids in IBD in a Chinese population. *J Dig Dis*. 2015;16:22–30.

42. Bouzidi A, Mesbah-Amroun H, Boukercha A, et al. Association between MDR1 gene polymorphisms and the risk of Crohn's disease in a cohort of algerian pediatric patients. *Pediatr Res*. 2016;80:837–43.
43. Lung J, Liu KJ, Chang JY, et al. MBP-1 is efficiently encoded by an alternative transcript of the ENO1 gene but post-translationally regulated by proteasome-dependent protein turnover. *Febs J*. 2010;277:4308–21.
44. Mirlekar B, Ghorai S, Khetmalas M, et al. Nuclear matrix protein SMAR1 control regulatory T-cell fate during inflammatory bowel disease (IBD). *Mucosal Immunol*. 2015;8:1184–200.
45. Li C, Jiang S, Liu SQ, et al. Mesp2 enforces foxp3 expression to promote regulatory T cells' resilience to inflammation. *Proc Natl Acad Sci U S A*. 2014;111:E2807–16.
46. Ohkura N, Hamaguchi M, Morikawa H, et al. T cell receptor stimulation-induced epigenetic changes and foxp3 expression are independent and complementary events required for treg cell development. *Immunity*. 2012;37:785–99.
47. Li L, Boussiotis VA. The role of IL-17-producing foxp3+ CD4+ T cells in inflammatory bowel disease and colon cancer. *Clin Immunol*. 2013;148:246–53.
48. Méndez-Lucas A, Hyroššová P, Novellademunt L, et al. Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) is a pro-survival, endoplasmic reticulum (ER) stress response gene involved in tumor cell adaptation to nutrient availability. *J Biol Chem*. 2014;289:22090–102.
49. Vincent EE, Sergushichev A, Griss T, et al. Mitochondrial phosphoenolpyruvate carboxykinase regulates metabolic adaptation and enables glucose-independent tumor growth. *Mol Cell*. 2015;60:195–207.
50. Al-Jarallah A, Oriowo MA, Khan I. Mechanism of reduced colonic contractility in experimental colitis: role of sarcoplasmic reticulum pump isoform-2. *Mol Cell Biochem*. 2007;298:169–78.
51. Chiriac MT, Buchen B, Wandersee A, et al. Activation of epithelial signal transducer and activator of transcription 1 by interleukin 28 controls mucosal healing in mice with colitis and is increased in mucosa of patients with inflammatory bowel disease. *Gastroenterology*. 2017;153:123–38.e8.
52. Do JS, Visperas A, Sanogo YO, et al. An IL-27/lag3 axis enhances foxp3+ regulatory T cell-suppressive function and therapeutic efficacy. *Mucosal Immunol*. 2016;9:137–45.
53. Cheng SM, Li JC, Lin SS, et al. HIV-1 transactivator protein induction of suppressor of cytokine signaling-2 contributes to dysregulation of IFN{gamma} signaling. *Blood*. 2009;113:5192–201.
54. Nakagawa H, Sido JM, Reyes EE, et al. Instability of helios-deficient tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity. *Proc Natl Acad Sci U S A*. 2016;113:6248–53.
55. Takatori H, Kawashima H, Matsuki A, et al. Helios enhances treg cell function in cooperation with foxp3. *Arthritis Rheumatol*. 2015;67:1491–502.