Original Article

VEN-120, a Recombinant Human Lactoferrin, Promotes a Regulatory T Cell [Treg] Phenotype and Drives Resolution of Inflammation in Distinct Murine Models of Inflammatory Bowel Disease

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Abstract

Background and Aims: Inflammatory bowel disease [IBD] is characterised by a disruption of immune homeostasis, which is tightly regulated to protect against harmful pathogens yet not react to commensal antigens. Animal studies indicate that regulatory T cells [Treg] modulate the immune response to prevent IBD development. Lactoferrin [LF] is an endogenous anti-inflammatory pleiotropic protein secreted at high concentrations in colostrum and at mucosal sites. However, the effect of LF on specific T lymphocyte populations has not been studied. Here, we identify a novel mechanism by which a recombinant human LF, VEN-120, regulates T cell populations in health and disease.

Methods: Two murine models of intestinal inflammation, the dextran sodium sulphate colitis model and the TNF$^{\Delta ARE/+}$ model of ileitis, were used to study the anti-inflammatory and T cell modulating ability of VEN-120. Flow cytometry was used to evaluate T cell populations within the lamina propria and mesenteric lymph nodes, and to evaluate the effect of VEN-120 on CD4+ T cells in vitro.

Results: VEN-120 reduced inflammation in both models of IBD, accompanied by increased Tregs in the intestinal lamina propria. Treatment of CD4+ T cells in vitro resulted in an upregulation of Treg genes and skewing towards a Treg population. This in vitro T cell skewing translated to an increase of Treg homing to the intestinal lamina propria and associated lymph tissue in healthy mice.

Conclusions: These data provide a novel immunological mechanism by which VEN-120 modulates T cells to restrict inflammatory T cell-driven disease.

Key Words: VEN-120; lactoferrin; colitis; regulatory T cells

1. Introduction

Although the aetiology of inflammatory bowel disease [IBD; comprising ulcerative colitis, UC; and Crohn's disease, CD]

is still poorly defined, current hypotheses suggest that it may develop through a dysregulated mucosal immune response toward the commensal enteric flora in genetically susceptible

individuals.[1–3] This immune dysregulation results in an overproduction of pro-inflammatory cytokines such as tumour necrosis factor [TNF] by monocytes, macrophages, and T cells, thereby allowing for persistence of a hyperactivated immune response.[4,5] CD4+ T cells are critical drivers of pathogenesis in IBD, whereby an increase in activation and populations of CD4+ and CD8+ T cells are observed in patients with IBD.[6] More recently, it has been appreciated that over-activation of Th1/Th17 T cell phenotypes drives much of the chronic pathology in IBD.[7] On the other hand, it has been observed that the pro-inflammatory T cells can be tempered by the action of a subset of CD4+CD25+Foxp3+ cells, termed regulatory T cells [Treg], which exhibit suppressive effects on activated pro-inflammatory T cells, permitting resolution of inflammation and restoration of the homeostatic immune environment.

Lactoferrin [LF] is a single-chain iron-binding glycoprotein of ~ 80 kDa that belongs to the family of human transferrins.[8] LF is present in myriad mucosal fluids,[9–13] but is most predominant in human milk, particularly in the colostrum during early lactation, where it has been suggested to promote the healthy growth and development of the gastrointestinal [GI] tract,[14] promote the growth of commensal bacteria, and deter the establishment of pathogenic bacteria and viruses.[15–17] LF has been previously used for its beneficial effects in several models of human health including dermal inflammation,[18,19] wound healing,[20] and infectious diseases.[19,21,22]

Preclinical studies have shown the protective effects of orally administered human LF [hLF] in mice[23] and bovine LF [bLF] in rats in chemically induced models of colitis.[24,25] Though these studies have demonstrated the ability of LF to decrease the expression of cytokines that are associated with acute inflammation in these models, such as TNF and interleukin [IL]-6, the mechanism of action for LF has yet to be described.

In this study, we sought to determine the mechanism by which LF affects inflammation. To do this, we hypothesised that the beneficial effect of LF may be the result of its effect on T cell populations, specifically the Treg phenotype. Here we demonstrate the ability of LF [VEN-120] to induce a Treg phenotype in CD4+ cells in vitro, and in vivo, using two relevant models of disease, namely: the long-established DSS murine model of chemically induced colitis and the TNF$^{\Delta ARE/+}$ model, which recapitulates CD-like ileitis. With this model, we investigated the ability of VEN-120 to drive a Treg phenotype in vivo at the expense of Th17 populations and to ameliorate disease activity.

2. Materials and Methods

2.1. Chemicals and reagents
Recombinant human lactoferrin [rhLF, VEN-120] was obtained from Ventria Bioscience [Fort Collins, CO, USA]. Cytokines [IL-2, IL-6, IL12, TGF-β] were obtained from Peprotech [Rocky Hill, NJ, USA]. Phorbol 12-myristate 13-acetate [PMA], ionomycin, brefeldin A, and monensin were obtained from Sigma Aldrich [St Louis, MO, USA].

2.2. Animals
A colony of TNF$^{\Delta ARE/+}$ mice were bred in-house with age- and sex-matched wild type littermates used as controls. Faecal samples from these mice were consistently negative for *Helicobacter*, protozoa, and helminthes All animals were handled in accordance with procedures approved by the University of Colorado Institutional Animal Care and Use Committee.

2.3. Chemically induced acute murine colitis model
Eight-week old C57/BL6 mice [Jackson Laboratories, Bar Harbor, ME, USA] were treated with dextran sodium sulphate [DSS] ad libitum [3% w/v 36–50 kDa, MP Biomedicals, Santa Ana, CA, USA] in drinking water for 7 days. DSS groups received daily gavage of VEN-120 [500 mg/kg] or PBS. Body weight was assessed daily to assess disease severity. At the time of sacrifice, spleen, mesenteric lymph nodes, and segments of colonic tissue were excised for flow cytometric analysis of leukocyte subsets, colon lengths were assessed, and colon sections were processed for histology, RNA extraction, and cytokine analyses to assess disease activity. Histology was evaluated and scored by a trained pathologist blinded to the conditions of the experiments, according to a previously described scoring system.[26] Colonic tissues were snap-frozen for polymerase chain reaction [PCR].

2.4. TNF$\Delta^{ARE/+}$ murine model of CD-like ileitis
VEN-120 was solubilised in PBS, sterile filtered, and administered either orally by gavage or by micro-osmotic pump [Alzet #1002, flow rate:0.25uL/h, DURECT Corporation, Cupertino, CA, USA] implanted subcutaneously on the back, posterior to the scapulae, to 20-week old TNFΔ$^{ARE/+}$ mice. Dosing was determined based on a DSS dose response curve [100 mg^{-1} g/kg/day], identifying 500 mg/kg/day as the optimum dose in mice [data not shown]. Following the course of VEN-120 administration, mice were sacrificed and ileums longitudinally transected and processed for standard haematoxylin-eosin staining after fixation in 10% formaldehyde. Histological severity of inflammation was determined based upon an established colitis recording system scored by a trained pathologist[26] blinded to the treatment.

2.5. Intestinal barrier permeability assay
After 14 days' treatment with VEN-120 or BSA control, TNF$^{\Delta ARE/+}$ mice were gavaged with 150 μl of 80 mg/ml FITC-labelled dextran [4 kDa, FD4] and blood was collected 4 h later by submandibular bleeding. Serum was separated from blood after clotting and fluorescence measured.[27] Values were determined by interpolating from a standard curve of FD4.

2.6. Lymphocyte isolation
Single-cell suspensions were obtained by gently pressing the mesenteric lymph nodes [MLN] or spleen against a 70-μm cell strainer. Splenic red blood cells were lysed by 3-min incubation in ammonium chloride lysing reagent [ACK Lysis Buffer; Invitrogen, Carlsbad, CA, USA]. Intestinal segments were opened along the mesentery, and luminal content was rinsed off with phosphate-buffered saline solution before cutting into 1-cm sections in phosphate-buffered solution containing 15 mM HEPES and 1 mM EDTA with vigorous agitation on a vortex mixer. The tissue was then passed through a 70-μm tissue strainer, and the process was repeated until the wash remained clear. The remaining lamina propria was digested in 1 mg/mL collagenase type VIII [C9722; Sigma Aldrich, St Louis, MO, USA] for 10 min in an orbital shaker at 270 rpm and 37°C. Tissues were vortexed briefly and filtered to remove any remaining undigested material, and cells were counted before flow cytometric evaluation. CD4+ cells were then isolated by positive selection using EasySep™ Mouse CD4 Positive Selection [StemCell Technologies, Vancouver, BC, Canada].

2.7. Real-time PCR analysis
CD4+ cells were isolated from the spleens and mesenteric lymph nodes of healthy C57/BL6 mice. RNA was harvested from CD4+

lymphocytes obtained from the spleens of healthy C57/BL6 mice using Qiagen RNEasy kits. cDNA was subsequently synthesised from 0.5 µg total RNA with RT^2 First Strand Kit [Qiagen, Valencia, CA, USA]. qPCR was carried out using primers provided with the Mouse T Helper Cell Differentiation RT^2 Profiler™ PCR Array [Qiagen], per the manufacturer's instruction. Data analysis was carried out using $\Delta\Delta_{CT}$ method with normalisation of the raw data to endogenous control genes provided with the array.

2.8. Flow cytometry

Cells from indicated compartments were incubated with fluorescently labelled anti-mouse antibodies against: CD4 [GK1.5, BioLegend, San Diego, CA, USA]; IFN-γ [XMG1.2], IL-17a [FFA21], CD25 [PC61.5], FoxP3 [FJK-16s; BD Biosciences, San Jose, CA, USA]; or corresponding isotype controls. Intracellular staining was performed by use of the FoxP3 staining kit [eBioscience, San Diego, CA, USA], according to the manufacturer's instructions. Intracellular cytokine staining was performed following 4 h of stimulation with PMA [50 ng/mL], ionomycin [1 µg/mL], and brefeldin and monensin. Flow cytometric analysis was performed using a BD FACSCanto II [BD Biosciences, San Jose, CA, USA]. FACS was performed by use of a BD FACSAria III [BD Biosciences]. Further analyses were performed by use of FlowJo software [Tree Star, Ashland, OR, USA].

2.9. Statistical analysis

All murine colitis and ileitis studies were performed using a minimum of six mice per group and were repeated a minimum of three times. Statistical analyses were performed using Student's *t* test or repeated measures analysis of variance with Graphpad Prism Data Analysis software [GraphPad Software, La Jolla, CA]. Data were expressed as mean ± standard error of the mean [s.e.m]. Statistical significance was set at $p < 0.05$.

3. Results

3.1. VEN-120 ameliorates chemically-induced acute murine colitis

Previous studies have indicated that LF can ameliorate disease activity in chemically-induced models of colitis.[23–25] We hypothesised that one result of the anti-inflammatory role of VEN120 would be to stimulate the regulatory T cells. Although not considered a T cell-based colitis model, there are nonetheless data suggesting that T cells and specifically Tregs can play a role in the protection from DSS colitis.[28–30] We sought to investigate whether VEN-120 was efficacious in a similar model of colitis. For this purpose, 8-week old C57/BL6 mice were treated with either 500 mg/kg VEN-120 or vehicle [bovine serum albumin, 500 mg/kg] for 7 days while receiving 3% DSS ad libitum in drinking water. Treatment with VEN-120 significantly attenuated weight loss at Days 6 and 7 post induction of colitis compared with vehicle-treated animals [$p < 0.01$, Figure 1A]. The anti-inflammatory effect of VEN-120 administration was evident on gross examination of the colons from the DSS-treated mice, whereby the shortening of colons in vehicle-treated mice was significantly reversed in VEN-120 treated mice [5.0 ± 0.28 cm vs 5.9 ± 0.29 cm; $p < 0.05$, Figure 1B].

Blinded histopathological analysis of the colons from vehicle-treated mice revealed a loss of epithelial integrity and crypt architecture, absence of goblet cells, immune cell infiltration, and hypertrophy of the mucosa muscularis, which was largely reversed in mice treated with VEN-120. Evaluation and scoring of injury and inflammation revealed a significant decrease in histopathological

score from vehicle-treated animals [11.31 ± 0.54] compared with the VEN-120 treatment group [5.271 ± 0.69; $p < 0.0001$; Figure 1C]. This reduction in tissue injury, loss of crypt, architecture, and epithelial integrity, along with the reduction in inflammatory infiltrate, are represented by haematoxylin and eosin [H&E] micrographs [Figure 1D].

3.2. VEN-120 administration induces anti-inflammatory cytokines in CD4$^+$ T cells during chemically induced colitis

In an effort to understand the potential for VEN-120 to affect the inflammatory environment in vivo, we assessed the cytokine profile of CD4$^+$ T cells in the DSS mice treated with either vehicle or with VEN-120. Lymphocytes were isolated from the mesenteric lymph nodes [MLN] and lamina propria [LP] and evaluated by flow cytometry. Figure 2 demonstrates that treatment with VEN-120 resulted in a decrease in the frequency of CD4$^+$ T cells that express pro-inflammatory cytokines, IL-17 [LP: 11.8 ± 0.17% vs 8.7 ± 0.69%, MLN: 18.34 ± 1.3% vs 12.0 ± 0.01%, Figure 2A, B] and IFNγ [LP: 14.78 ± 1.61% vs 6.47 ± 0.34%, Figure 2C], as well as a concomitant increase in CD4$^+$ cells that produce the anti-inflammatory cytokine IL-10 [LP: 8.3 ± 0.09% vs 13.39 ± 1.11% and MLN: 2.33 ± 0.72% vs 4.7 ± 0.75%], which was evident in the LP, but not the MLN of VEN-120 treated mice [Figure 2E, F].

To investigate the role of VEN-120 in the regulation of Treg in the DSS model of colitis, cell populations were evaluated from both DSS-treated groups to assess for the presence of CD4$^+$Foxp3$^+$ cells. By investigating the burden of all CD4$^+$ T cell subtypes, we were able to demonstrate that VEN-120 reduced cellularity at the draining lymph nodes [MLN: 28.4 ± 2.39% vs 20.3 ± 0.5%], but not at the local site of inflammation [LP: 13.28 ± 0.88% vs 13.41 ± 1.2%], indicative of an overall decrease in inflammatory infiltrates following VEN-120 administration [Figure 3A, B]. However, of the populations of CD4$^+$ T cells that are present at both tissue sites, we demonstrate an increase in Foxp3$^+$ CD4$^+$ T cells [LP: 19 ± 0.83% vs 28.7 ± 2.2% and MLN: 8.8 ± 0.5% vs 12 ± 0.49%], indicative of an induced population of Tregs in response to VEN-120 administration [Figure 3C, D]. Taken together, these data indicate an overall decrease in inflammation and subsequent decrease in inflammatory cytokine output at the source of inflammation in the DSS-mediated model of colitis.

3.3. VEN-120 drives a Treg cell phenotype in vitro

To ascertain the potential for VEN-120 to differentially modulate the phenotype of naïve murine Th cells, we evaluated its effects on skewing conditions of Treg and Th1 cells in vitro. Murine naïve CD4$^+$CD25$^-$ T cells were isolated and placed in naïve T cell conditions, or Treg conversion cultures, for 3 days as described in the methods. During this incubation time, T cells were incubated in the absence or presence of 10 nM VEN-120. Cells were then restimulated with PMA/ionomycin in the presence of intracellular transport inhibitor brefeldin A and assayed by flow cytometric intracellular cytokine staining to identify IL-10$^+$ cells. VEN-120 significantly induced an increase in IL-10 expression in both Th0 and Treg skewing conditions [Figure 4A]. In order to investigate the ability for VEN-120 to drive the canonical transcription factor associated with Treg, we placed CD4$^+$ cells in Treg conversion culture in the presence or absence of VEN-120. Following nuclear staining and subsequent flow cytometric analysis, we were able to identify a significant increase in Foxp3$^+$ cells upon treatment with VEN-120 [Figure 4B]. This finding was further supported by the finding that the majority of genes positively regulated by VEN-120 treatment were those
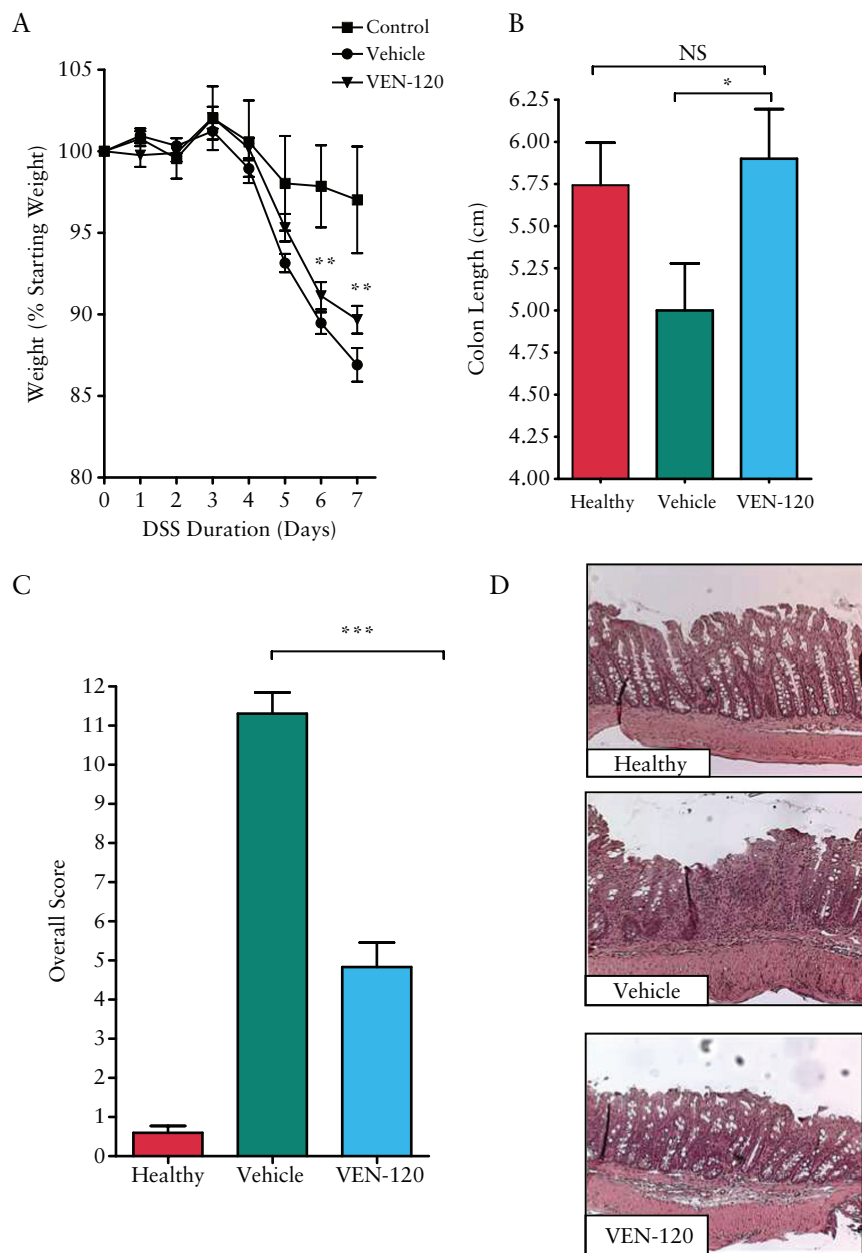
Figure 1. VEN-120 reduces gross inflammatory indices and histopathological damage in a DSS model of colitis. A] Weight loss measurements demonstrated a protection of mice following administration of VEN-120 [*$p < 0.05$ two-way ANOVA, with Bonferoni's post-test]. B] Administration of VEN-120 resulted in a protection of mucosal injury, as demonstrated by preservation of colon length [*$p < 0.05$, Student's t test]. C] VEN-120 reduces overall injury and inflammation score in DSS colitis [***$p < 0.001$, Student's t test]. D] H&E staining of the colon reveals a loss of epithelial integrity, loss of crypt architecture, absence of goblet cells, and immune cell infiltration upon insult with DSS alone, which is rescued by VEN-120 administration at 500 mg/kg/day, representative images, $n = 7$. DSS, dextran sodium sulphate; H&E, haematoxylin and eosin; ANOVA, analysis of variance.

thought to drive Treg, notably *foxp3* and nuclear factor of activated T cell genes [*nfatc1, nfatc2*]. Furthermore, another subset of genes were decreased which are associated with the development of pro-inflammatory T cell subtypes, most notably Il-17, as assessed using a T helper cell differentiation array with pre-determined primer sets corresponding to specific T cell subsets [Supplementary Figure 1, available at as Supplementary data at *ECCO-JCC* online].

3.4. VEN-120 promotes Treg homing to the gut and associated lymph nodes

Following the observation that VEN-120 can affect the fate of naïve T cells in vitro, treatment effects on the T cell repertoire in vivo were

subsequently determined. Healthy C57/BL6 mice were treated either with sham injection [PBS], VEN-120 delivered subcutaneously by osmotic pump [200 mg/kg/day], or by gavage at a dose of 500 mg/kg/day for 14 days. Upon termination of the experiment, tissues were collected [intestinal lamina propria, LP; mesenteric lymph nodes, MLN; axillary lymph nodes, AxLN; peripheral blood mononuclear cells, PBMC; and spleen], and evaluated by flow cytometry for expression of CD4, CD25, and Foxp3. Figure 5 demonstrates that either route of administration of VEN-120 resulted in a marked increase in Treg populations at the intestinal LP, followed by at the MLN and at the spleen. Treg induction did not appear to occur in the lymph tissue adjacent to the site of insertion of the osmotic pump [AxLN], nor
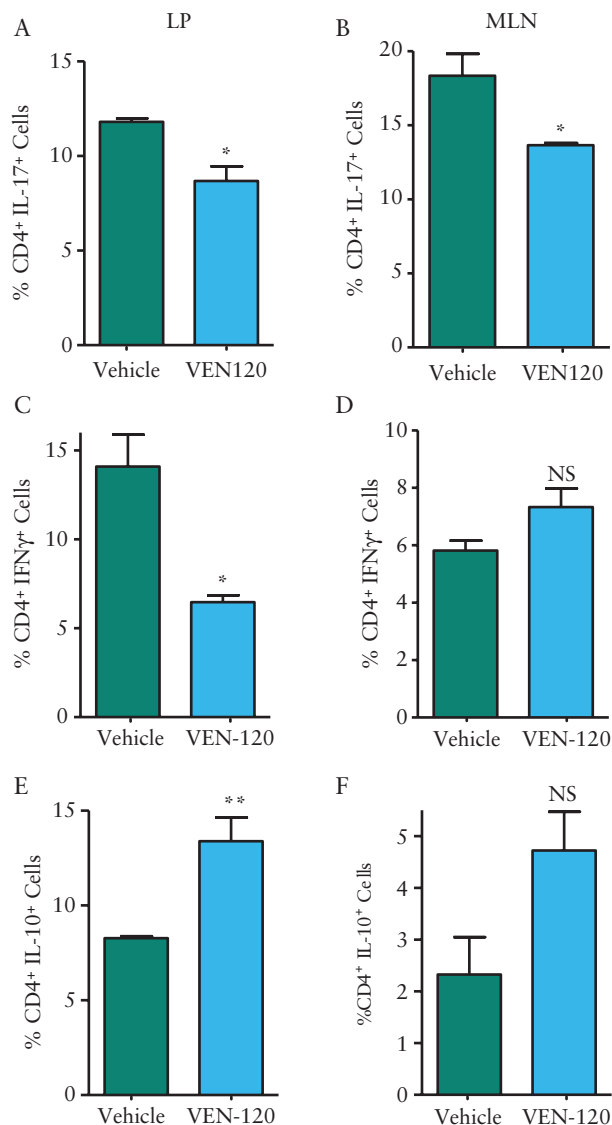
Figure 2. VEN-120 promotes a shift in balance of CD4+ T cells in a DSS model colitis. Flow cytometric analysis demonstrated that VEN-120 reduced expression of the pro-inflammatory cytokine IL-17 in: A] LP CD4+ cells; and B] MLN CD4+ cells with concomitant reduction in the pro-inflammatory cytokine IFNγ in: C] LP CD4+ cells, with no effect demonstrated at: D] the MLN. Conversely, IL-10 producing CD4+ cells were demonstrated to be increased in: E] MLN of mice administered VEN-120. However, in CD4+ cells isolated from: F] colonic LP, no significant effect on IL-10 production was demonstrated, although an increased trend was identified. [*$p < 0.05$; **$p < 0.01$, one-way ANOVA, $n = 7$]. MLN, mesenteric lymph nodes; LP, lamina propria; ANOVA, analysis of variance.

within the circulating blood. This suggests that VEN-120 modulates Treg cell populations and directs them to the intestinal tissues in the absence of disease, regardless of route of administration.

3.5. VEN-120 administration reduces severity of disease in a TNF$^{ΔARE/+}$ model of ileitis

In an effort to demonstrate that the protective effects of VEN-120 are not restricted to a single model, and to interrogate the broad spectrum of disease phenotypes that IBD encompasses, the TNF$^{ΔARE/+}$ murine ileitis model was used. TNF$^{ΔARE/+}$ mice represent a relevant chronic model of Crohns disease [CD] generated by deletion within
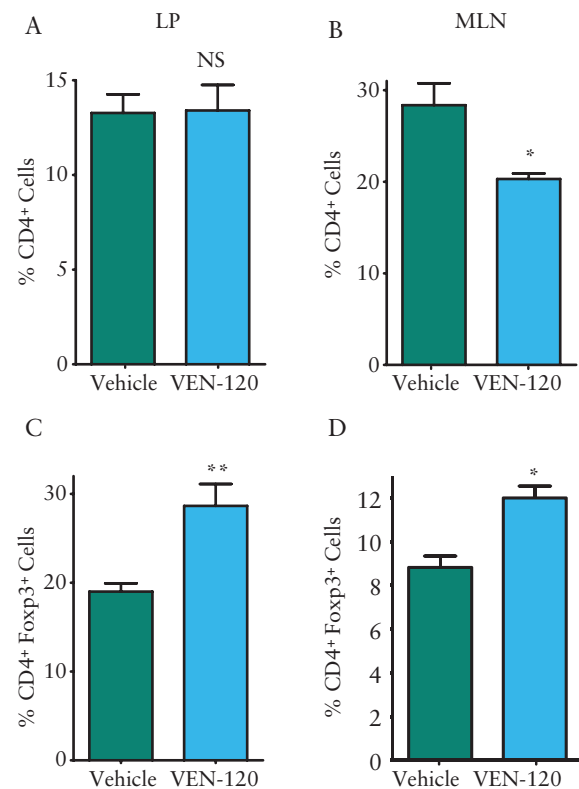
Figure 3. VEN-120 decreases overall T cell burden and promotes Treg phenotype in a DSS model of colitis. Flow cytometric analysis demonstrated CD4+ cell infiltration to be reduced in the VEN-120 treated mice versus the vehicle control at the B] MLN but not A] LP. Of the T cell populations present, an increased frequency in Tregs [CD4+Foxp3+] was observed in mice treated with VEN-120 at both the C] LP and D] MLN. [*$p < 0.05$; **$p < 0.01$, one-way ANOVA, $n = 7$]. DSS, dextran sodium suphate; MLN, mesenteric lymph nodes; LP, lamina propria; ANOVA, analysis of variance.

the AU-rich element [ARE] of the TNF gene in mice[31] [ieTNF$^{ΔARE/+}$]. In order to test the immunoregulatory effect of VEN-120 in these mice, and to remove any potential confounding effects that VEN-120 may have on the gut microbiome and direct effects on the intestinal epithelium, 20-week old TNF$^{ΔARE/+}$ mice were treated for 14 days by subcutaneous delivery with VEN-120 or vehicle [BSA, 200 mg/kg/day] using a 2-week Alzet osmotic pump. As a monitor of disease activity, the effect of VEN-120 treatment on the intestinal barrier function was assessed as described in the Methods section. Figure 6A demonstrates a significant reduction [$p < 0.05$] in permeability of FD4 by roughly 50% from the intestine, suggesting a protective effect of VEN-120 at the site of inflammatory injury. To further demonstrate the protective effects of VEN-120, mice were sacrificed at the end of the 14-day treatment period and tissues harvested for analysis. Herein, we were able to demonstrate that the moderate-to-severe inflammation observed in the sham-treated mice was reversed in the VEN-120 treated mice [Figure 6B, C]. Although VEN-120 did not completely reverse the tissue inflammation over the 14-day treatment period, it significantly reduced inflammatory scoring across all parameters [chronic and active inflammation and preservation of villous architecture]. As an indicator of the anti-inflammatory effects of VEN-120, we harvested various lymphoid tissues [lamina propria, LP; mesenteric lymph nodes, MLN; and spleen, SP] and analysed the infiltration of naïve T cells [CD4+CD44^{Low}CD62L^{High}] at these sites by flow cytometry. Figure 6D demonstrates the ability of VEN-120
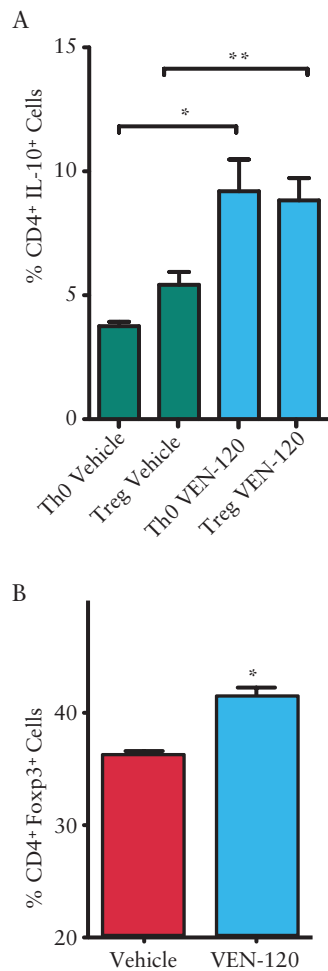
Figure 4. Conversion of CD4+CD25Neg T cells under polarising conditions is skewed by VEN-120 to a pro-regulatory phenotype. A] Isolated CD4+CD25Neg T cells were cultured in either naïve [Th0] or Treg polarising conditions in the presence of 10 nM VEN-120 or vehicle control. VEN-120 significantly induced an increase in IL-10 expression in both Th0 and Treg skewing conditions. B] VEN-120 drives the expression of the Treg transcription factor, Foxp3, under Treg skewing conditions [*$p < 0.05$; **$p < 0.01$, one-way ANOVA $n = 7$]. ANOVA, analysis of variance.

to significantly decrease the number of naïve T cells in all tissues analysed [$p < 0.01$]. Furthermore, in an analysis of unsorted lymphocytes, there was a significant decrease in cellularity at the LP, with an observed increase in cellularity at the MLN and spleen, which may be due to a temperospatial phenomenon of T cells migrating away from the local site of injury to the draining lymph nodes following resolution of inflammation [Figure 6E].

3.6. Oral VEN-120 reduces severity of disease in a TNF$^{\Delta ARE/+}$ model of ileitis

Twelve-week-old TNF$^{\Delta ARE/+}$ mice were given 200 µl by mouth [p.o.] of 500 mg/kg/day of VEN-120 for 14 days. As a positive control, 5 mg/kg anti-TNF monoclonal antibody [IFX; infliximab, Janssen Biotec, Inc.] was given intraperitoneally twice weekly. Upon termination of the experiment, mice were euthanised and tissues collected for further analysis. Mice treated with VEN120 had a significant reversal of pathology, as analysed by a pathologist in a blinded manner. Administration of VEN-120 resulted in improved histopathological scores, to a similar extent as those that were observed with
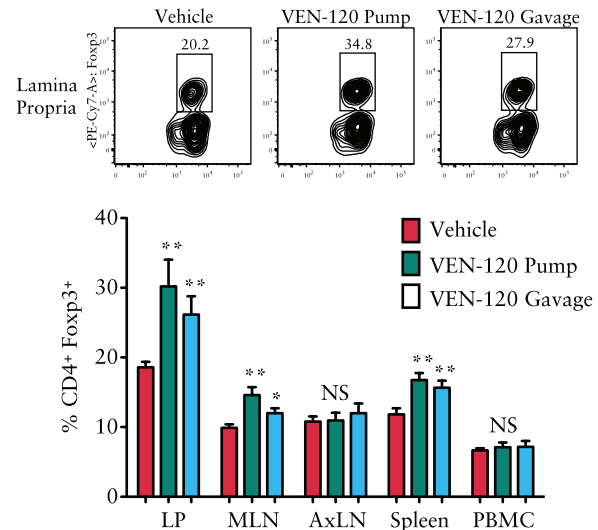
Figure 5. S.Q. and oral administration of VEN-120 results in Treg homing to intestinal tissues and associated lymphoid organs. Healthy, 8-week old C56/BL6 mice were treated with VEN-120 S.Q. by osmotic pump [200 mg/kg/day] or gavage [500 mg/kg/day] for a 14-day period. Flow cytometry revealed the ability of VEN-120 to induce accumulation of Treg at the LP, the MLN, and the spleen. No significant induction of Treg was noted at the AxLN or PBMC. [**$p > 0.01$, one-way ANOVA, n = 7]. S.Q., subcutaneous; MLN, mesenteric lymph nodes; LP, lamina propria; ANOVA, analysis of variance; AxLN, axillary lymph nodes; PBMC, peripheral blood mononuclear cells.

the administration of IFX [Figure 7A]. Most notable was the preservation of normal tissue architecture, with a clear restoration of villus height and the presence of mucus-secreting goblet cells [unstained cells embedded within the mucosal epithelium, Figure 7B].

3.7. VEN-120 drives Treg phenotype in TNF$^{\Delta ARE/+}$ model of ileitis

To gain further insights into the role of CD4+ T cells, we isolated T cells from the LP and MLN, and assessed their phenotypes by flow cytometry. By determining the burden of all CD4+ T cell subtypes, we were able to demonstrate that VEN-120 reduced cellularity both locally at the LP and at the MLN [Figure 7C and D, respectively]. This indicates an overall decrease in inflammatory activity in response to VEN-120 administration. In order to characterise the inflammatory profile of T cells at the site of inflammation, we stained for the cytokine markers of Treg and Th17 cells, IL-10 and IL-17, respectively. Here, we demonstrated that VEN-120 significantly induced IL-10 expression with a concomitant decrease in IL-17 in LP CD4+ cells [Figure 7E and G, respectively]. However, we were unable to demonsrate a similar effect on IL-10 and IL-17 at the MLN [Figure 7F and H, respectively]. Together, these data demonstrate that in a mouse model of IBD, oral administration of VEN-120 decreases the severity of TNF-driven disease as evidenced by a protection of tissue at the histopathological level.

4. Discussion

IBD affects an estimated 1.4 million people in the USA,[32,33] and is associated with high morbidity and decreased quality of life.[34–36,37] Corticosteroids are often used in the short term to achieve symptomatic relief and decreased inflammation, yet are ineffective in mucosal healing and not appropriate for long-term maintenance therapy.[38] Over the past two decades, monoclonal antibodies
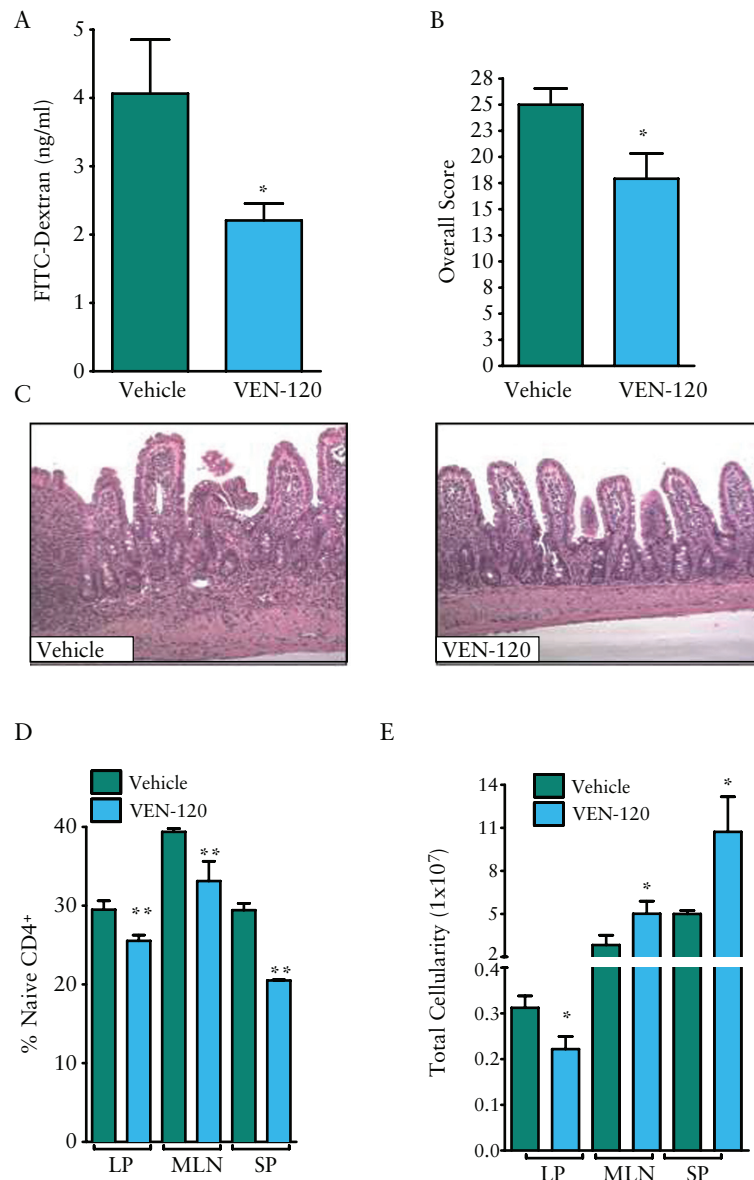
Figure 6. Subcutaneous administration with VEN-120 attenuates chronic murine ileitis. A] VEN-120 treatment of TNF$^{\Delta ARE/+}$ mice resulted in a significant decrease in FD4 transport across the intestinal epithelial barrier. B] Two-week treatment of TNF$^{\Delta ARE/+}$ mice with VEN-120 results in a significant improvement in tissue architecture and a significant decrease in histological indices of inflammation. C] Representative H&E staining demonstrates improved architectural appearance and decreased chronic and acute inflammation. D] Flow cytometric analysis demonstrating a significant reduction in the influx of naïve CD4$^+$ cells known to perpetuate inflammation in this model at the ileal LP, MLN and at the spleen. [*$p < 0.05$; **$p < 0.01$, one-way ANOVA, $n = 6$]. MLN, mesenteric lymph nodes; LP, lamina propria; ANOVA, analysis of variance; H&E, haematoxylin and eosin.

against TNF have revolutionised the treatment and management of IBD.[39–41] However, more than one-third of patients show no response to induction therapy and, for up to 50% of responders, anti-TNF therapy becomes ineffective over time.[42–46] Additionally, anti-TNF therapy has multiple concerning side effects including increased risk of infection, anaphylaxis, and increased incidence of malignancies.[47] Therefore, the development of a novel therapeutic approach, with minimal side effects, for the long-term treatment of IBD represents a significant unmet medical need. The anti-inflammatory potential of LF has been extensively studied in various models of disease. In experimental models of sepsis and rheumatoid arthritis, LF has been demonstrated to exert protection by inhibiting the production of pro-inflammatory cytokines [TNF, IL-6, and IL-1β], and stimulating anti-inflammatory/pro-restitution cytokines [IL-10 and IL-4].[48–51]

Moreover, studies have demonstrated the protective effects of orally administered human LF [hLF] in mice[23] and bovine LF [bLF] in rats in chemically induced models of colitis.[24,25] In each of these studies, administration of LF was associated with a decrease in TNF at the site of tissue injury. Interestingly, in both the DSS[24] and TNBS[25] models of colitis in rats, the Treg-associated cytokine, IL-10, was observed to be increased in colonic tissue following LF treatment. However, the scope of these studies did not ascertain the source of the cytokine in the whole-colon lysates. Although DSS colitis is not a classical T cell-driven model of disease, the induction of IL-10 by LF indicates a potential involvement of Treg as an important mediator of Treg's suppressive function,[52] and Treg has been shown to supress inflammation in this model.[28–30] In a clinical study, bLF was shown to reduce the incidence of necrotising enterocolitis [NEC] in preterm infants. This study identified an increase in Treg cells in the
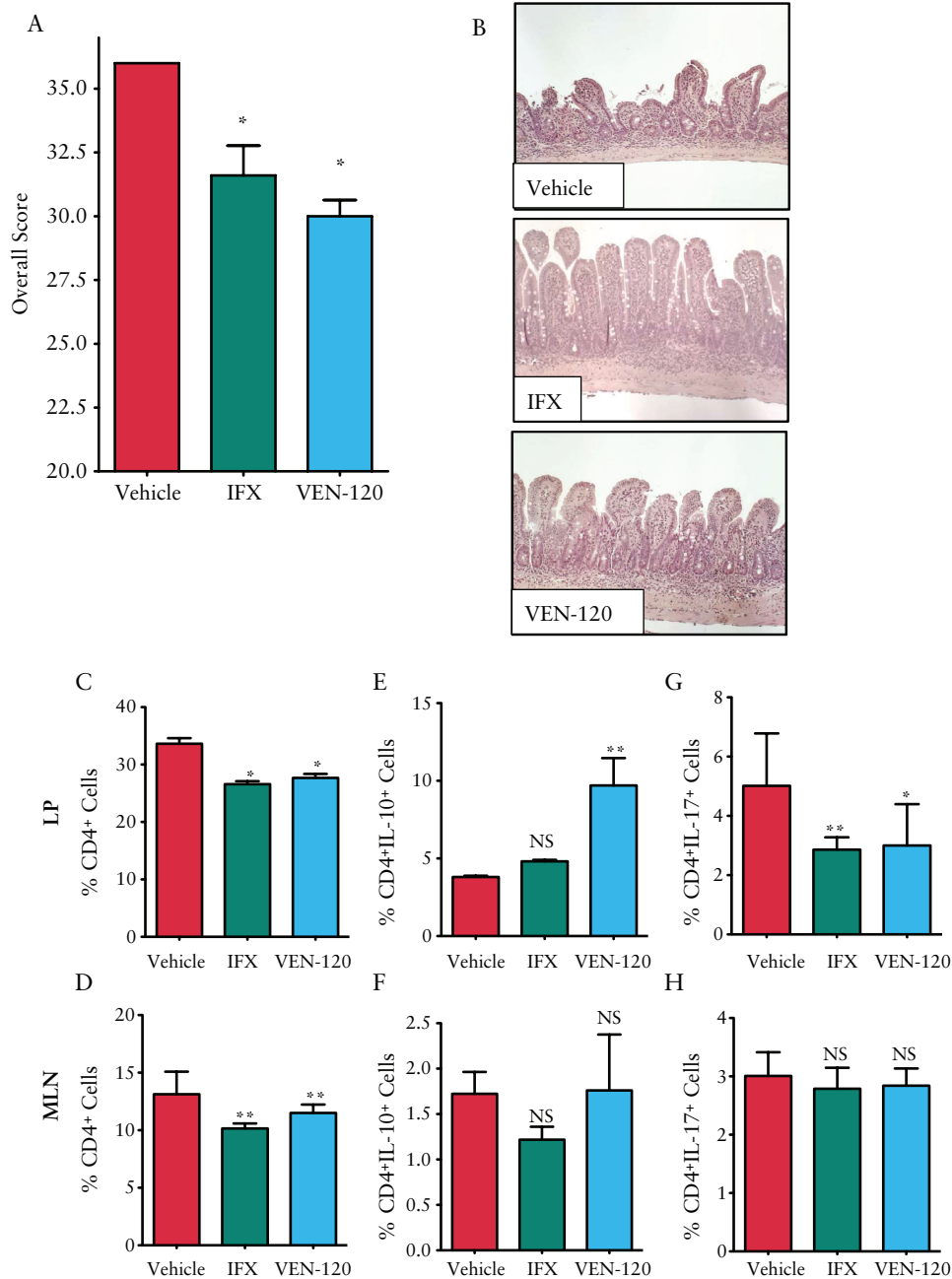
Figure 7. Oral administration with VEN-120 attenuates chronic murine ileitis. A, B] H&E staining demonstrates improved architectural appearance and decreased chronic and acute inflammation following oral administration of VEN-120 or subcutaneous anti-TNF antibody, infliximab [IFX] [*$p < 0.05$; ***$p < 0.0001$, Student's t test]. Flow cytometric analysis demonstrating that VEN-120 administration significantly decreases CD4$^+$ burden at C] the LP, and D] at the MLN in TNF$^{\Delta ARE/+}$ mice. Further interrogation of these T cells identified a significant increase in IL-10 at the E] LP but not the F] MLN of TNF$^{\Delta ARE/+}$ mice treated with VEN-120. A significant reduction in IL-17 expressing cells was demonstrated at G] the LP but not H] the MLN of TNF$^{\Delta ARE/+}$ mice treated with VEN-120 [*$p < 0.05$; **$p < 0.01$, one-way ANOVA, $n = 6$]. MLN, mesenteric lymph nodes; LP, lamina propria; ANOVA, analysis of variance; H&E, haematoxylin and eosin; TNF, tumour necrosis factor.

group of patients that received the bLF, although no experimental evidence was generated in order to directly link bLF adminstration to the increase in Tregs—as Treg populations were noted to rise with age.[53] It is possible that the increase in Treg numbers was simply due to decreased inflammation in the bLF-treated patients. Collectively, these past studies failed to address the direct effect of LF treatment on specific immune cells known to modulate inflammatory pathology.

This current study is the first to describe a novel mechanism by which LF regulates inflammation. Here we interrogate the T cell populations at various peripheral lymphoid tissues in both a chemically

induced model of colitis and a TNF-driven model of CD-like ileitis. Our initial experiments demonstrated that VEN-120 can ameliorate DSS-induced colitis in mice, as has been demonstrated in previous studies with native forms of bovine or human lactoferrin.[23] However, our suggested mechanism of action has demonstrated direct effects of VEN-120 on the immune compartment, with decreased infiltrating CD4$^+$ cells demonstrated at the draining lymph nodes of VEN-120 treated animals, although not evident at the site of local inflammation [LP]. This may be indicative of the late-stage response of accumulation of CD4$^+$ cells at the LP in the DSS model, which

may be evident in a more chronic disease or at a later time point sampling. Furthermore, when phenotyping the cells present at the MLN and LP of VEN-120 treated mice, we were able to demonstrate significant increases in Foxp3⁺ cells, indicative of VEN-120's ability to drive the expression of the canonical Treg transcription factor. VEN-120 was found to induce Tregs at the expense of pro-inflammatory Th17, with a concomitant amelioration of histopathological manifestations in both models. Interestingly, this effect of VEN-120 was found to be similar in magnitude to the administration of the anti-TNF monoclonal antibody (anti-TNF clone CA2, [infliximab]) in the TNF-driven CD-like ileitis model.

Subsequent experiments aimed at further elucidating this novel mechanism noted the significant increase in Treg frequency in the intestinal LP and the draining MLNs in healthy mice treated orally or via osmotic pump with VEN-120. However, no significant increase in Treg was noted in the peripheral blood or in the axillary lymph nodes adjacent to the site of implantation of the pump. Collectively, these data suggest the ability of VEN-120 to activate a tolerogenic phenotype, which is likely a function of LF's importance in the infant's early stages of development of a healthy gut immune system which can accommodate commensal bacteria.[54] Furthermore, these studies agree with other previously published reports that Treg homing to the gut is required for immunological tolerance,[55] and that specific effect on Tregs by factors such as retinoic acid affects their ability to preferentially home to distinct tissues and increase their protective effects in a model of T-cell induced murine colitis.[56–58]

Although LF may act to alter the gut microbiome in health and disease, we show that in vitro T cell experiments and subcutaneous administration of LF result in direct skewing of T cell populations and less severe disease in vivo. Together, these data suggest that LF may act without direct action on the gut microbiome. However, given the importance of specific microbial species in the context of IBD, further investigations are required to assess the role LF may play in influencing the microbial landscape.

The induction of immune tolerance is currently a topic of intense interest in several disease areas. For this reason, several groups are investigating the therapeutic potential of cell therapy using Tregs to ameliorate autoimmune disorders such as acute graft-versus-host disease [GVHD][59,60] and type I diabetes.[61] The use of an oral therapy consisting of an endogenous protein with a proven safety profile[19,62] to induce Tregs may be a more attractive approach to treating autoimmune disease and may be associated with lower cost. Furthermore, LF has been shown to exhibit resistance to proteolytic breakdown and loss of iron at low pH, thereby allowing it to retain its native structure and function.[63]

Whereas VEN-120 was shown to significantly influence the phenotypic skewing of naïve T cells towards Treg and away from pro-inflammatory Th17 cells, studies have not addressed the upstream mechanisms responsible for this event, either receptor engagement or intracellular signalling events. The molecular mechanisms through which LF exerts its anti-inflammatory effects are not completely understood but appear in part to occur through the modulation of transcription factors, such as the inhibition of nuclear factor kappa B [NFκB] signalling pathways.[64–68] These events may be downstream of a number of putative LF receptors such as Toll-like receptor 4 [TLR4],[67] low-density lipoprotein receptor-related protein-1 [LRP-1],[69] intelectin-1 [also known as omentin-1],[70] and CXCR4.[71] As an extension of these potential signalling events, VEN-120 was found to induce the transcription of various genes that are associated with the development of Treg. Most notable were the induction of *foxp3, nfatc1, nfatc2,* and *il2ra* genes. Interestingly, this is in agreement with

the observation that VEN-120 acts to drive Treg in vivo and attenuate disease pathology in models whose activity is largely influenced by the overabundance of pro-inflammatory T cell subtypes and in which the induction of Treg could result in amelioration of disease. Although the scope of this current trial did not investigate the events from receptor binding to gene and protein regulation, future efforts are warranted to more clearly delineate the mechanism by which LF drives an anti-inflammatory response.

Although we demonstrate that LF acts through the promotion of Tregs to restrict disease, it cannot be ignored that LF may act to influence diverse cell types to achieve normal immune function and homeostasis. It has also been reported that bLF acts as a potent anti-inflammatory agent on monocytes by triggering a tolerogenic-like programme during their differentiation into dendritic cells [DC].[62] Other groups have suggested that LF may act to preserve barrier function in epithelial cells in vitro; however, we have been unable to demonstrate an ability of VEN-120 to prevent decreases in transepithelial resistance in Caco-2 and T84 transwell following inflammatory insult [data not shown]. These studies identify a complex role for LF in immunomodulation, which may represent the requirement for initial activation of the inflammatory response in an orchestrated manner, together with the requirement for a dampening of inflammation to prevent a sustained pathophysiological outcome. Numerous studies have identified an increase in faecal LF in patients with active IBD,[72,73] and it has been suggested as a potential biomarker to monitor disease progression. Although the levels of LF are elevated in these patients, there are many possible explanations as to the lack of benefit from this secreted LF. This increased LF output could be due to increased neutrophil degranulation at the site, or distal to the area of inflammation, where it may not be exerting an effect due to mechanical washout in the setting of diarrhoea. Additionally, it is conceivable that there is an increased loss of LF into the luminal contents during flareup of inflammation which results in increased faecal LF levels. Thus, augmenting LF by either systemic or topical administration may result in a reconstitution of sufficient concentrations of LF and thereby augment its activity, as has been suggested with other proteins elevated in inflammation such as alpha-1-antitrypsin,[74] grehlin,[75] and adrenomedullin.[76]

Regardless of additional mechanisms that may be important for the anti-inflammatory abilities of LF, the importance of VEN-120 and LF to induce Treg populations may be of particular interest when designing future human trials. In light of our current findings, we speculate that the use of LF and various related peptides and isoforms may be deleterious in the treatment of diseases whose aetiology is driven in part by immune evasion as may be seen in certain neoplasms and sepsis. The role of Treg signalling has been demonstrated to result in higher rates of proliferation and increased disease progression in models of breast,[77] pancreatic,[78] and lung cancer.[79] Indeed, this may in part explain the failure of LF- based therapies in several clinical trials. When patients were administered talactoferrin alpha, a recombinant form of hLF to treat severe sepsis, the 28-day mortality rate was higher in talactoferrin-treated patients, although not statistically significant. However, in-hospital mortality rates were significantly higher in talactoferrin-treated patients than in patients in the placebo group.[80] Furthermore, talactoferrin conferred no advantages in 6-month survival, progression-free survival, or disease control rate in patients with advanced non-small cell lung cancer.[81]

The data presented here demonstrate for the first time that lactoferrin acts directly to modulate the immune response in a TNF-driven model of Crohn's-like ileitis and DSS-induced colitis, with

rescue of normal intestinal physiology as demonstrated by enhanced gut barrier function. Furthermore, VEN-120 reverses the severe chronic-stage pathology seen in both TNF$^{\Delta ARE/+}$ and DSS mice, with reversal of tissue damage and a decrease in associated T cell infiltration. Taken together, these data suggest that LF acts by skewing the phenotype of CD4$^+$ cells away from a pro-inflammatory Th17 phenotype and towards a tolerogenic Treg phenotype to modulate inflammation. This mechanism of action through which rhLF can be used to target inflammation is relevant not only to models of IBD, but potentially to other diseases in which excessive inflammation drives chronic pathologies such as rheumatoid arthritis and multiple sclerosis.

Funding

Conflict of Interest

CM and RA are employees of Ventria Bioscience. CC, TN, and EdZ report no conflict of interest relating to the submitted work.

Author Contributions

The authors listed herein had significant input into the concept and design of the study [CM, CC, RA, EdZ], acquisition of data [CM, TN], analysis and interpretation of data [CM, CC, PJ, EdZ], drafting the article or revising it critically for important intellectual content [CM, CC, RA, EdZ], and final approval of the version to be submitted [CM, RA, EdZ].

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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