Title: Can we target endogenous anti-inflammatory responses as a therapeutic strategy for inflammatory bowel disease?

Authors and Affiliations:

Ross John Porter BSc (Hons)[1], Caroline Andrews DVM[2], Daniel Paul Brice BSc (Hons)[1], Scott Kenneth Durum PhD[2] and Mairi Hall McLean MBChB (Hons) PhD[1]

[1] School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Scotland, UK

[2] Laboratory of Molecular Immunoregulation, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, USA

Corresponding Author:

Dr. Mairi H. McLean

The Institute of Medical Sciences

Foresterhill, Aberdeen

Scotland, UK, AB25 2ZD

Telephone: 01224 437319

Email: m.h.mclean@abdn.ac.uk

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ABSTRACT

Inflammatory bowel disease (IBD) describes chronic relapsing remitting inflammation of the gastrointestinal tract including ulcerative colitis and Crohn's disease. The prevalence of IBD is rising across the globe. Despite a growing therapeutic arsenal, current medical treatments are not universally effective, do not induce lasting remission in all, or are accompanied by short and long-term adverse effects. Therefore, there is a clinical need for novel therapeutic strategies for IBD. Current treatments for IBD mainly manipulate the immune system for therapeutic gain by inhibiting pro-inflammatory activity. There is a robust endogenous immunoregulatory capacity within the repertoire of both innate and adaptive immune responses. An alternative treatment strategy for IBD is to hijack and bolster this endogenous capability for therapeutic gain. This review explores this hypothesis and presents current evidence for this therapeutic direction in immune cell function, cytokine biology, and alternative mechanisms of immunoregulation such as microRNA, oligonucleotides and the endocannabinoid system.

Keywords: Inflammatory Bowel Disease, immunoregulation, cytokine, ulcerative colitis, Crohn's disease, treatment.

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INTRODUCTION

Inflammatory bowel disease (IBD) describes relapsing, remitting chronic inflammation of the gastrointestinal tract. The main phenotypes are ulcerative colitis and Crohn's disease. The prevalence of IBD remains highest in Europe and the United States, and continues to rise across the globe.[1] The aetiology of IBD is complex and involves dynamic interplay between genetics, the immune system and environmental exposures including intestinal microbial dysbiosis.[1] Advances in IBD research has widened therapeutic opportunities and impacted on disease treatment and natural progression.

Current treatments for IBD aim to inhibit pro-inflammatory immune responses, targeting both inflammatory cells and cytokines. There are several agents available to achieve this including steroids, thiopurines and biologic anti-tumour necrosis factor (TNF) and anti-integrin therapies. However, not all patients are responsive to these drugs.[2] There are emerging novel biologics for IBD for example anti-p40 biologic targeting interleukin (IL)-12 and IL-23 offering multiple cytokine inhibition. Trials have suggested that these new biologics are not globally effective across all IBD patient cohorts.[3] Furthermore, currently available drugs are associated with serious adverse side effects including susceptibility to infection, bone marrow suppression, liver dysfunction and increased risk of malignancy.[2] Therefore, there is a clinical need for newer, safer treatments for IBD.

There is a robust endogenous immunoregulatory capacity within the repertoire of both innate and adaptive immune responses. An alternative treatment strategy for IBD could be to hijack and bolster this endogenous capability for therapeutic gain. This review explores this hypothesis and presents current evidence for this therapeutic direction.

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IMMUNE CELL POPULATIONS

Regulatory T-Cells

Regulatory T cells (Tregs) are vital contributors to immune homeostasis. There are various subsets of Tregs, including IL-10 producing Tr1 cells, $CD4^+CD25^+Foxp3^+$ or $CD4^+CD25^-Foxp3^+$ Tregs and IL-35 producing T-cells.[4] $Foxp3^+$ Tregs include thymic-derived Tregs (for central tolerance) and peripheral induced $CD4^+$ T-cells (for local tolerance). Thymic-derived Tregs naturally express Foxp3 and peripheral $CD4^+$ T-cells can upregulate Foxp3 under certain circumstances, such as in response to pro-inflammatory cytokines.[4]

In the intestine, Tregs secrete anti-inflammatory cytokines and modulate T-cell function to exert immunosuppressive activity. Tregs also downregulate co-stimulatory molecules on antigen presenting cells to maintain immune tolerance through expression of T-cell receptors specific for enteric flora and dietary antigens. Imbalance between Treg and effector T-cell populations is an important mechanism underlying IBD pathogenesis.[4,5]

IBD patients in remission express more $CD4^+CD25^{high}$ and $Foxp3^+$ Tregs in their peripheral bloodstream, compared to patients with active disease.[6] These cells maintain their suppressive role, determined by proliferative suppression of $CD4^+C25^-$ T-cells, correlated with disease activity and CRP levels.[6,7] In the gastrointestinal mucosa, patients with IBD express more $CD4^+CD25^{high}$ and $Foxp3^+$ Tregs compared to non-inflamed mucosa.[6] Compared to normal mucosa, patients with ulcerative colitis express more $Foxp3^+$ Tregs in their inflamed and non-inflamed mucosa. Expression appears predominantly in the lamina propria.[8] In mesenteric lymph nodes of patients with ulcerative colitis, $CD4^+CD25^+$ cells express Foxp3 and suppress the proliferation of autologous $CD4^+CD25^+$ cells.[8] Overall, IBD is associated with reduced peripheral and modestly increased Tregs in the colonic mucosa. Whilst many

questions remain unanswered in Treg biology, the question relevant to this review is potential promotion of the potent anti-inflammatory activity of Tregs as a therapeutic strategy for IBD.

Promotion of the anti-inflammatory functions of Tregs can be achieved indirectly through multiple diverse actions and there is a broad base of investigative work in the literature to this effect. Intestinal dysbiosis plays a pivotal role in IBD pathogenesis.[1] Indeed, bacterial components are important for Treg function, for example, spore-forming clusters IV and XIVa of the Clostridium genus promote Treg accumulation in the colons of mice.[9] Hence, manipulation of intestinal bacteria may bolster the mucosal Treg population and promote a tolerogenic microenvironment. Butyrate, an energy source for colonocytes, can promote Treg activity and suppress serum and colonic mucosal levels of IL-17, leading to amelioration of TBNS-induced colitis in rats.[10] Lactoferrin, an endogenous pleiotropic protein secreted at mucosal sites, can modulate inflammation by shifting the phenotype of $CD4^+$ T-cells away from Th17 and towards Tregs.[11]

Oral immune therapies introduce low dose antigens to the gut to manipulate the local host immune system and this strategy can preferentially expand mucosal Treg populations to promote oral antigenic tolerance. There have been numerous promising pre-clinical studies and a few clinical trials that have suggested benefit in IBD, as comprehensively reviewed elsewhere.[12] An example of this strategy is the oral administration of non-absorbable autologous colonic protein-derived antigens (Alequel). This ameliorated immune-mediated colitis in mice and induced remission in human Crohn's disease in phase I and II trials.[13–16] The mechanism is not fully understood but likely involves promotion of Treg cells, upregulation of IL-10, and an altered $CD4^+/CD8^+$ ratio.

Another example of enhancing Treg responses as part of a wider immunosuppressive repertoire is the use of anti-CD3 monoclonal antibody biologics, such as otelixizumab. The

mechanism of action is multifactorial, including inhibition of antigenic stimulation by internalisation of the CD3/TCR complex inhibiting antigenic stimulation, promotion of effector T cell apoptosis, and amplification of TGFβ signalling to create a tolerogenic microenvironment via promotion of Treg and tolerogenic dendritic cell populations.[17] These novel biologics have been assessed in different autoimmune conditions, with particular focus in Type 1 diabetes mellitus. In a murine model of IBD, use of an anti-CD3 antibody attenuated T cell transfer-induced enterocolitis.[18] These agents have been trialled in humans in both acute severe ulcerative colitis[19–21] and Crohns disease.[22] Although initial small studies were optimistic, larger studies showed this agent was not effective in acute severe UC and was associated with an unacceptable toxicity profile. Different biologics to the CD3 receptor are in clinical development and emerging and this is likely to continue as a future area of therapeutic development in IBD.

Can we use regulatory T cells directly as a therapy? Adoptive transfer of autologous Tregs has emerged as another promising therapeutic avenue in IBD. This strategy of enhancing endogenous cell based immunoregulation has been shown to be effective in pre-clinical models.[23–28] For example, adoptive transfer of $CD4^+CD25^+$ Tregs in a T cell transfer model of murine IBD significantly reversed clinical and histological markers of colitis by migrating to the intestine, proliferating and directly interacting with effector T cells.[26] Challenges in application of this treatment to humans include *in vitro* cell expansion to achieve an effective dose, potential plasticity of the expanded cells towards a pro-inflammatory phenotype, and ensuring an effective and consistent homing to the GI tract on infusion. Canavan and colleagues published data to show successful isolation, characterisation and expansion of a specific Treg population deemed suitable for this

application.[29] A clinical trial (TRIBUTE) to assess these cells in human Crohn's disease is in the early stages of recruitment (UK Trials Gateway; ISRCTN97547683).

Another group reported data from a 12-week, open-label, multi-centre phase 1/2a clinical trial to investigate the safety and efficacy of adoptive transfer of a different Treg subset, namely intravenous ovalbumin-specific Tr1 Tregs in humans, expanded from peripheral mononuclear blood cells, in refractory Crohn's disease.[30] This treatment was efficacious in 40% of patients, dose-dependent (lower dose was more efficacious) and was well tolerated with few adverse effects. A larger multi-centre international randomised placebo controlled study of this antigen specific Treg therapy (Ovasave) began but terminated early (NCT02327221). The early termination of this study was reportedly due to manufacturing difficulties and the trial is to recommence recruitment (https://www.biopharma-reporter.com/Article/2016/05/27).

The therapeutic potential of this cell population may extend beyond quashing inflammation to hindering colitis-associated colon cancer, making further study of these populations paramount.[35]

Immunoregulatory B-Cells

B cells are an integral part of the adaptive immune system that produce antibodies, secrete cytokines, and can also communicate with other cells including Tregs to maintain gut homeostasis,.[36] In humans, therapies that target B-cells, for example rituximab, are not effective in IBD and can clinically exacerbate, or even induce colitis.[37] This observation affirms an important, protective role of B-cells in maintaining intestinal homeostasis.

In recent years, interest has grown around a 'regulatory' phenotype that can secrete IL-10, IL-35 and transforming growth factor (TGF)-β to maintain peripheral tolerance.[38] In this

review, IL-33 and IL-35 are latter discussed as anti-inflammatory cytokines with a therapeutic potential worth exploiting for therapeutic gain. IL-10-producing B cells are present in the gastrointestinal mucosa of patients with active Crohn's disease and ulcerative colitis compared to healthy controls.[39]

Sattler and colleagues reported that intraperitoneal IL-33 injection into $IL10^{-/-}$ mice exacerbated colitis whereas IL-33-treated wildtype mice (with intact IL-10 receptors) didn't develop colitis.[40] Upon investigating the underlying mechanism, there was no change in the frequency of $CD25^{+}$ or $Foxp3^{+}$ Tregs, but increased IL-10 producing B cells ($Breg^{IL\text{-}33}$) in wildtype, but not $IL10^{-/-}$ mice. Adoptive transfer of these $B^{IL\text{-}33}$ cells transferred into $IL\text{-}10^{-/-}$ mice led to histological and clinical improvements in colitis. Taken together, this important study suggests that IL-10 deficiency reduces the function of this immunoregulatory cell population.

In support of these findings, intravenous adoptive transfer of peritoneal cells into mice with DSS-induced colitis improved disease activity scores, mucosal recovery and survival rates.[41] The mechanism is likely through dual activity of IL-10 and TGF-β secreted by natural regulatory B cells (and also macrophages).[41] Adoptive transfer of these immunoregulatory B cells may therefore be an attractive treatment in the future but this needs further assessment in pre-clinical models of IBD alongside a greater understanding of the biological function of the cells in the human gastrointestinal tract.

Tolerogenic Dendritic Cells

Dendritic cells are professional antigen presenting cells that induce immune tolerance within the gastrointestinal mucosa to luminal contents.[42]

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The mechanisms that underlie the anti-inflammatory role of dendritic cells is not fully defined. Their influence over pro-inflammatory T-cells and Treg development is likely vital to protective functions. For example, $CD103^+$ dendritic cells isolated from mesenteric lymph nodes can induce the differentiation of naïve $CD4^+$ T-cells into $Foxp3^+$ Tregs.[43] $CD103^+$ dendritic cells are downregulated during experimental colitis and produce thymic stromal lymphopoietin in response to TLR activation, which suppresses the secretion of IL-17 from T-cells and promotes $Foxp3^+$ Treg development.[44] In human IBD, patients with Crohn's disease have more $CD103^+$ lymphocytes in their lamina propria compared to patients with ulcerative colitis or healthy controls.[45]

The anti-inflammatory capabilities of $CD103^+$ dendritic cells are reduced in murine colitis models.[46] The ability of $CD103^+$ dendritic cells to promote the development of $Foxp3^+$ Tregs is significantly impaired in T-cell induced colitis in $RAG^{-/-}$ mice and, instead, $CD103^+$ dendritic cells appear to favour the development of interferon-γ-producing $CD4^+$ T-cells in the inflammatory microenvironment.[46] This is discouraging when considering the application of dendritic cells as cell therapy for IBD. Nonetheless, there have been some encouraging data. Vasoactive intestinal peptide-induced dendritic cells can induce Tregs, which show a protective and therapeutic value in trinitrobenzene sulfonic acid (TNBS)-induced murine colitis.[47] Granulocyte-Colony Stimulating Factor (G-CSF) induces IL-10-secreting T-cells in the peripheral blood, as well as plasmacytoid dendritic cells in the lamina propria in human Crohn's disease patients who clinically respond to G-CSF therapy.[48]

Colonic microbiota play a role in the pathogenesis of IBD.[1] These bacteria impact on mucosal dendritic cell function, and there is evidence that this interaction can lead to promotion of immunoregulatory mechanisms including oral tolerance.[49] $CD103^+CD11b^+$ dendritic cells can recognise flagellin in the lamina propria and upregulate IL-23 which induces

epithelial cells to express RegIII-γ, a bactericidal c-type lectin, in response to TLR-5 activation.[50] Mice treated with caecal bacterial antigen-pulsed regulatory dendritic cells secrete high levels of IL-10 and ameliorate colitis in T-cell transfer-induced murine colitis.[51] In the same study, regulatory dendritic cells also induced the differentiation of naive $CD4^+$ T-cells into $CD4^+CD25^+Foxp3^+$ T-cells *in vitro* and *in vivo*. These findings support the growing hypothesis that the gut microbiota can shape dendritic cell populations. The question is whether this can be targeted for therapeutic gain in IBD.

In keeping with this hypothesis, *Bifidobacterium infantis* increases $CD103^+$ and retinoic acid metabolising dendritic cells that induce $Foxp3^+$ Tregs and suppress Th17 cells.[52] This partially explains why *Bifidobacterium infantis* can suppress IL-17 production in DSS-induced murine colitis and induce IL-10 *in vitro*.[53] Taken together, it would appear that adoptively transferred tolerogenic dendritic cells could prove therapeutically beneficial for IBD and this continues to be explored in pre-clinical models. However, it is clear that dendritic cell plasticity and function in the gastrointestinal mucosa is complex with conflicting data and further work will be required to translate to human disease. In a single-centre, phase 1 trial, 9 patients with treatment-refractory Crohn's disease were treated with autologous transfer of tolerogenic dendritic cells. These cells were derived from blood monocytes in vitro and delivered by ultrasound guided intra-peritoneal injection. The treatment was well-tolerated, induced remission in 1 patient and a showed good clinical response in 2 patients.[54] Additional clinical trials of this therapy are recruiting (Intralesional Tolerogenic Dendritic cells in Crohn's Disease Treatment (TolDecCDintra), NCT02622763).

Further mechanistic studies are required to better understand dendritic cell biology in the gastrointestinal tract and identify the target patient population most likely to benefit from this cell therapy.

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Innate Lymphoid Cells

Innate lymphoid cells (ILC) are a recently discovered population of immune cells.[55] ILCs are sub-classified into type 1, type 2 and type 3, based on transcription factor association, cell surface marker expression and cytokine response.[55] Unlike T-cells and B-cells ILCs do not express recombined antigen-specific receptors.[56] The biology of ILCs has been summarised elsewhere.[55–57] In summary, group 1 ILCs constitutively express transcription factor T-bet and are activated in response to IL-12 and IL-18. They secrete type 1 cytokines including interferon-γ. Their key role is to promote immunity to intracellular bacteria, viruses and parasites. Group 2 ILCs express GATA3 and ROR-α and are activated in response to alarmins such as IL-25, IL-33 and thymic stromal lymphopoietin. They secrete type 2 cytokines including IL-4, IL-5 and IL-13. Their key role is to promote immunity to helminths. Group 3 ILCs constitutively express ROR-γt and are activated in response to IL-23, IL-1α and IL-1β. They secrete IL-17, and IL-22. Their key role is to promote immunity to extracellular bacteria, especially to maintain tolerance to commensal gut microbiota.

In IBD, patients with Crohn's disease have an increased expression of intraepithelial type 1 ILCs within the colonic mucosa.[58] These cells produce interferon-γ and worsen colitis in anti-CD40-induced murine colitis, so may be contributing to disease pathogenesis.[58] Suppressing the expression and function of such cells may be therapeutically advantageous. Group 3 ILCs play a role in maintaining intestinal homeostasis and could be a promising cellular target. Specifically, a subset termed IL-22 producing NCR^+ (CD56NKp44) group 3 ILCs, make up around 5% of normal colonic lymphocytes, resist mucosal infection and promote epithelial barrier integrity.[59] Although present in small numbers relative to other lymphocytes, ILCs exert a potent effect on immune responses.[60] The NCR^+ subtype confers

protection in DSS-induced and T-cell transfer murine colitis, through increased secretion of IL-22 resulting from retinoic acid signaling[61] or expression of aryl hydrocarbon receptor (essential for the maintenance and function of type 3 innate lymphoid cells).[62]

The emergence of this new cell type with a key role in the arena of gastrointestinal mucosal immunity offers a potential new therapeutic target in IBD. However, a significant amount of pre-clinical investigation and a better characterisation of their role in the human GI tract in health and disease is required before translational studies can be considered and developed. Caution is warranted as ILCs are central to the development of inflammation associated colonic cancer through IL-22 stimulation of epithelial cell proliferation.[63]

ANTI-INFLAMMATORY CYTOKINES

IL-10

Therapeutically enhancing interleukin (IL)-10, the quintessential anti-inflammatory cytokine, showed promising efficacy in pre-clinical models of IBD, but unfortunately human trials to date have failed to show a direct translational benefit in human patients.[64] This is likely to reflect the differences in biology from murine models to human disease alongside considerations in patient recruitment, disease characterisation and cytokine delivery. As such, there still is interest at progressing this therapeutic strategy forward. For example, a phase 1 double-blind randomised control trial to assess safety, tolerability and pharmacology of IL-10 was commenced, but prematurely terminated in 2016 due to potential risk of further dosing in healthy subjects (NCT02711462). Currently, a phase 2a double-blind randomised control trial is underway to assess IL-10 as an add-on therapy to infliximab in patients with active ulcerative colitis (NCT03269695).

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Back in the laboratory, success of pre-clinical studies have fuelled the race to identify novel anti-inflammatory cytokines with translational capability to bolster the immunoregulatory capacity of the immune system to sequester inflammation, often by indirectly upregulating IL-10.

IL-27

The known biology of IL-27 has recently been reviewed.[65] In brief, interleukin-27 (IL-27) is a heterodimeric type-1 cytokine expressed by many cell types including dendritic cells, macrophages, plasma cells, epithelial cells and endothelial cells. IL-27 is composed of Epstein-Barr virus-induced gene 3 and p28 subunits, and the IL-27 receptor is composed of two subunits, namely gp130 and IL27Rα. Upon receptor binding, Janus Kinase/STAT, PI3K/Akt or MAPK signalling pathways are activated. This results in phosphorylation of STAT1, STAT3, STAT5 and STAT6. The best known downstream effects of IL-27 involve regulating T-cell responses (for example, inhibition of the lineage commitment of Th17 cells by blocking retinoic acid receptor-related orphan receptor (ROR)-γt, preventing Fas-mediated activation-induced cell death, and inhibiting Th2 differentiation and cytokine expression through increasing T-bet and suppressing GATA-3 expression) and inducing IL-10 production from Tr1 T regulatory cells.

An observational, cross-sectional study of 54 IBD patients reported that IL-27 mRNA is increased in patients with active Crohn's disease compared to inactive disease with higher mucosal IL-27 protein expression in patients with active ulcerative colitis, compared to tissue controls or active Crohn's disease.[66] The biological consequences of this expression profile in the inflamed mucosa is unclear, although a protective effect is suspected through extrapolation of IL-27 mediated immunosuppressive capabilities.[65]

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There have been several murine studies investigating the role of IL-27 in different colitis models. In T-cell transfer models, IL-27 is essential for enhancing Treg function and enabling IL-10 secreting B-cells to attenuate colitis.[67,68] In DSS models, colitis is more severe and survival reduced in IL-27-$R^{-/-}$ mice.[69] IL27-$R^{-/-}$ in $RAG^{-/-}$ mice also suffer more severe colitis compared to $RAG^{-/-}$ mice with intact IL-27 receptors.[69] Hanson and colleagues demonstrated that mucosal delivery of IL-27, by a *Lactococcus lactis* bacterial vector, is therapeutically beneficial in both T-cell transfer and DSS models of murine colitis, and is dependent upon increased IL-10 production by T-cells in the gastrointestinal mucosa.[70] Mucosally delivered IL-27 attenuated innate cell driven acute murine colitis through IL-10 dependent, T cell independent mechanisms including inhibition of chemokine gradient and neutrophil infiltration.[71] These data suggest that IL-27 promotes immunosuppression through IL-10 amplification. However, neutralisation of IL-27 worsens colitis in IL-$10^{-/-}$ mice indicating other mechanisms are also important.[72] Furthermore, IL-27 can suppress Th17 and Th1 differentiation independent of IL-10.[72] Treating murine colitis (induced by both TNBS and DSS) with single-chain human IL-27, delivered by subcutaneous osmotic pumps, clinically improves colitis through suppression of IL-17 producing T-helper cells.[73] Therefore, IL-27 appears capable of attenuating colitis by IL-10 dependent and independent mechanisms.

However, there is conflicting murine data in the literature that require further consideration. It has been reported that IL-27 promotes colitis, enhancing IL-6 and IL-1β production and promoting Th17 differentiation; loss of IL27Rα has been associated with clinical improvements in murine colitis models.[74]

Most of this data arises from preclinical models. The role of IL-27 in human IBD requires characterisation before IL-27 directed therapy for IBD can be further explored in the clinical arena.

IL-33

Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines and is constitutively expressed in the nucleus of most epithelial, endothelial and stromal cells where it regulates gene expression through stability of chromatin structure.[75] IL-33 can sequester NF-κB to dampen pro-inflammatory responses and can be released into the extracellular environment to function as an 'alarmin' or damage associated molecular pattern.[75] Immune cells can actively secrete IL-33 in response to pro-inflammatory cytokines such as TNF, IL-1β, IL-3 and IL-4.[75] Given the complexities of pro- versus anti-inflammatory effects, the role of IL-33 in IBD remains unclear. Again, most data have been derived from pre-clinical models of IBD and the translational impact of this to the human disease is not yet defined.

In the gastrointestinal tract, from an anti-inflammatory perspective, IL-33 and its receptor ST2 are expressed on colonic Tregs.[76] IL-33 promotes TGF-β1-medited Treg differentiation and the accumulation of Tregs in an inflammatory environment.[76] IL-33 promotes IgA production (important for maintaining gut-microbial homeostasis) and prevents IL-1α-dependent colitis and colitis-associated colon cancer.[77] Intraperitoneal-delivered recombinant IL-33 in DSS-induced murine colitis clinically, macroscopically and histologically improves inflammation through reducing Th17 and Th1 cell populations in the lamina propria, effectively switching from a Th1 to Th2-mediated response.[78] Conversely, IL-33 can drive eosinophilic infiltration and potent mucosal adaptive immune responses which lead to chronic ileitis.[79] Genetic ablation of, or monoclonal antibodies against, ST2 can significantly improve colitis in murine models. Administration of IL-33 has also been shown to increase intestinal permeability.[80]

Is there any information from human studies? In a large paediatric and adult patient cohort, Latiano and colleagues reported that single nucleotide polymorphisms in the IL-33 gene (such as rs3939286) are associated with IBD susceptibility.[81] Paediatric carriers of the IL-33 polymorphism were 44% more likely to respond to steroids compared to those who did not carry the risk allele. Adult patients carrying the risk allele were more likely to have extensive, severe disease. Patients with IBD also had increased IL-33 mRNA expression in inflamed mucosa.[81] Studies have confirmed a significant relationship between increased IL-33 expression, increased severity of disease and ulcerative colitis disease phenotype and, similar to IL-33, ST2 expression is increased in the intestinal mucosa in patients with UC and is positively associated with disease activity: ST2 has been proposed as an activity biomarker for predicting clinical outcomes in UC.[82,83]

Although we know IL-33 is increased in human IBD, the biological consequence of this is unclear. It would appear that IL-33 plays an important role in IBD pathogenesis, although the underlying mechanisms are not yet characterised in vivo.

IL-35 and IL-37

Interleukin 35 (IL-35) and interleukin 37 (IL-37) are two recently discovered cytokines whose anti-inflammatory properties have attracted interest in IBD. IL-35 is a member of the IL-12 family and is produced by $CD4^{+}Foxp3^{+}$ Tregs, activated B-cells and likely tolerogenic dendritic cells. IL-35 regulates IL-10 producing B cells and suppresses Th17 responses *in vitro*.[84] IL-37 is a member of the IL-1 cytokine family and, although cytokines in this family tend to be pro-inflammatory, IL-37 can function as part of a negative feedback mechanism to limit inflammation in an IL-10-independent fashion.[85]

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Gene and protein expression of IL-35 is higher in colonic mucosa from adult patients with IBD, compared to healthy controls.[86] IL-37 expression in infiltrating immune cells and intestinal epithelial cells is also increased.[87] Although reduced levels of the cytokines themselves have been reported in the peripheral blood of IBD patients compared to healthy controls,[86] other studies reveal an increased presence of circulating IL-35-expressing $CD4^+$ and $CD8^+$ T-cells, and increased IL-37-producing $CD14^+$ monocytes, -$CD56^+$ natural killer cells and -$CD19^+$ B-cells in patients with IBD, compared to healthy controls or patients with inactive disease.[39]

Gene expression of major splice variant IL-37b is increased in the inflamed mucosa in patients with ulcerative colitis and Crohn's disease, and is undetectable in normal colonic mucosa.[88] Expression is predominantly in the epithelia and infiltrating immune cells of the inflamed mucosa.[89] The consequence of this is not clear but a clue may come from a study where *IL-37b* gene transfer enhanced the therapeutic effects of mesenchymal stromal cells in the resolution of DSS-induced murine colitis.[89] In DSS-induced colitis in a transgenic mouse expressing human IL-37 under a CMV promoter, IL-37 expression was only detectable in the colon when the epithelial barrier had been disrupted.[90] Although IL-37 increases IL-10, the protective effects of IL-37 do not disappear upon blocking IL-10R in IL-37 transgenic mice – the protective effects are not dependent upon IL-10 production and are yet uncharacterised.

There is limited data on the biological function of IL-35 in the gastrointestinal tract. Collison and colleagues reported that IL-35 can induce the generation of a unique subpopulation of Tregs, - IL-35 producing Tregs - *in vitro* and *in vivo,* that can function independent of IL-10 and TGF-β.[91] Both IL-35 and IL-27 belong to the IL-12 family of heterodimeric cytokines, share the Epstein-Barr virus induced gene protein 3 subunit, and are upregulated in IBD.[65,84] In this regard, Wirtz and colleagues investigated the differential role

of these two cytokines through DSS-induced colitis, TNBS-induced colitis, T-cell transfer and spontaneous colitis models in IL-27p28$^{-/-}$ and EB13$^{-/-}$ mice.[92] The study found IL-27p28 (the unique subunit of IL-27)-deficient mice were similar to wild-type mice whereas Epstein-Barr virus induced gene protein 3 (shared subunit)-deficient mice developed severe or worsened colitis, with decreased survival. In addition, the administration of single chain IL-35 prevented the development of colitis through suppressing Th1 and Th17 responses.[92]

IL-37 and IL-35 thus offer promising therapeutic potential in modulating inflammation in IBD, independent of IL-10. Again, the role of these cytokines in human IBD requires characterisation before the therapeutic potential can be fully understood. Perhaps these cytokines will offer the greatest efficacy in patients with an IL-10 high expression phenotype.

ENDOGENOUS MOLECULAR TARGETS TO REGULATE IMMUNE RESPONSES

miRNA

A micro RNA (miRNA) is a small, single-stranded, non-coding, hairpin ribonucleic acid (RNA) approximately 22 nucleotides long. Transcribed miRNA binds to a specific mRNA and induces its degradation. One of the first microarray-based studies in this field found that patients with ulcerative colitis had a differential expression of miRNAs, distinct from those expressed in patients with irritable bowel syndrome, Crohn's disease, microscopic colitis or infectious colitis.[93] There was also differential expression of miRNAs in patients with active ulcerative colitis compared to latent disease. Specifically, miRNA-192 was reduced during active ulcerative colitis. Subsequently, a differential expression of miRNA between ileal and colonic Crohn's disease has been reported.[94] This endogenous method of post-transcriptional gene regulation is therefore of interest in IBD pathogenesis and possibly highlights an opportunity to finely tune inflammatory activity.

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miR-192 targets the chemokine macrophage-inflammatory peptide 2α, which increases the migration of neutrophils and lymphocytes to the intestinal mucosa.[93] miR-141 can suppress expression of the chemokine CXCL12β and thus CXCL12β-mediated T-cell, neutrophil and macrophage migration to the inflamed gut.[95] Therapeutic upregulation of miRNA-141 in TNBS-induced murine colitis reduces leukocyte recruitment to inflamed intestinal mucosa.[95] miR-146 is upregulated upon TLR activation and targets the IL-1 receptor associated kinase and TNF receptor-associated factor 6, upstream of NF-κB, to limit inflammation in a negative feedback fashion.[96]

Upregulation of certain miRNAs may limit inflammation. For example, miR-155 is thought essential for Treg development and, whilst insufficient to independently control Treg function, it can augment other miRNAs in doing so.[97]

miR-10a suppresses Th17 and Th1 responses and downregulates the expression of IL-12/IL-23p40 and NOD2 on dendritic cells. This reduces the secretion of IL-12 and IL-23.[98] Interestingly, miRNA-10a is decreased in the inflamed colonic mucosa and peripheral blood mononuclear cells in patients with IBD, and is later upregulated following anti-TNF-α treatment.[98] This observation may partly explain the therapeutic mechanism of anti-TNF biologic therapy.

NOD2 plays a critical role in modulating a protective inflammatory response and in maintaining intestinal homeostasis.[99] NOD2 polymorphisms in Crohn's disease commonly lead to epithelial barrier dysfunction, chronic inflammation and likely impact microbial dysbiosis.[99] miR-146a is a nitrous oxide-triggered, downstream messenger of NOD2 signalling that promotes pro-inflammatory responses, through sonic hedgehog signalling, and expression of the transcription factor NUMB.[100] miRNA-192, 495, 512 and 671 were found to decrease NOD2, downregulate NF-κB and inhibit IL-8 and CXCL3 mRNA in colonic epithelial

HCT116 cells.[101] However, miR-192 was not associated with NOD2 regulation in a paediatric cohort.[102] Nonetheless, further characterisation is required and miRNA may be a therapeutic target for patients with NOD2 mutation phenotypes of IBD.

miRNAs also offer an opportunity to therapeutically target the intestinal epithelium directly. In human ulcerative colitis and DSS-induced murine colitis, miR-150 targets the transcription factor c-Myb which, leads to decreased apoptosis,[103] suggesting that upregulation of miR-150 may be therapeutic to protect the epithelial barrier.

miRNAs with a pro-inflammatory role also exist, including miRNA-301a which increases TNF-α production and Th17 cell differentiation.[104] In these circumstances, an anti-sense miRNA may have therapeutic benefit. Proposed RNA interference targets are summarised in Table 1.

Clinical studies exploring this treatment strategy are lacking for now. A phase 1 trial examining miRNA-RX34 in liver cancer was prematurely terminated after 5 immune-related serious events were reported (NCT01829971). A better understanding of the clinical safety and utility, along with identification of miRNAs that upregulate immunosuppressive host responses, will be of future interest as a novel future therapy in IBD.

Oligonucleotides

An oligonucleotide describes a short, synthetic DNA or RNA molecule. The therapeutic rationale is similar to miRNA – mucosal delivery binds to target nucleotide sequences and stimulates or inhibits effector pathway gene expression. The most successful studies to date using this strategy in IBD have exploited the TGFβ pathway. TGFβ is a multi-functional cytokine with immunosuppressive effects, produced by many immune and non-immune cells.[105] TGFβ is important in controlling many cellular functions, including proliferation,

differentiation, cell-cycle regulation, wound healing and angiogenesis. Loss of the TGFβ-signalling pathway is associated with IBD.[106,107] The endogenous regulatory protein SMAD7 blocks TGFβ-mediated inhibition of NF-κB, through activating NF-κB inhibitor-α.[108,109] In Crohn's disease, TGFβ signalling is inhibited by high levels of SMAD7 protein, resulting in unregulated production of pro-inflammatory cytokines.[109] Anti-sense SMAD7 oligonucleotide (Mongersen) has been developed as a therapeutic agent for use in IBD, blocking mRNA of SMAD7, inhibiting protein production and removing mucosal presence of this endogenous regulator of TGFβ signalling. Mongersen appeared safe and capable of improving endoscopic and clinical markers of colitis, as well as inducing remission in patients with active Crohn's disease in Phase II trials.[109–111] Following this, a phase 3 double-blind randomised control trial commenced, investigating the efficacy of Mongersen as a maintenance therapy in Crohn's disease (NCT02641392). However, this was prematurely discontinued based on lack of emerging benefit and further developments are awaited.

Toll-like receptor 9 (TLR9) is expressed in numerous antigen presenting cells including dendritic cells, B-cells and macrophages.[112] Activation of TLR9 occurs upon binding of cytosine-guanine nucleotide sequences present on microbial DNA fragments and synthetic oligonucleotides. In intestinal epithelial cells, if the apical surface is stimulated, the non-canonical NF-κB pathway is activated, resulting in release of anti-inflammatory molecules such as IL-10.[113–115] In the laboratory, the anti-microbial peptide cathelicidin and synthetic oligonucleotides are capable of inducing this pathway.[116] While both show translational potential, human studies concentrate on synthetic oligonucleotides. A double-blind placebo-controlled randomised control trial of a TLR9 agonist in 131 patients with moderate to severe ulcerative colitis was performed.[114] While not sufficient to induce clinical remission compared with placebo, TLR9 oligonucleotide DIMS0150 induced symptomatic remission, mucosal

healing and histological improvement in colitis at 4 weeks. A previous open-label phase 2a trial showed that, of the 22 patients enrolled, oral administration of TLR9 oligonucleotide BL-7040 induced remission in 2 patients and mucosal healing in 8 patients. Mucosal neutrophil and IL-6 levels were also reduced.[115] While these findings are modest, additional trials are required.

Therefore, oligonucleotides can inhibit (SMAD7) or stimulate (TLR9) critical pathways to bolster immunoregulatory capability for therapeutic gain in IBD.

The Endocannabinoid System

This system involves multiple endogenous ligands, enzymes and molecules responsible for, cannabinoid biosynthesis and cellular uptake.[117] Δ9-tetrahydrocannabinol signals through two G-protein coupled cannabinoid receptors, namely CB1 and CB2.

CB1 and CB2 signalling pathways reduce intestinal inflammation.[117] Inflammation can upregulate endogenous cannabinoid levels which impact intestinal motility. Although both receptors seem to be important, they are differentially expressed in both normal and inflamed intestinal mucosa.[118,119] In healthy colon, CB1 receptors are expressed on the epithelium, smooth muscle and submucosal myenteric plexus where they contribute to wound healing. Both CB1 and CB2 receptors are expressed on plasma cells in the lamina propria. In IBD, patients have increased expression of colonic epithelial CB2 receptors.[118] CB2 receptors are expressed on macrophages and the immune cell infiltrate during colitis whereas CB1 receptors are upregulated in enteric neurons and endothelium.[118–120] Clearly, regulating intestinal motility is not the only important function of this system, there is also immunoregulatory capability.

Can we target endogenous anti-inflammatory responses as a therapeutic strategy for inflammatory bowel disease?

CB2 agonists can ameliorate cytokine-mediated colonic inflammation in explant tissue, without affecting epithelial permeability.[121] They can also reduce reactive oxidative species production within intestinal epithelial cells and ameliorate murine experimental colitis.[122] Central cannabinoid receptors have a greater intestinal anti-inflammatory effect compared to peripheral receptors.[123]

CB2 agonism could be a new therapeutic direction for IBD. Activation of the CB2 receptor doesn't trigger the psychotropic side effects associated with cannabinoids, making this a clinically attractive therapeutic target.[124]

There are several ways to bolster the endogenous endocannabinoid system including promoting biosynthetic enzymes and inhibiting CB1 and CB2 ligand degradation pathways. For example, blocking cellular uptake and intracellular degradation pathways through VDM11 (an endocannabinoid membrane transport inhibitor), a fatty acid amide hydrolase inhibitor, or a combination of these reduces intestinal inflammation through increasing the concentration of anandamide, a fatty-acid neurotransmitter degraded by fatty acid amide hydrolase inhibitors.[125,126]

N-acylethanolamine-hydrolysing acid amidase inhibitors increase an endogenous anti-inflammatory fatty acid known as N-palmitoylethanolamine. N-palmitoylethanolamine has a high affinity for the endocannabinoid receptors and can subsequently reduce colonic inflammation and improve colitis through targeting the S100B/TLR-4 axis on enteric glial cells, reducing the downstream inflammatory effects of NF- κB.[127] Enteric glial cells are astrocyte-like cells found in enteric ganglia of the submucosal and myenteric plexus that promote epithelial barrier repair.[128] Glial regulation of epithelial barrier function can become disrupted in IBD and cannabidiol has been shown to restore glial-immune homeostasis to ameliorate inflammation.[129] This therapeutic strategy is summarised in Figure 1.

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Translating endocannabinoid *in vitro* and *in vivo* studies into clinical practice is controversial given associations with cannabis misuse. The efficacy of cannabis cigarettes on patients with treatment-refractory Crohn's disease has been assessed in a small randomised trial and did not induce remission.[130] A more recent randomised control trial of oral canabidiol was safe in Crohn's disease, but with no statistical efficacy demonstrated.[131] A double-blind randomised control trial of 36 patients with Crohn's disease is planned for July 2018 to determine whether oral cannabidiol is a safe and effective adjunct therapy for symptomatic relief in Crohn's disease (NCT03467620). The intestinal endocannabinoid system offers promise as a therapeutic target in IBD.

WHERE DO WE GO FROM HERE?

Our understanding of the immune system has advanced considerably over the past few years, providing insight into ways we may be able to harness its endogenous checks and balances for therapeutic gain (Figure 2). However, these mechanisms are complex and potential adverse effects must be considered.

There are several novel treatments to bolster the endogenous anti-inflammatory activity close to or recruiting to clinical trials currently and these agents may enter the future therapeutic arena for IBD if shown to be efficacious with a satisfactory side effect profile.

In addition, there are other novel strategies to treat IBD emerging that may exert effect through partial enhancement of the endogenous immunosuppressive mechanisms and not discussed here. An example of this is mesenchymal stem cell therapy that is mainly immunosuppressive through inhibition of many pro-inflammatory cells types, such as dendritic cells, B cells, effector T cells and NK cells. They also act however to promote Treg and immunoregulatory macrophage subsets, enhancing immunosuppressive capability.[132]

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There is significant variability at an individual patient level in IBD disease activity, progression over time and response to treatment. Better characterisation of disease phenotypes will better inform research and treatment decisions in the era of personalised medicine.

Can we target endogenous anti-inflammatory responses as a therapeutic strategy for inflammatory bowel disease?

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Can we target endogenous anti-inflammatory responses as a therapeutic strategy for inflammatory bowel disease?

Table 1: miRNA manipulation for therapeutic consideration.

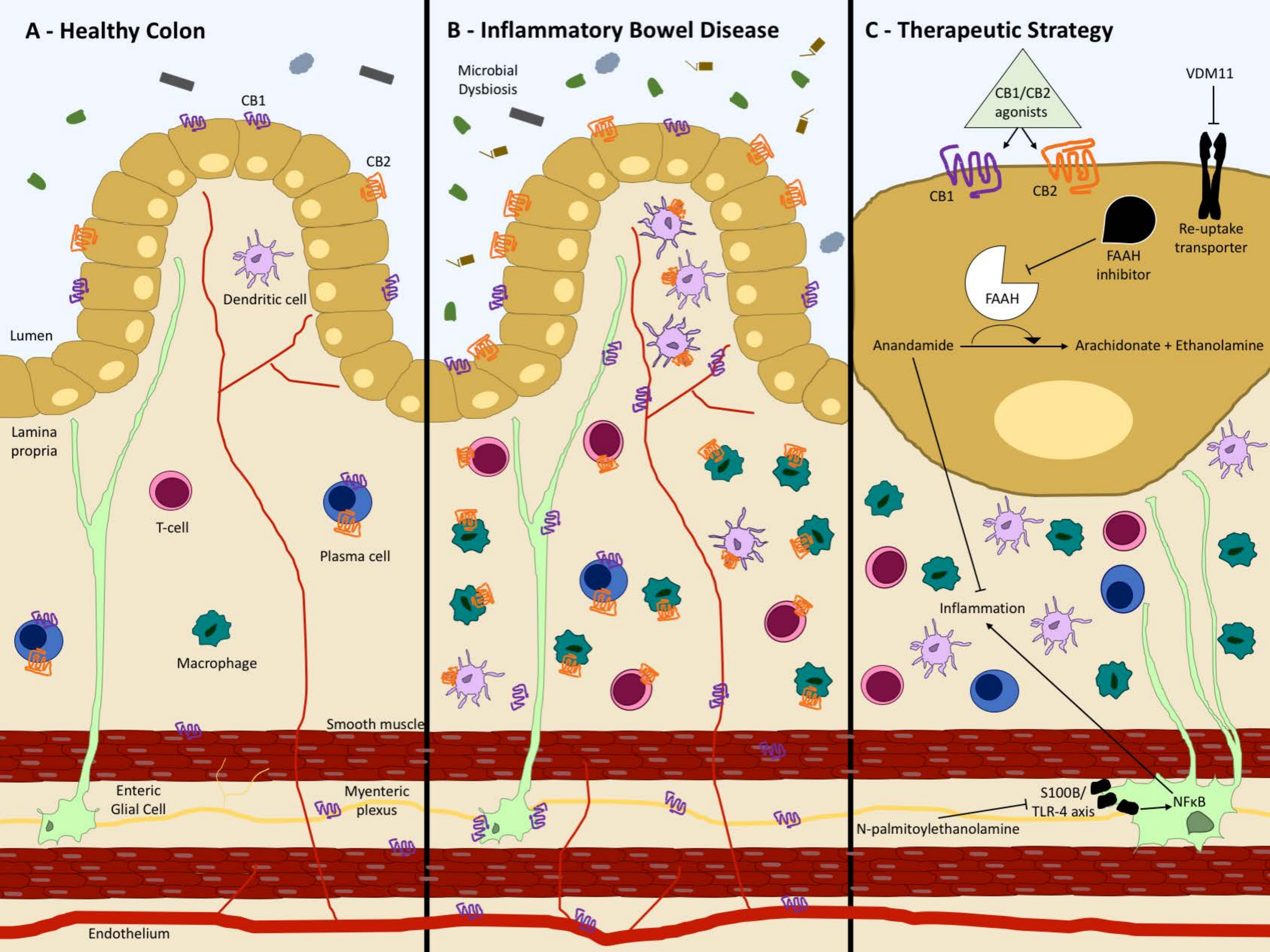
miRNA	Proposed Direction of modulation	Potential Therapeutic Effect
miR-192	Increase	Decreases migration of neutrophils and lymphocytes to the intestinal mucosa
miR-141	Increase	Reduce CXCL12β-mediated T-cell, neutrophil and macrophage migration to the inflamed bowel
miR-146	Increase	Reduce inflammation, downstream of IL-1 receptor, through blocking NF-kB pathway
miR-146a	Decrease	Decrease inflammation downstream of NOD2
miR-155	Increase	Promote Treg function
miR-10a	Increase	Reduced inflammation by suppressing Th17 and Th1 responses and secretion of IL-12 and IL-23
miR-192 miR-495 miR-512 miR-671	Increase	Downregulate NF-κB and inhibit IL-8 and CXCL3
miR-150	Increase	Decreases apoptosis
miR-301a	Decrease	Reduce TNF-a production and Th17 cell differentiation

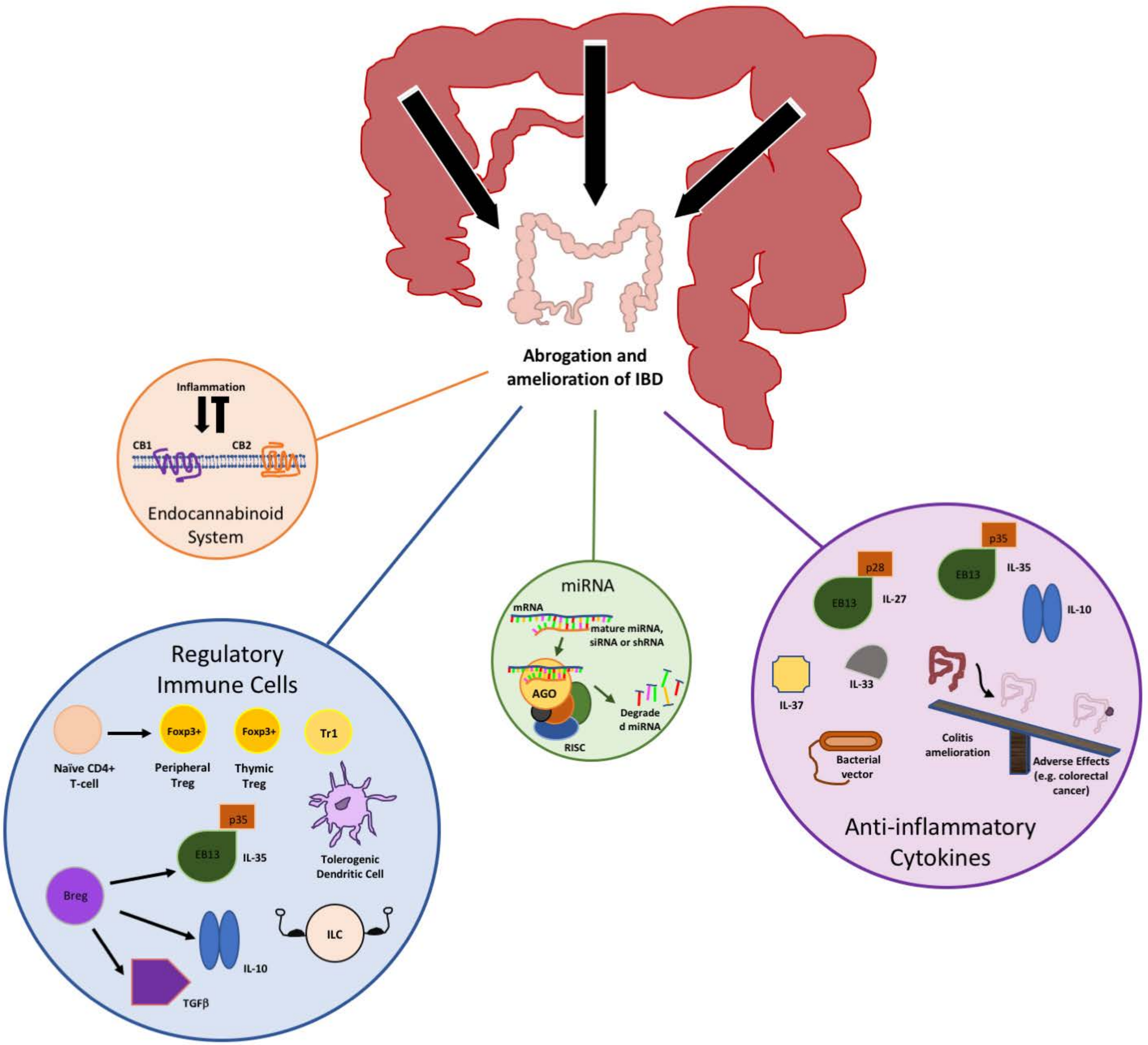
Can we target endogenous anti-inflammatory responses as a therapeutic strategy for inflammatory bowel disease?

FIGURE LEGENDS

Figure 1 - Targeting the endocannabinoid system in IBD. A - In the non-inflamed colon, CB1 is expressed on colonic epithelial cells, smooth muscle cells and the submucosal myenteric plexus. CB1 and CB2 are expressed on plasma cells in the lamina propria. B – In IBD, CB1 is upregulated on enteric neurons and endothelium whereas CB2 is expressed on the inflammatory cell infiltrate and is upregulated on colonic epithelial cells. C - CB1 and CB2 ligand degradation pathways can be inhibit by VDM11 or a fatty acid amide hydrolase inhibitor which increases the concentration of anandamide, which helps ameliorate inflammation in the gut. N-palmitoylethanolamine can also inhibit inflammation by sequestering NF- kB by targeting the S100B/TLR-4 axis on enteric glial cells.

Figure 2 - Strategies to promote endogenous immune-mediated anti-inflammatory activity in IBD.


A - Healthy Colon
CB1
CB2
Dendritic cell
Lumen
Lamina propria
T-cell
Plasma cell
Macrophage
Smooth muscle
Enteric Glial Cell
Myenteric plexus
Endothelium
B - Inflammatory Bowel Disease
Microbial Dysbiosis
C - Therapeutic Strategy
CB1/CB2 agonists
VDM11
CB1
CB2
Re-uptake transporter
FAAH inhibitor
FAAH
Anandamide
Arachidonate + Ethanolamine
Inflammation
S100B/ TLR-4 axis
NFκB
N-palmitoylethanolamine


Abrogation and amelioration of IBD
Inflammation
IT
CB1
CB2
Endocannabinoid System
Regulatory Immune Cells
Naïve CD4+ T-cell
Foxp3+
Peripheral Treg
Foxp3+
Thymic Treg
Tr1
Tolerogenic Dendritic Cell
p35
EB13
IL-35
Breg
IL-10
ILC
TGFβ
miRNA
mRNA
mature miRNA, siRNA or shRNA
AGO
RISC
Degrade d miRNA
p35
EB13
IL-35
p28
EB13
IL-27
IL-10
IL-33
IL-37
Bacterial vector
Colitis amelioration
Adverse Effects (e.g. colorectal cancer)
Anti-inflammatory Cytokines