IBD MANAGEMENT - NOVEL TARGETS AND THERAPEUTIC PERSPECTIVES

EDITED BY: Luca Antonioli, Barbara Romano, Corrado Blandizzi and Matteo Fornai

PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-771-3
DOI 10.3389/978-2-88963-771-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

IBD MANAGEMENT - NOVEL TARGETS AND THERAPEUTIC PERSPECTIVES

Topic Editors:
Luca Antonioli, University of Pisa, Italy
Barbara Romano, University of Naples Federico II, Italy
Corrado Blandizzi, University of Pisa, Italy
Matteo Fornai, University of Pisa, Italy

Citation: Antonioli, L., Romano, B., Blandizzi, C., Fornai, M., eds. (2020). IBD Management - Novel Targets and Therapeutic Perspectives. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-771-3

Table of Contents

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership

et al. reported that treatment with etrolizumab induced the internalization of $\alpha4\beta7$ integrin, and that this event functionally impaired $\beta7$-dependent cellular adhesion to MAdCAM-1. Of note, the authors demonstrated that the internalization of $\alpha4\beta7$ integrin was higher with etrolizumab as compared with vedolizumab (Lichnog et al.). Likewise, the trafficking of lymphocytes represents another interesting progress that can now be targeted by a small molecule (Pérez-Jeldres et al.). Sphingosine-1-phosphate receptor agonism, operated *via* fingolimod, KRP-203, ozanimod, etrasimod or amiselimod is another novel strategy that acts, in part, by interfering with lymphocyte recirculation, through the blockade of lymphocyte egress from lymph nodes (Pérez-Jeldres et al.).

In recent years, the perspective for innovative IBD therapies is changing. Indeed, it is emerging that novel pharmacological approaches to IBD management are refocusing their attention toward the modulation of the interplay between the innate and the adaptive components of the immune system (Vadstrup and Bendtsen, 2017; Bassoy et al., 2018; Stojanovic et al., 2018). In this context, the natural killer group 2, member D (NKG2D) receptor is emerging as an attractive target in IBDs. The NKG2D receptor is a type II transmembrane protein expressed by both innate and adaptive immune cells, including natural killer (NK) cells, $CD8^+$ T cells, invariant NKT cells, $\gamma\delta$ T cells, and some $CD4^+$ T cells under certain pathological conditions (Stojanovic et al., 2018). In particular, when activated, both macrophages and DCs upregulate NKG2D, thereby enabling them with additional mechanisms for regulating lymphocyte responses (Mao and Rieder, 2019). On this basis, blocking NKG2D would be another new mechanism of action for moderate to severe CD patients, as highlighted by the evidence about a significant increase in clinical remission in CD patients treated with an anti-NKG2D antibody (Vadstrup and Bendtsen, 2017).

The IL-36 cytokine family, produced predominantly by epithelial cells, acts on several cells including the immune cells, epithelial cells, and fibroblasts and is increased in IBD patients, thus representing another interesting target to manage bowel inflammation (Bassoy et al., 2018). In this regard, anti-IL36R antibodies are entering phase II trials in patients with moderate to severe ulcerative colitis (UC) (Mao and Rieder, 2019).

The termination of inflammation is governed by endogenous molecular factors collectively referred to as 'mediators of resolution' of inflammation. There is now strong evidence to suggest that failed resolution may underpin autoimmune and inflammatory diseases, including IBDs, and could thus be targeted to curb inflammation (Sugimoto et al., 2019). The field of resolution pharmacology represents an intriguing way worthy of being pursued for the management of inflammatory disorders, changing the paradigm of 'fighting inflammation' to 'targeting and advancing inflammation resolution' (Sugimoto et al., 2019). Over the last few years, increasing efforts have been addressed toward the characterization of proresolving mediators, allowing to identify novel molecular targets useful to design resolution-based therapies for IBDs (Sugimoto et al., 2019).

The ways forward for the resolution of inflammation can be different. Several authors have identified the antisense oligonucleotide technology as a specific, rapid, and potentially high-throughput approach (Di Fusco et al.; Scarozza et al.).

It is also emerging that the hallmarks of mitochondrial dysfunction, including oxidative stress and altered ATP production, are evident in the intestines of patients with IBD (Novak and Mollen, 2015). In this regard, it is widely acknowledged that the mitochondria are capable of regulating the proinflammatory responses of cells through the activation of a molecular complex known as the NLRP3 inflammasome (Novak and Mollen, 2015). Recently, Pellegrini et al. showed that direct NLRP3 inhibition can be a suitable strategy for the treatment of bowel inflammation. Indeed, INF39, a novel NLRP3 inhibitor, was found to be more effective than caspase-1 inhibition or IL-1β receptor blockade in reducing systemic and bowel inflammatory alterations in DNBS-colitis (Pellegrini et al.).

Overall, this Research Topic is providing new insights into novel pharmacological entities that are already present or are facing the therapeutic landscape for the management of IBDs. These range from innovative antibodies or small molecules aimed at stemming inflammatory cytokines pivotally involved in the IBD pathophysiology to strategies aimed at disrupting the vicious circle that occurs among cells of the innate and acquired immunity, as well as to intriguing approaches aimed at correcting defective function of proresolution mechanisms to rectify chronic inflammatory conditions. If successful, the impact of all these approaches will improve significantly not only the management of IBDs but also the quality of life of individuals suffering from these disorders.

AUTHOR CONTRIBUTIONS

AL, FM, BR, CP and CB participated to write the Editorial.

REFERENCES

Bassoy, E. Y., Towne, J. E., and Gabay, C. (2018). Regulation and function of interleukin-36 cytokines. *Immunol. Rev.* 281, 169–178. doi: 10.1111/imr.12610

Jairath, V., and Feagan, B. G. (2020). Global burden of inflammatory bowel disease. *Lancet Gastroenterol. Hepatol.* 5 (1), 2–3. doi: 10.1016/S2468-1253(19)30358-9

Kim, D. H., and Cheon, J. H. (2017). Pathogenesis of Inflammatory Bowel Disease and Recent Advances in Biologic Therapies. *Immune Netw.* 17, 25–40. doi: 10.4110/in.2017.17.1.25

Mao, R., and Rieder, F. (2019). Cooling Down the Hot Potato: Anti-Interleukin 36 Therapy Prevents and Treats Experimental Intestinal Fibrosis. *Gastroenterology* 156, 871–873. doi: 10.1053/j.gastro.2019.02.007

Novak, E. A., and Mollen, K. P. (2015). Mitochondrial dysfunction in inflammatory bowel disease. *Front. Cell Dev. Biol.* 3, 62. doi: 10.3389/fcell.2015.00062

Stojanovic, A., Correia, M. P., and Cerwenka, A. (2018). The NKG2D/NKG2DL Axis in the Crosstalk Between Lymphoid and Myeloid Cells in Health and Disease. *Front. Immunol.* 9, 827. doi: 10.3389/fimmu.2018.00827

Sugimoto, M. A., Vago, J. P., Perretti, M., and Teixeira, M. M. (2019). Mediators of the Resolution of the Inflammatory Response. *Trends Immunol.* 40, 212–227. doi: 10.1016/j.it.2019.01.007

Vadstrup, K., and Bendtsen, F. (2017). Anti-NKG2D mAb: A New Treatment for Crohn's Disease? *Int. J. Mol. Sci.* 18 (9), E1997. doi: 10.3390/ijms18091997

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

After manuscript acceptance, the author list of this article was amended to include an additional author. The Handling Editor AAI acknowledges a shared affiliation and past co-publications with this added author BR. This was declared to the Editorial Office during article production. The review process met the standards of a fair and objective review.

frontiers in Pharmacology

REVIEW
published: 08 October 2018
doi: 10.3389/fphar.2018.01107

Revisiting the Role of Thiopurines in Inflammatory Bowel Disease Through Pharmacogenomics and Use of Novel Methods for Therapeutic Drug Monitoring

*Sheng Zhang Lim and Eng Wee Chua**

Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

Azathioprine and 6-mercaptopurine, often referred to as *thiopurine compounds*, are commonly used in the management of inflammatory bowel disease. However, patients receiving these drugs are prone to developing adverse drug reactions or therapeutic resistance. Achieving predefined levels of two major thiopurine metabolites, 6-thioguanine nucleotides and 6-methylmercaptopurine, is a long-standing clinical practice in ensuring therapeutic efficacy; however, their correlation with treatment response is sometimes unclear. Various genetic markers have also been used to aid the identification of patients who are thiopurine-sensitive or refractory. The recent discovery of novel Asian-specific DNA variants, namely those in the *NUDT15* gene, and their link to thiopurine toxicity, have led clinicians and scientists to revisit the utility of Caucasian biomarkers for Asian individuals with inflammatory bowel disease. In this review, we explore the limitations associated with the current methods used for therapeutic monitoring of thiopurine metabolites and how the recent discovery of ethnicity-specific genetic markers can complement thiopurine metabolites measurement in formulating a strategy for more accurate prediction of thiopurine response. We also discuss the challenges in thiopurine therapy, alongside the current strategies used in patients with reduced thiopurine response. The review is concluded with suggestions for future work aiming at using a more comprehensive approach to optimize the efficacy of thiopurine compounds in inflammatory bowel disease.

Keywords: inflammatory bowel disease, azathioprine, 6-mercaptopurine, pharmacogenomics, therapeutic drug monitoring

OPEN ACCESS

Edited by:
Luca Antonioli,
Università degli Studi di Pisa, Italy

Reviewed by:
Ignacio Catalan-Serra,
Norwegian University of Science and Technology, Norway
Niels Vande Casteele,
University of California, San Diego, United States

***Correspondence:**
Eng Wee Chua
cew85911@ukm.edu.my

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 June 2018*
Accepted: *10 September 2018*
Published: *08 October 2018*

Citation:
Lim SZ and Chua EW (2018) Revisiting the Role of Thiopurines in Inflammatory Bowel Disease Through Pharmacogenomics and Use of Novel Methods for Therapeutic Drug Monitoring. Front. Pharmacol. 9:1107. doi: 10.3389/fphar.2018.01107

INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic, chronic inflammatory disorder caused by dysregulation of the gut immune response (Matricon et al., 2010). It is categorized into Crohn's disease (CD) and ulcerative colitis (UC), which differ in their anatomical site and pattern of inflammation, and the layers of the gastrointestinal wall that are affected. The etiology of IBD is unclear, but environmental factors appear to trigger immunological hyperactivity in genetically susceptible individuals. Exposure to microbes helps the immune system to establish its repertoire

of defensive responses against foreign agents. A hygienic environment presumably provides fewer stimuli for maturing the immune system, thereby lowering immune tolerance. For instance, IBD has been found to be more prevalent in developed countries (Economou and Pappas, 2008). Recently, the gut microbiome has also been linked to the development of IBD, and new IBD therapy such as fecal microbiota transplantation is being explored (Weingarden and Vaughn, 2017).

Corticosteroids, immunomodulators, and biologics comprise the mainstay of IBD treatment. While biologics offer advantages in terms of safety and efficacy, the high cost and the need for parenteral administration limit their clinical usage. Therefore, immunomodulators, particularly thiopurines, are more commonly used in IBD for maintenance therapy. Despite their widespread use, thiopurines are often associated with adverse drug reactions and treatment failures. The variability in response to thiopurine compounds relates to interindividual pharmacokinetic differences. In addition, ethnic differences in the prevalence of certain genetic polymorphisms in the thiopurine-related pathways could affect thiopurine responsiveness (Lee et al., 2015). Understanding this underlying pharmacogenetics of the thiopurine pathway could be useful in helping clinicians to optimize therapy individually for better safety and efficacy. In this review, we will focus on the influences of ethnicity-specific genetic markers on thiopurine response and explore possible mechanisms that underpin thiopurine resistance.

THE PLACE OF THIOPURINES IN IBD THERAPY

Azathioprine and 6-mercaptopurine are the two most commonly used thiopurine compounds in IBD management. Because of their slow onset, requiring at least 12–17 weeks of continuous therapy to produce noticeable effect (Prefontaine et al., 2010), thiopurines have a specific role in IBD, i.e., one of maintaining long-term remission. The recommended dose is 1.5–2.5 mg/kg for azathioprine and 0.75–1.5 mg/kg for 6-mercaptopurine (Gomollón et al., 2017; Harbord et al., 2017). At initial stages of IBD therapy, thiopurines are combined with a short course of a steroid or an anti-TNF agent for rapid induction of remission. Several studies have shown that thiopurines lengthened remission in UC or CD patients. A meta-analysis reported that 73% of CD patients treated with azathioprine were able to maintain remission over a period of 6–18 months, as compared with 62% in a placebo group (RR 1.19, 95% CI, 1.05–1.34) (Chande et al., 2015). Likewise, UC patients given azathioprine were found to have a lower disease relapse rate of 44% as compared with 65% among those treated with a placebo (RR 0.68, 95% CI, 0.54–0.86) (Timmer et al., 2016). However, the strength of the findings presented in both studies is limited by inadequate data and unknown risk of bias (Chande et al., 2015; Timmer et al., 2016). Therefore, the role of azathioprine monotherapy in IBD remains a point of discussion among clinicians. Nonetheless, the European Crohn's and Colitis Organisation released a consensus statement to support the use of thiopurines as a monotherapy or an adjunct to infliximab in CD and steroid-dependent UC, noting that thiopurines were significantly more effective than aminosalicylates in curbing flares of UC (Gomollón et al., 2017; Harbord et al., 2017).

Thiopurines are also commonly used as adjunct therapy in a step-down strategy for treating severe CD. An intensive drug regimen that includes an anti-TNF agent is used at the beginning of therapy and subsequently de-escalated when remission is attained. In an open randomized trial that investigated the step-down approach, 60% of the patients who received a combination of immunosuppressants were in steroid-free remission compared with 35.9% in a conventional step-up treatment group at week 26 (D'Haens et al., 2008). The Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease reported similar findings that the infliximab-azathioprine combination was superior to infliximab or azathioprine monotherapy at inducing and maintaining steroid-free remission and mucosal healing (Colombel et al., 2010). Similarly, the UC SUCCESS trial reported that 39.7% of the UC patients receiving the infliximab-azathioprine combination achieved steroid-free remission at week 16, as compared with ∼22–23% patients receiving infliximab or azathioprine alone (Panaccione et al., 2011). The effectiveness of the combination therapy has been ascribed to the protective effect of azathioprine against the formation of antibodies that impede the action of infliximab (Colombel et al., 2010; Panaccione et al., 2014). The beneficial interaction between thiopurines and anti-TNF agents lowers the requirements for the effective drug concentrations and has important implications for therapeutic drug monitoring in IBD, which will be discussed in another section of this review.

MONITORING SIDE EFFECTS

Prior to starting thiopurine therapy, a clinical assessment should be performed to ascertain whether patients are at risk of developing opportunistic infection or adverse drug reactions. Patients' medical and vaccination history is important in establishing their current immunological status. Serological screening for hepatitis B virus, hepatitis C virus, varicella zoster virus, human immunodeficiency virus, and Epstein-Barr virus should be done as recommended by the European Crohn's and Colitis Organisation. Screening for tuberculosis can be done by chest radiography and tuberculin skin test. Furthermore, vaccination for pneumococcal disease and influenza is required prior to the start of treatment and inactivated trivalent influenza vaccine should be given annually (Rahier et al., 2014). Patients should also be informed that live vaccines are contraindicated once the therapy is started and they should notify their physicians about their condition when getting treatment for other illnesses (Warner et al., 2018).

The common adverse drug reactions associated with thiopurines are leukopenia, hepatotoxicity, pancreatitis, and gastric intolerance (Warner et al., 2018). A full blood count and a liver function test should be conducted before starting a thiopurine and continued every 2 weeks for the first 2 months. If patients respond well to thiopurines, routine full blood counts

and liver function tests are recommended every 3 months throughout therapy (Goel et al., 2015). Close monitoring of elderly patients is highly recommended, especially during the first 3 months of therapy as they are at higher risk of developing adverse reactions. A case-controlled study conducted by the Spanish Working Group on Crohn's Disease and Ulcerative Colitis involving a large cohort of 48,752 adult IBD patients reported that patients older than 60 years receiving thiopurines suffered a significantly higher rate of adverse reactions and treatment discontinuation, compared with those younger than 50 years of age (Calafat et al., 2018).

Another concern over thiopurine therapy is an increased risk of malignancy, specifically lymphoproliferative disorder. Previous studies reported a four- to fivefold increase in the risk of malignancy compared with the general population, although the absolute risk remains very low (Kandiel et al., 2005; Beaugerie et al., 2009). Recently, a large prospective observational study reported a sevenfold increase in the risk of developing a myeloproliferative disorder for those who were previously exposed to thiopurines, but no increased risk for patients who were receiving ongoing thiopurine therapy (Lopez et al., 2014). A rare form of aggressive lymphoma known as hepatosplenic T-cell lymphoma has been associated with the use of thiopurines in CD patients, and the majority of them were young male patients with a median age of 22 years old (Ochenrider et al., 2010). The event rate was so low that it was impossible to estimate the risk; however, regular monitoring is recommended especially for young male CD patients who are currently on or have previously been exposed to a thiopurine. In addition, non-melanomatous skin cancer is another malignancy associated with thiopurine therapy. A systematic review has concluded that thiopurine use in IBD patients was associated with increased risk of non-melanomatous skin cancer with a hazard ratio of 2.1–2.28. However, the authors also cautioned that IBD *per se* is a risk factor for skin cancer, and younger patients were particularly at risk (Hagen and Pugliano-Mauro, 2018). More recently, a large retrospective analysis reported that the rate of malignancy for patients aged > 50 years receiving thiopurine therapy was 18.2%, significantly higher than the rate of 3.8% for patients < 50 years. Treatment duration more than 4 years was also shown to carry a greater risk for malignancy (Beigel et al., 2014). Overall, the consensus is that the benefit of thiopurine therapy outweighs the risk of malignancy or other adverse effects. The findings of those studies provide important information for clinicians to consider when deciding to initiate or withdraw thiopurine treatment. Elderly patients seem notably prone to thiopurine toxicity; therefore, the benefits of thiopurine therapy should be weighed more carefully against its risks for this vulnerable group.

THE THIOPURINE PATHWAY

The thiopurine compounds are a group of antimetabolites that structurally resemble endogenous purines. Azathioprine is a prodrug that is converted into 6-mercaptopurine by glutathione transferases (GST); alternatively, the conversion can be non-enzymatic. As non-enzymatic conversion accounts for less than 1% of the biotransformation based on an *in vitro* study (Eklund et al., 2006), the role of *GST* polymorphisms was thought to be insignificant in causing variation in azathioprine pharmacokinetics. However, null expression of a highly polymorphic GST subtype, GSTM1, was correlated to reduced azathioprine response in young IBD patients (Stocco et al., 2014). After entry into cells, 6-mercaptopurine is converted by a series of metabolic steps into 6-thioguanine nucleotides (6-TGNs). Other pathways competing with the formation of 6-TGN are methylation and oxidation of 6-mercaptopurine, catalysed by thiopurine *S*-methyltransferase (TPMT) and xanthine dehydrogenase (XDH), respectively (**Figure 1**).

The immunosuppressive effects of thiopurines are produced from three pathways. First, 6-TGN can be converted into deoxy-6-thioguanosine, which causes cell apoptosis by incorporation into DNA and inhibition of DNA-processing enzymes such as topoisomerase and DNA ligase that maintain base-pair stability and DNA dynamics (Somerville et al., 2003). Second, 6-TGNs, in particular thioguanosine triphosphate (TGTP), inhibit the activity of the GTPase Rac1, which regulates T-lymphocyte proliferation, and repress immune responses (Tiede et al., 2003; Poppe et al., 2006). The third route to immunosuppression involves methyl-thioinosine monophsophate (meTIMP), which inhibits phosphoribosyl pyrophosphate amidotransferase, an enzyme that catalyzes the first step of *de novo* purine synthesis (Karim et al., 2013).

THE ROLE OF TPMT MONITORING

The American Gastroenterological Association recommends that TPMT activity should be tested prior to the start of thiopurines and the dose adjusted according to TPMT status (Feuerstein et al., 2017). Owing to its role in methylating and inactivating thiopurines (**Figure 1**), TPMT influences the risk of severe and potentially fatal myelosuppression among patients receiving standard doses of azathioprine or 6-mercaptopurine. Individuals with TPMT deficiency should avoid thiopurine treatment or, if deemed necessary, start with < 10% of the standard initiation dose. Heterozygotes or individuals with intermediate enzyme activity should be given half of the usual dose (Coenen et al., 2015). Deleterious *TPMT* genetic polymorphisms are primarily responsible for the interindividual variability in TPMT enzyme activity. The common alleles causing TPMT deficiency are *TPMT*2*, *TPMT*3A*, *TPMT*3B*, and *TPMT*3C* (Salavaggione et al., 2005). The degree of enzyme deficiency depends on whether one or two gene copies are defective. In Caucasians, approximately 10% of the population had at least one of the defective alleles, with *TPMT*3A* being the most common allele. A small proportion (0.3–0.5%) of the Caucasian population are homozygous for the non-wild type allele or completely deficient in TPMT function (Collie-Duguid et al., 1999; Hon et al., 1999). The prevalence of defective *TPMT* alleles varies with ethnicity. As opposed to Caucasians, Asians are much less likely to be TPMT-deficient, with less than 5% of the population having at least one defective allele and almost none of them being a homozygote

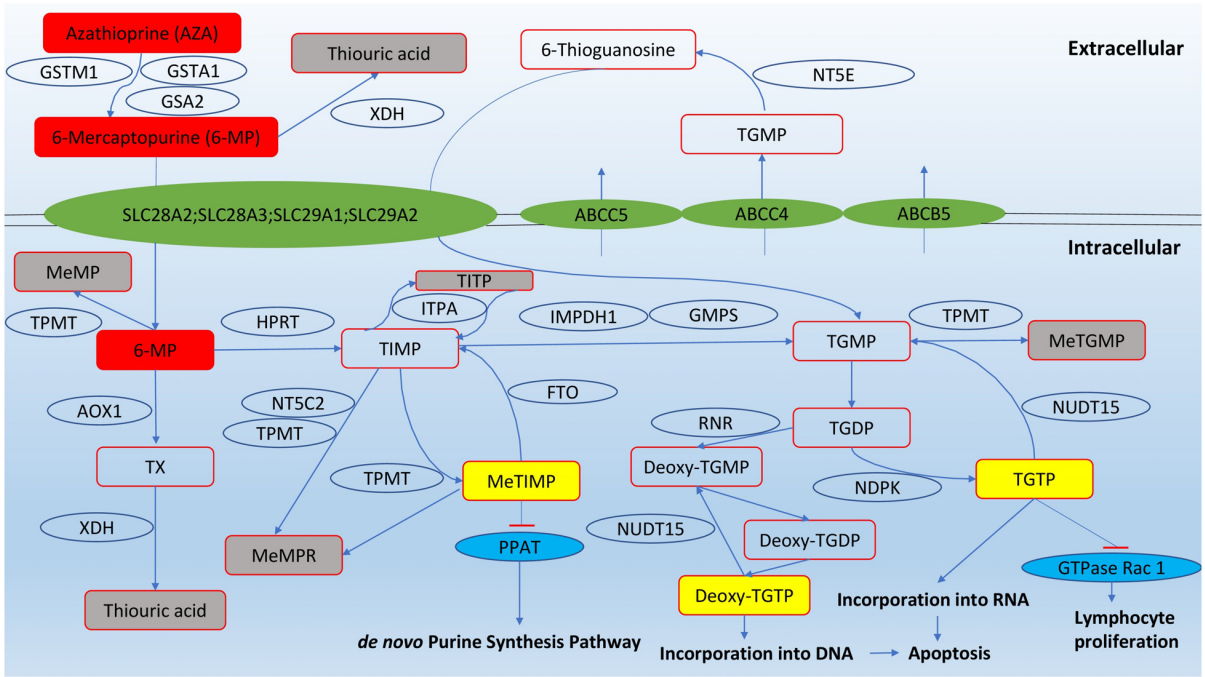

FIGURE 1 | Pathways of thiopurine disposition and action (adapted from Zaza et al., 2010). Cytosolic 5′-nucleotidase II (NT5C2); ecto-5′-nucleotidase (NT5E); fat mass and obesity-associated protein (FTO); nudix hydrolase 15 (NUDT15); glutathione S-transferase mu 1 (GSTM1); glutathione S-transferase alpha 1 (GSTA1); Glutathione S-transferase alpha 2 (GSTA2); thiopurine methyltransferase (TPMT); xanthine dehydrogenase (XDH); hypoxanthine guanine phosphoribosyltransferase (HPRT); inosine triphosphate pyrophosphatase (ITPA); inosine monophosphate dehydrogenase type 1 (IMPDH1); guanosine monophosphate synthetase (GMPS); aldehyde oxidase type 1 (AOX1); phosphoribosyl pyrophosphate amidotransferase (PPAT); ribonucleotide reductase (RNR); nucleotide diphosphate kinase (NDPK); methyl-mercaptopurine (meMP); thioxanthine (TX); methyl-mercaptopurine ribonucleotides (meMPR); thioinosine monophosphate (TIMP); thioinosine triphosphoate (TITP); methyl-thioinosine monophosphate (meTIMP); thioguanine monophosphate (TGMP); methyl-thioguanine monophosphate (meTGMP); thioguanine diphosphate (TGDP); thioguanine triphosphate (TGTP).

(Collie-Duguid et al., 1999; Kham et al., 2008). Therefore, TPMT testing in Asians may not be as useful as in Caucasians and it is not included in the routine therapeutic work-up for Asian IBD patients (Lee et al., 2015).

THIOPURINE METABOLITES MONITORING

The level of 6-TGN is often measured in clinical studies to gauge the success of thiopurine therapy. To date, much of the work on establishing a therapeutic 6-TGN cut-off has been performed in Caucasian patients. The accepted therapeutic range has stayed around 230–450 pmol/8 $\times$ 10^8 RBC (red blood cell) since its derivation more than a decade ago (Gisbert et al., 2006; Osterman et al., 2006). A popular practice at the clinics is adjusting the dosage and aiming for the target levels to enhance the probability of disease remission in patients with active disease or those who experience side effects (Osterman et al., 2006; Feuerstein et al., 2017; Vande Casteele et al., 2017). However, because of substantial inter- and intra-population differences, defining a threshold 6-TGN level that universally predicts positive clinical outcomes is difficult (Wright et al., 2004; Teml et al., 2007). The observed differences arise from a lack of a standard method for 6-TGN testing, variable study designs, or the intricacies of thiopurine pharmacology that we have yet to understand fully (Konidari et al., 2014).

Age, Ethnicity, and Anti-TNF Agents

Many studies have documented age- and ethnicity-related differences in the 6-TGN concentrations needed to produce a clinical benefit. A meta-analysis of fifteen studies involving pediatric IBD patients concluded that even though there was positive correlation between clinical outcomes and 6-TGN levels, a definitive therapeutic cut-off could not be ascertained (Konidari et al., 2014). A retrospective cohort study has reported that lower 6-TGN levels of 180–355 pmol/8 $\times$ 10^8 RBC were adequate for Chinese adults to maintain remission (Mao et al., 2017). This coincides with a prior observation that Asians are more prone to thiopurine-induced leukopenia (Lee et al., 2015). These reports suggest that Asians are more sensitive than Caucasians to thiopurines.

The effective 6-TGN levels may also be altered by concurrent drug therapy. The combination of thiopurines with anti-TNF agents is becoming a paradigm for managing IBD. Several benefits of the regimen have emerged from a cross-sectional study involving IBD patients who maintained remission with a combination of infliximab and azathioprine

or infliximab alone. The study showed that patients with 6-TGN levels > 125 pmol/8 × 10^8 RBC had a significantly higher median infliximab trough level than those with 6-TGN levels < 125 pmol/8 × 10^8 RBC (13.4 mcg/mL vs. 4.3 mcg/mL). These patients also had a higher mucosal healing rate and were less likely to produce anti-infliximab antibodies than those who did not reach the cut-off of 125 pmol/8 × 10^8 RBC (Yarur et al., 2015). In a more recent study that examined the combination of thiopurines with anti-TNF agents, the action of both infliximab and adalimumab was augmented when 6-TGN levels exceeded 125 pmol/8 × 10^8 RBC (Kelly et al., 2016). The authors attributed the synergistic effect to the ability of thiopurines to prevent immune responses against infliximab or adalimumab, thus resulting in the higher levels of the two drugs (Colombel et al., 2010; Yarur et al., 2015). A randomized open-label trial [DIAMOND] comparing the effectiveness of adalimumab-azathioprine combination with adalimumab monotherapy found that the combination therapy was significantly more effective than adalimumab alone in achieving the desired endoscopic response at week 26 (84.2% vs. 63.8%); however, the difference diminished at week 52 (79.6% vs. 69.8%) (Matsumoto et al., 2016). Taken together, the findings from the two trials suggest that infliximab-azathioprine may be more suitable than adalimumab-azathioprine in maintaining mucosal healing. The difference in clinical efficacy between infliximab and adalimumab may be due to variation in their pharmacokinetic and pharmacodynamic properties, which can affect the outcome of their interaction with other molecules. Therefore, the benefit observed with the use of infliximab is probably not a class effect, and the clinical benefit of each anti-TNF agent should be individually tested (Fiorino and Danese, 2016).

Aside from the uncertainties about the target range (Reinshagen et al., 2007; González-Lama et al., 2011; Dassopoulos et al., 2014), there is a lack of clear evidence for whether it is beneficial to guide thiopurine dosing based on routine thiopurine metabolites monitoring as compared with conventional weight-based dosing (Feuerstein et al., 2017; Vande Casteele et al., 2017). Therefore, the American Gastroenterological Association does not recommend routine thiopurine metabolites testing. The association, however, conditionally recommends reactive thiopurine metabolite testing in patients experiencing adverse drug reactions or active IBD related symptoms to guide treatment changes, though the quality of evidence is very low (Feuerstein et al., 2017; Vande Casteele et al., 2017). Thiopurine metabolite monitoring is also valuable in detecting non-adherence or underdosage, or identifying patients with aberrant metabolic profiles (Stocco et al., 2010; Gilissen et al., 2012).

Methods for Measuring Thiopurine Metabolites and Their Limitations

Different methods of 6-TGN measurement can produce discrepant results, thus rendering the findings from many studies not directly comparable (Shipkova et al., 2003; Simsek et al., 2017). For instance, the method reported by Dervieux et al. (2005) gave concentrations that were 2.6-fold those detected by the conventional method (Lennard and Singleton, 1992). The discrepancy may be due to differences in sample preparation and improved detection sensitivity of HPLC technologies. Standardizing the method of 6-TGN measurement seems an obvious solution to the problem, but even the studies which used the same method came to conflicting conclusions. Some studies showed a significant correlation between serum 6-TGN levels and clinical responses, while others showed no relationship. The heterogeneity of these studies in terms of their designs (retrospective vs. prospective), duration of thiopurine treatment, measures of clinical outcome, and differences in baseline patient characteristics may have contributed to the inconsistency (Konidari et al., 2014).

In current clinical practice, thiopurine metabolite measurement is performed using a method which cannot distinguish the mono-, di-, and triphosphates of 6-TGN and meMPR (methyl-mercaptopurine ribonucleotide) (**Figure 1**). TGTP is the predominant phosphate form of thiopurines in RBC and is responsible for their bioactivity (Vikingsson et al., 2009), but measuring all nucleotides together could be useful to provide additional insights into the association between metabolite levels and therapeutic responses. A threshold serum TGTP level of 100 pmol/8 × 10^8 RBC predicts a positive response to treatment, and patients with an elevated fraction of thioguanosine diphosphate (TGDP) have an attenuated response (Neurath et al., 2005). New methods have been developed to distinguish the three phosphorylated forms of 6-TGN. However, these methods are complex, require extra steps of oxidation, and suffer from relatively low accuracy (50–80%) (Vikingsson et al., 2013) when compared with the conventional method (∼99%) (Lennard and Singleton, 1992).

In most studies published to date, the thiopurine metabolites have been measured in RBCs. However, RBCs are not the site of action of thiopurines. Lacking a nucleus, they do not have all the enzymes required for thiopurine metabolism (Duley and Florin, 2005). This might explain the poor correlation between RBC 6-TGN levels and therapeutic responses as the measurement does not reflect the action of thiopurines in their target sites, i.e., white blood cells and bone marrow. The 6-TGN levels in white blood cells are, however, difficult to measure as the isolation of these cells is often confounded by RBC contamination (Bergan et al., 1997; Duley and Florin, 2005). To overcome this bottleneck, a new method has been developed to quantify the amount of DNA-incorporated 6-TGN, deoxy-6-thioguanosine, by using liquid chromatography-mass spectrometry (Coulthard et al., 2016).

GENETIC MARKERS OF ADVERSE EFFECTS

The Novel Asian-Dominant Gene NUDT15

The network of thiopurine metabolism is complex, and the activity of each pathway therein varies across ethnicity (**Table 1**). In this section, we will focus on several genes that have a major

TABLE 1 | Various genes shown to influence thiopurine responsiveness.

Classification	Candidate genes	Gene variants, changes in gene expression, or phenotype	Outcome	Reference
Thiopurine transport	Influx transporters:			
	i) *SLC28A2*, *SLC28A3*, *SLC29A1*, *SLC29A2*	Downregulation	Thiopurine resistance demonstrated in human cell lines	Fotoohi et al., 2006; Peng et al., 2008; Karim et al., 2011
	Efflux transporters:			
	i) *ABCC5*	Overexpression	Thiopurine resistance demonstrated in human cell lines	Wijnholds et al., 2000; Wielinga et al., 2002
	ii) *ABCC4*	Overexpression	Thiopurine resistance demonstrated in human cell lines	Peng et al., 2008
		rs3765534; rs146708960	Decreased protein expression and increased thiopurine sensitivity	Janke et al., 2008; Krishnamurthy et al., 2008; Ban et al., 2010
	iii) *ABCB5*	rs2031641 G/G	Thiopurine hypermethylation with high 6-MMP levels	Blaker et al., 2012
	Extracellular enzyme:			
	i) *NT5E*	Multiple functional variants affecting expression	Increased expression correlated with enhanced thiopurine sensitivity and higher 6-TGN levels, and vice versa	Li et al., 2010
Thiopurine metabolism	Pro-drug conversion:			
	i) *GSTM1*	Gene deletion; abolished gene expression	Reduced azathioprine therapy response; low 6-TGN/dose ratio for azathioprine	Stocco et al., 2014
	6-TGN synthesis:			
	i) *HPRT1*	Enzyme activity	High enzyme activity correlated with leukopenia and higher 6-TGN levels	Ding et al., 2012
	ii) *IMPDH1*	Enzyme activity	Enzyme activity inversely correlated with meTIMP concentrations; no association with 6-TGN levels	Haglund et al., 2011
		IMPDH1 P3 Promoter insertion 91–83insGAGCAGTAG	Azathioprine resistance	Roberts et al., 2007
	Inactivation pathway:			
	i) *TPMT*	Enzyme activity	High enzyme activity predicted treatment failures	Ansari et al., 2002; Cuffari et al., 2004
		A complex array of polymorphisms, most notably rs1800462 (**2*); rs1800460 (**3B*); rs1142345 (**3C*); *TPMT *3A* haplotype that comprises rs1800460 (**3B*) and rs1142345 (**3C*)	Thiopurine toxicities	Salavaggione et al., 2005
	ii) *AOX1*	rs55754655	Reduced response towards azathioprine; higher requirements for azathioprine dose	Smith et al., 2009; Kurzawski et al., 2012
	Mixed roles:			
	i) *ITPA*	rs1127354	Low enzyme activity; potentially increased 6-TGN levels	Stocco et al., 2009
	Dephosphorylation of active metabolites:			
	i) *NUDT15*	rs116855232	Enzyme deficiency impairing dephosphorylation of TGTP and deoxy-TGTP	Yang et al., 2014; Moriyama et al., 2016
	TGDP to TGTP conversion:			
	i) *NDPK*	Enzyme activity	Reduced enzyme activity could lower TGTP/TGDP ratios; potentially less effective treatment	Karner et al., 2010

(Continued)

TABLE 1 | Continued

Classification	Candidate genes	Gene variants, changes in gene expression, or phenotype	Outcome	Reference
Indirect pathway: modulation of enzyme activity	Synthesis of SAM			
	i) *TYMS, MAT1A, MAT2A, MTHFR*	A multitude of variants affecting enzyme activity	The variants could affect SAM production that is important in maintaining TPMT stability; the effect was more pronounced in individuals heterozygous for one of the defective *TPMT* alleles	Karas-Kuzelicki et al., 2010; Milek et al., 2012; Karas-Kuželički et al., 2014
	Molybdenum cofactor activity:	A collection of variants affecting enzyme activity	Significant association with low TPMT activity	Coelho et al., 2016
	i) *MOCOS*		Lowered activity of molybdenum cofactor for XDH; required lower doses of azathioprine (rs594445)	Kurzawski et al., 2012
Others	Endogenous purine synthesis:			
	i) *PRPS1*	Several non-synonymous variants	Reduced feedback inhibition of *de novo* purine synthesis; thiopurine resistance	Li et al., 2015
	Unspecific pathways:			
	i) *FTO*	rs79206939	Reduce FTO protein activity; higher risks of thiopurine-induced leukopenia	Kim et al., 2016
	ii) *PACSIN2*	rs2413739	Altered TPMT activity; increased sensitivity of cells to thiopurines via interaction with Rac1	de Kreuk et al., 2011; Stocco et al., 2012
	Genes identified through profiling:			
	i) *CD1D, CTSS, DEF8, FAM46A, FAM156A, FAR1, GNB4, HVCN1, IMPDH2, LAP3, MAP3K1, PLCB2, SLX1A, SMAP2, TGOLN2, TOX4, TUSC2, UBE2A*	A diverse collection of variants	Correlated well with disease activity and metabolite profiles	Haglund et al., 2013

6-MMP, 6-methylmercaptopurine; 6-TGN, 6-thioguanine nucleotide; meTIMP, methyl-thioinosine monophosphate; TGDP, thioguanine diphosphate; TGTP, thioguanine triphosphate; SAM, S-adenosyl methionine; TPMT, thiopurine methyltransferase; XDH, xanthine dehydrogenase; FTO, fat mass and obesity-associated.

influence on the development of thiopurine-related adverse effects in Asians. *NUDT15* is a gene that encodes a purine-specific Nudix hydrolase which is responsible for the hydrolysis of nucleosides-diphosphates. The enzyme is hypothesized to dephosphorylate the thiopurine active metabolites TGTP and deoxy-TGTP, thus hindering the binding of TGTP to Rac1 and incorporation of deoxy-TGTP into DNA (Moriyama et al., 2016).

Unlike other biomarkers discovered thus far, the *NUDT15* variants have been consistently found to have an unfavorable effect on thiopurine metabolism and therefore clinical response. These variants are highly penetrant and sensitive predictors of thiopurine toxicity that are comparable to their *TPMT* counterparts (Yang et al., 2014, 2015). The summation of the influences of individual *NUDT15* variants follows the additive model of genetic inheritance, whereby the severity of the aberrant phenotype is proportional to the number of risk alleles found in the gene (Moriyama et al., 2016). Homozygous carriers of *NUDT15* variants are extremely intolerant of thiopurine compounds and have been shown to be able to tolerate $< 10\%$ of a standard dose of mercaptopurine in acute lymphoblastic leukemia patients (Yang et al., 2015). Another study based on *NUDT15*-knockdown cell lines treated with thiopurine compounds showed a significant increase in TGTP levels, with a higher TGTP/TGMP ratio and a higher percentage of TGTP in 6-TGN (Moriyama et al., 2016).

The discovery of the impact of *NUDT15* variants on thiopurine sensitivity marks a significant milestone in the treatment of Asian IBD patients. The most studied marker of thiopurine toxicity to date, TPMT deficiency, has been well established in the Western population; however, *TPMT* mutations are rare and of limited relevance in Asians (Zhang et al., 2004; Gearry and Barclay, 2005; Kham et al., 2008; Lee et al., 2015). Instead, *NUDT15* has now become known as a major genetic determinant of thiopurine response in the Oriental population (Moriyama et al., 2016; Zhang et al., 2017). A non-synonymous variant in the gene, Arg139Cys (rs116855232), is a primary contributor to impaired enzyme activity and thiopurine-related adverse reactions such as myelosuppression and alopecia (Kakuta et al., 2015; Yang et al., 2015; Asada et al., 2016; Chiengthong et al., 2016; Shah et al., 2016; Zhu et al., 2016). The variant is frequently found in East Asians (9.8%) and Hispanics (3.9%), but it is rare in Europeans (0.2%) and absent in Africans (Yang et al., 2015).

Recognizing the risk of excessive immunosuppression and consequently early leukopenia in NUDT15-deficient individuals, the Korean Association for the Study of Intestinal Diseases has recommended that *NUDT15* genotypes should be tested before initiating thiopurine therapy (Lee et al., 2015). However, the cost-effectiveness of the strategy has not been systematically evaluated; so, the recommendation to deploy it at the clinics may have been premature. Furthermore, the interpretation of *NUDT15* genotypes is complicated in patients carrying multiple functional variants, where different heterozygous haplotypes can confer subtle variation in enzyme activity. The current proposal is to reduce the dose of thiopurines for patients carrying deleterious *NUDT15* variants; however, the extent of dosage reduction remains vague, and this has further limited the usefulness of the genotype-guided dosing strategy (Moriyama et al., 2016).

Beyond NUDT15

The discovery of *NUDT15* has nevertheless rekindled the interest in the influence of ethnicity on thiopurine response. There is a long-standing assumption that Asians are in general less able to tolerate thiopurine drugs; for instance, the average lower doses of azathioprine, ranging from 1 to 2 mg/kg/day, were reported to be sufficient to achieve clinical efficacy and the target 6-TGN levels in Asian IBD patients, as compared with the conventional dosage of 1.5–2.5 mg/kg/day (Andoh et al., 2008; Komiyama et al., 2008; Kim et al., 2009; Lee et al., 2009; Shi et al., 2016). This observation, in tandem with the emergence of *NUDT15*, has quickened the search for other markers that could also help to predict thiopurine efficacy in Asians. Most recently, a genome-wide association study in East Asian IBD patients has discovered a coding variant in the fat mass and obesity-associated (FTO) protein, Ala134Thr or rs79206939. The variant diminishes FTO activity and predisposes individuals harboring the variant to thiopurine-induced leukopenia (Kim et al., 2016). FTO is a member of the AlkB family of Fe(II)/α-ketoglutarate-dependent dioxygenases, which demethylate DNA and RNA (Fedeles et al., 2015). The demethylating action of the FTO protein may serve to counteract meTIMP, a potent inhibitor of purine biosynthesis (Fotoohi et al., 2010), and curb excessive impairment of cell replication. FTO is also responsible for regulating other genes in hematopoiesis, and a reduction in FTO activity can lead to severe myelosuppression (Kim et al., 2016). Interestingly, Ala134Thr is common in Koreans (5.1%) but much less so in Caucasians (< 0.1%) (Kim et al., 2016). In addition, a functional variant of ABCC4 (E857K), an efflux transporter that extrudes thiopurine metabolites from lymphocytes, is prevalent in Japanese IBD patients (>18%) and increases thiopurine sensitivity (Krishnamurthy et al., 2008; Ban et al., 2010).

The effect of variants in other genes seems less clear. A inosine triphosphate pyrophosphatase (ITPA) variant, 94C > A (rs1127354), which is associated with low enzyme activity (Sumi et al., 2002), is more common in Asians (14–19%) than Caucasians (6–7%) (Marsh et al., 2004). ITPAase is involved in the interconversion of thioinosine monophosphate (TIMP) into inactive thioinosine triphosphate (TITP), and low ITPA enzyme activity can increase the 6-TGN levels and the risk of hematological toxicities in acute lymphoblastic leukemia patients (Stocco et al., 2009; Hareedy et al., 2015); however, a meta-analysis of six studies has concluded that ITPA 94C > A was not significantly associated with any of the reported adverse drug reactions (Van Dieren et al., 2007), and more recent studies have shown that low ITPA enzyme activity was not always correlated with side effects from thiopurine therapy (Shipkova et al., 2011; Chiengthong et al., 2016). Furthermore, the role of hypoxanthine-guanine phosphoribosyltransferase (HPRT) may also be ethnicity-specific, as only one clinical report in Chinese IBD patients demonstrated a substantial correlation with thiopurine responsiveness, in contrast to none in Europeans (Ding et al., 2012).

Thiopurine Hypermethylation and Therapeutic Resistance

Thiopurine compounds are associated with a high rate of non-response, which occurs in approximately 50% of patients given the drugs (Fraser et al., 2002). The resistance to thiopurine therapy can be explained by an individual's inability to produce sufficient 6-TGNs to achieve therapeutic levels of the active metabolite (Dubinsky et al., 2000; Cuffari et al., 2001; Fraser et al., 2002; Osterman et al., 2006; Moreau et al., 2014).

The 6-TGN under-production in a subgroup of IBD patients is largely believed to be due to thiopurine hypermethylation (Dubinsky et al., 2002). These individuals are noted for their skewed thiopurine metabolism (*shunters*) whereby thiopurine compounds are preferentially metabolized into methyl-mercaptopurine (meMP) and meMPR (**Figure 1**), resulting in inadequate 6-TGN levels and therefore treatment resistance (Dubinsky et al., 2000; Hindorf et al., 2004). A high meMP level of ∼5700 pmol/8 × 10^8 RBC has been found to cause hepatotoxicity and other thiopurine-induced adverse drug reactions (Dubinsky et al., 2000; Gisbert et al., 2007a; Jharap et al., 2010). Currently, two cut-off 6-TGN/meMP ratios that define hypermethylation have been proposed, i.e., 20:1 (Van Egmond et al., 2012) and 11:1 (Dubinsky et al., 2002; Smith et al., 2012). There is no consensus on which of the two ratios is better at identifying metabolic shunters.

The role of TPMT in inducing thiopurine hypermethylation has been a subject of great interest. It was initially suggested that unusually high activity of TPMT may shift the metabolic machinery toward thiopurine methylation; this has been reported to result in low 6-TGN levels and unfavorable clinical response in IBD and acute lymphoblastic leukemia patients (Lennard et al., 1990; Ansari et al., 2002; Bloomfeld and Onken, 2003; Cuffari et al., 2004). Such enhanced TPMT function has not originated from DNA variation in the gene *per se*, but in those involved in the modulation of TPMT activity. A gene polymorphism in *PACSIN2* (rs2413739) has been noted to increase thiopurine-induced hematological toxicity through, in part, its ability to modulate TPMT activity. Cell lines carrying rs2413739 had

higher TPMT activity and were more sensitive to thiopurine compounds (Stocco et al., 2012). *PACSIN2* encodes a lipid-binding protein that interacts with Rac1, the molecular target of thiopurines (de Kreuk et al., 2011). Other genes such as *MOCOS*, *MAT1A*, *MAT2A*, and *TYMS*, though not directly involved in thiopurine metabolism, have also been implicated in thiopurine resistance because of their TPMT activity-altering action (Karas-Kuzelicki et al., 2010; Milek et al., 2012; Coelho et al., 2016). However, the hypothesis that TPMT activity is governed by multiple genes has been disproved by findings from a recent genome-wide association study, which has shown that TPMT was a monogenic trait, and that TPMT activity was not affected by non-*TPMT* markers (Liu et al., 2017). Nevertheless, the study has not ruled out regulation of TPMT activity by an unknown epigenomic pathway or factor. Other studies have also countered the utility of measuring TPMT activity for predicting 6-TGN levels and clinical response (Dubinsky et al., 2002; Lennard, 2002; Smith et al., 2012; Van Egmond et al., 2012). The contradicting results from various studies suggest that thiopurine resistance may be a multifactorial phenomenon, in which the exact role of TPMT is clouded by the influences of multiple and possibly obscure pathways.

Our incomplete understanding of the mechanism of thiopurine resistance is worsened by the existence of different phosphate forms of the thiopurine metabolites. A high level of TGTP is desired, as it has a high affinity toward Rac1, and should lead to favorable treatment outcomes (Neurath et al., 2005). The level of TGTP can be increased by nucleotide diphosphate kinase (NDPK), which converts TGDP into TGTP. In theory, variation in the activity of the enzyme could alter TGDP/TGTP ratios and influence thiopurine responsiveness. However, a study of 37 subjects found no correlation between the NDPK activity and the concentrations of the different phosphate forms (Karner et al., 2010). This study, however, is limited by its small sample size. Moreover, high levels of TGDP do not always mean unfavorable outcomes, as the diphosphates can be converted by ribonucleotide reductase into deoxynucleotides, which exert their cytotoxic effect by incorporating into DNA (Zaza et al., 2010).

In addition, meTIMP, which is a precursor of meMPR and conventionally thought to contribute to adverse drug reactions, acts on the purine synthesis pathway and has a different mode of action from TGTP in causing immunosuppression. For instance, in an experiment that used a leukemic cell line, increased formation of meTIMP from TIMP augmented thiopurine sensitivity, but the di- and triphosphates had no effect (Karim et al., 2013). The discovery of the ensemble of phosphorylated thiopurine metabolites provides a possible explanation for the lack of a clear association between TPMT activity and thiopurine resistance. High TPMT activity causes a universal increase in the different phosphorylated forms of the methylated metabolites, which have contrasting actions (Karim et al., 2013); however, they are not routinely discriminated by conventional HPLC detection (Lennard and Singleton, 1992; Vikingsson et al., 2013).

Because of the uncertain role of TPMT and related mechanisms, the involvement of other pathways and enzymes that may affect 6-TGN generation has gained more attention. In some instances, the link of those pathways to thiopurine responsiveness is not immediately apparent. Serving as key enzymes in the conversion of 6-mercaptopurine to 6-TGN, inosine-5′-monophosphate dehydrogenase and HPRT are obvious, though not firmly established, predictors of clinical response (Pieters et al., 1992; Roberts et al., 2007; Haglund et al., 2008; Ding et al., 2012; Moon and Loftus, 2016). Phosphoribosyl pyrophosphate synthetase 1 (PRPS1), whose function is to promote *de novo* purine biosynthesis, can increase the production of endogenous purines, which in turn compete with thiopurine drugs for the same enzymatic pathway, inhibiting their bioactivation (Li et al., 2015).

Another well-known pathway of thiopurine metabolism is the production of inactive metabolites thiouric acid, which involves XDH and aldehyde oxidase (AOX). 6-Mercaptopurine is converted to thiouric acid through sequential metabolism of thioxanthine intermediates involving both AOX and XDH (Choughule et al., 2014). A cohort study of 192 IBD patients has reported that the presence of an AOX1 variant rs55754655 together with high TPMT activity predicted a lower chance of patients responding to azathioprine treatment (Smith et al., 2009). This observation is indirectly supported by another study involving kidney transplant recipients, where the carriers of rs55754655 required a higher dose of azathioprine to maintain immunosuppressive effect at 3, 6, and 12 months after transplantation (Kurzawski et al., 2012).

The transmembrane transporters have also been shown to regulate the intracellular levels of 6-TGN. The down-regulation of influx transporters of 6-mercaptopurine including SLC28A2 (CNT2), SLC28A3 (CNT3), SLC29A1 (ENT1) and SLC29A2 (ENT2) has been shown to render lymphocyte-derived cell-lines resistant to thiopurines, owing to decreased uptake of thiopurine metabolites into the cells (Fotoohi et al., 2006; Peng et al., 2008; Karim et al., 2011). In addition, increased activity of efflux proteins has been associated with thiopurine resistance. ABCC4 and ABCC5 are the two transporters responsible for the efflux of thiopurine intermediates and metabolites (Wijnholds et al., 2000; Wielinga et al., 2002; Peng et al., 2008; Smith et al., 2010). The cells that overexpressed the transporters have been found to contain low levels of 6-TGN and other thiopurine metabolites (Krishnamurthy et al., 2008). The efflux mechanism is counteracted by *thiopurine cellular circulation*, whereby thiopurine nucleotides extruded by ABCC4 will be salvaged by an extracellular enzyme NT5E through hydrolysis into nucleosides, before being transported back into the cells by nucleoside transporters (Li et al., 2010). Several other subtypes of the efflux transports also exist. The action of each transporter is substrate-specific, giving rise to their differing roles in regulating the meMP/6-TGN ratio intracellularly. For instance, inactivating polymorphisms of ABCB5 have been found to cause accumulation of the methylated metabolites but not 6-TGNs inside cells (Blaker et al., 2012).

Finally, several genetic markers and enzymes have been reported to cause thiopurine resistance, but the mechanisms are

obscure. Variants in the intronic or flanking regions of the genes directly involved in the thiopurine pathway were correlated with unfavorable treatment response, but the functional impact of the variants was unclear (Matimba et al., 2014). Even less clear is the significance of non-thiopurine pathway-related genetic markers, which have been associated with poor outcomes of thiopurine treatment (Haglund et al., 2013; Matimba et al., 2014).

STRATEGIES TO OVERCOME THIOPURINE RESISTANCE

Dose-Splitting Regimen

A novel dose-splitting strategy has been used to correct unfavorable response to thiopurine therapy in lieu of the conventional weight-based dosing approach. Dividing the total daily dose of azathioprine or 6-mercaptopurine in thiopurine-resistant patients has been shown to reduce meMP levels and the development of adverse drug reactions, without compromising the levels of 6-TGNs and the clinical efficacy of thiopurines (Shih et al., 2012). This approach not only eliminates the need for the addition of allopurinol, it may even allow further upward titration of thiopurine doses to achieve the desired efficacy with little to no risk of adverse drug reactions (Bradford and Shih, 2011). The proposed rationale of the split-dose strategy is that the reduced dose of thiopurines in each administration produces suboptimal substrate affinity for TPMT, which prevents induction of TPMT activity during treatment (Weyer et al., 2001).

Thiopurine-Allopurinol Combination Therapy

The current clinical practice in managing thiopurine shunters is through combining low-dose azathioprine or 6-mercaptopurine with allopurinol. The thiopurine dose is reduced by 25–50%, and 100 mg of allopurinol is added (Amin et al., 2015; Fong et al., 2015), though a lower dose of allopurinol has been suggested to be equally efficacious yet safer, with a lower risk of leukopenia and opportunistic infection (Ansari et al., 2008; Govani and Higgins, 2010; Curkovic et al., 2013). Several studies that tested the use of the strategy in IBD patients have reported a significant reduction of the meMP concentration alongside an increase in the 6-TGN level (Sparrow et al., 2005; Ansari et al., 2010; Appell et al., 2013). In the assessment of clinical outcomes, the combination therapy has proven to be safe and efficacious in maintaining long-term steroid-free remission, with improved IBD disease activity scores in thiopurine shunters (Sparrow et al., 2007; Leung et al., 2009). Also, the combination therapy may be especially valuable to patients with extreme thiopurine sensitivity, resolving hepatotoxicity that some patients experienced during the conventional thiopurine monotherapy (Smith et al., 2012; Beswick et al., 2014; Johnson et al., 2014).

The mechanism of allopurinol in optimizing thiopurine therapy is complex and may lie in the ability of allopurinol to inhibit XDH and saturate or limit the methylating capacity of TPMT (Elion, 1989). XDH is involved in the conversion of 6-mercaptopurine to inactive thiouric acid (**Figure 1**). The inhibition of this auxiliary pathway by allopurinol should allow a larger fraction of 6-mercaptopurine to be converted into 6-TGN and meMP, although two studies documented a contradictory decrease in the meMP levels (Sasaki et al., 1987; Oláh et al., 1994). In human RBCs, allopurinol is successively converted by several enzymes to oxypurinol riboside monophosphate, which is a 6-oxo analog of 6-TIMP and a substrate of TPMT (Duley et al., 1985, 2005). Direct TPMT inhibition by allopurinol or oxypurinol has not been demonstrated *in vitro* (Sparrow et al., 2005, 2007). Instead, it has been proposed that allopurinol increases the production of thioxanthine, which acts as a TPMT inhibitor (Blaker et al., 2013).

Allopurinol may also directly heighten 6-TGN production. A prospective study involving IBD patients with aberrant metabolic profiles recorded an increase in HPRT activity following the thiopurine-allopurinol therapy but no effect on TPMT activity, suggesting that allopurinol may affect HPRT through an unknown mechanism (Seinen et al., 2013). Other as-yet undiscovered enzyme cofactors, targeted by allopurinol, may also play a role in thiopurine methylation (Leong et al., 2008).

Dose-Splitting Regimen or Thiopurine-Allopurinol Combination Therapy?

There is at present a lack of evidence to show which option is superior, except for one small cohort study in IBD pediatric patients. The study reported that patients treated with a combination of thiopurine and allopurinol were more likely to achieve the desired 6-TGN levels than those receiving the split-dose regimen (Chadokufa et al., 2016). However, the study is limited by a small cohort size and its focus on a pediatric population, whose pharmacokinetic profile differs from that of adult patients. In clinical practice, the choice of a treatment strategy for IBD patients is based on other factors including medical history, economic status, and therapeutic compliance.

OTHER FACTORS INFLUENCING THIOPURINE TREATMENT OUTCOME

Before prescribing thiopurines to the patients, several factors should be considered, as they can affect the outcome of thiopurine therapy. Thiopurines should be used with caution in smokers, as long-term smoking can upregulate CYP450 that can cause demethylation of meMP, potentially leading to a greater proportion of the drug being converted to 6-TGN, though this has not been clinically proven (Warner et al., 2016). On the other hand, excessive alcohol consumption during thiopurine treatment has been reported to cause peliosis hepatis (Elsing et al., 2007), as hepatotoxicity is a common adverse effect of both

alcohol and thiopurines. Furthermore, long-term consumption of alcohol could disrupt the methionine cycle, causing depletion of S-adenosylmethionine (Martinez-Chantar et al., 2002), which is essential to the catalytic activity of TPMT (Arenas et al., 2005). This may then predispose patients to thiopurine-induced toxicity, as 6-TGN levels may rise to a dangerous level. TPMT inhibition was also observed in patients who took a combination of a loop diuretic and a thiopurine (Lysaa et al., 1996). Similarly, increased 6-TGN and a higher rate of thiopurine intolerance were reported with the mesalamine-thiopurine combination therapy (Lees et al., 2008; Gao et al., 2012), and the withdrawal of mesalamine caused a drop in 6-TGN levels (Dewit et al., 2002; Gisbert et al., 2007b; Lees et al., 2008). Finally, in elderly patients, thiopurines are not an ideal choice of therapy, as the benefits of taking thiopurines for more than 5 years may not outweigh the risks (Lewis et al., 2000). This is due to the dysregulated immune function in elderly patients, leading to their increased susceptibility to infection, malignancies, and other adverse drug reactions (Gavazzi and Krause, 2002; Weng, 2006; Castelo-Branco and Soveral, 2013).

CONCLUDING REMARKS AND SUGGESTIONS FOR FUTURE WORK

We have not fully grasped the complexity of thiopurine pharmacology. Asians and Caucasians differ subtly in how they metabolize thiopurines. Through pharmacogenetic investigations, two major pharmacokinetic markers have emerged. Inactivating *NUDT15* variants impair the breakdown of Rac1-binding TGTPs and sensitize Asians to thiopurine toxicity (Moriyama et al., 2016). In Caucasians, *TPMT* variants resulting in low TPMT activity skews the thiopurine pathway to produce excessive 6-TGNs (Gearry and Barclay, 2005). Complete TPMT deficiency, for which TPMT genotyping would be most useful, is rare and irrelevant in Asians (Zhang et al., 2004; Kham et al., 2008).

Together, the two diverging routes of metabolism are gatekeepers of thiopurine toxicity. At the clinic, *NUDT15* or *TPMT* screening can inform initial decisions on treatment eligibility or drug dosages. Further fine-tuning of the treatment regime can then be guided by metabolite profiling. Such a tiered approach may reduce the development of costly adverse drug reactions, while preserving drug efficacy. However, the dosage recommendations for patients with NUDT15 deficiency are lacking, possibly because ascertaining the exact relation between *NUDT15* alleles and 6-TGN levels is challenging. When NUDT15 function is diminished, the resultant accumulation of TGTP is offset by a decrease in TGMP. The net outcome is that 6-TGN levels remain apparently unchanged (Moriyama et al., 2016).

Quantifying TGTPs and TGDPs by liquid chromatography is imprecise, owing to rapid interconversion of the two metabolites (Vikingsson et al., 2013). In routine metabolite testing, TGMP, TGDP, and TGTP are reported collectively as '6-TGNs'. Basing clinical decisions on 6-TGN levels alone can overlook NUDT15-deficient patients who are prone to thiopurine-induced myelosuppression. In these patients, 6-TGN levels may appear normal despite elevated TGTP (Moriyama et al., 2016).

Overall, surveys of thiopurine-related genes revealed that nucleoside phosphorylation has a noteworthy role in governing thiopurine sensitivity. The number of phosphate groups attached to thiopurine metabolites dictates their action, and only TGTP, dexoy-TGTP and meTIMP are bioactive. This may explain why some patients do not respond to optimal levels of 6-TGNs or suffer adverse effects from high levels of meMPs (Wright et al., 2004; Teml et al., 2007). A method needs to be devised with adequate resolving power for distinguishing the phosphorylated metabolites in blood samples. Then, we can revisit in detail how changes in NUDT15 or TPMT activity influence metabolite levels and drug efficacy, and possibly reset therapeutic thresholds. It is worth noting that no study has been conducted to ascertain the connection between the extent of *in vivo* 6-TGN and meMPR phosphorylation and thiopurine sensitivity in Asian IBD patients. Comparing the variation in the levels of these metabolites or the activity of enzymes such as NDPK between Asian and Caucasian patients may uncover new findings for optimizing the use of thiopurines across ethnic groups.

Prior genetic research has shed light on the possible origins of excess methylated thiopurine derivatives, i.e., altered cellular transport of the metabolites, impaired 6-TGN synthesis, and alternate routes of metabolism, driven by TPMT or other enzymes, that deviate from 6-TGN production (Fong et al., 2015). Pre-emptive multigene testing may help to identify patients who are unlikely to respond well to a thiopurine and therefore better suited for a biologic or the thiopurine-allopurinol regimen. We envision that the use of thiopurines would become increasingly selective to lower the likelihood of adverse effects, and the process of therapeutic drug monitoring in IBD would be refined further. For instance, the combined action of a thiopurine and an anti-TNF agent may change the definitions of therapeutic 6-TGN levels.

Future work should also focus on the pharmacodynamic aspects of thiopurines. With the advancement of high-throughput sequencing technologies, whole-genome or -exome sequencing has made it possible to examine potential factors that can affect the thiopurine pathway. We surmise that the causative factors may lie within the overarching Rac1 networks, which regulate cell proliferation. Finally, through a multipronged approach that enhances the efficacy of thiopurines, the place of the drugs should continue to remain firm in IBD management.

AUTHOR CONTRIBUTIONS

EWC conceived the outline of the manuscript and reviewed and edited the initial and final drafts of the manuscript. SZL wrote the manuscript.

FUNDING

This work was supported by a grant from the National University of Malaysia, Dana Impak Perdana (DIP-2016-017).

REFERENCES

Amin, J., Huang, B., Yoon, J., and Shih, D. Q. (2015). Update 2014: advances to optimize 6-mercaptopurine and azathioprine to reduce toxicity and improve efficacy in the management of IBD. *Inflamm. Bowel Dis.* 21, 445–452. doi: 10.1097/MIB.0000000000000197

Andoh, A., Tsujikawa, T., Ban, H., Hashimoto, T., Bamba, S., Ogawa, A., et al. (2008). Monitoring 6-thioguanine nucleotide concentrations in Japanese patients with inflammatory bowel disease. *J. Gastroenterol. Hepatol.* 23, 1373–1377. doi: 10.1111/j.1440-1746.2008.05419.x

Ansari, A., Elliott, T., Baburajan, B., Mayhead, P., O'Donohue, J., Chocair, P., et al. (2008). Long-term outcome of using allopurinol co-therapy as a strategy for overcoming thiopurine hepatotoxicity in treating inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 28, 734–741. doi: 10.1111/j.1365-2036.2008.03782.x

Ansari, A., Hassan, C., Duley, J., Marinaki, A., Shobowale-Bakre, E.-M., Seed, P., et al. (2002). Thiopurine methyltransferase activity and the use of azathioprine in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 16, 1743–1750.

Ansari, A., Patel, N., Sanderson, J., O'Donohue, J., Duley, J. A., and Florin, T. H. J. (2010). Low-dose azathioprine or mercaptopurine in combination with allopurinol can bypass many adverse drug reactions in patients with inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 31, 640–647. doi: 10.1111/j.1365-2036.2009.04221.x

Appell, M. L., Wagner, A., and Hindorf, U. (2013). A skewed thiopurine metabolism is a common clinical phenomenon that can be successfully managed with a combination of low-dose azathioprine and allopurinol. *J. Crohns Colitis* 7, 510–513. doi: 10.1016/j.crohns.2012.10.016

Arenas, M., Simpson, G., Lewis, C. M., Shobowale-Bakre, E.-M., Escuredo, E., Fairbanks, L. D., et al. (2005). Genetic variation in the MTHFR gene influences thiopurine methyltransferase activity. *Clin. Chem.* 51, 2371–2374. doi: 10.1373/clinchem.2005.053157

Asada, A., Nishida, A., Shioya, M., Imaeda, H., Inatomi, O., Bamba, S., et al. (2016). NUDT15 R139C-related thiopurine leukocytopenia is mediated by 6-thioguanine nucleotide-independent mechanism in Japanese patients with inflammatory bowel disease. *J. Gastroenterol.* 51, 22–29. doi: 10.1007/s00535-015-1142-4

Ban, H., Andoh, A., Imaeda, H., Kobori, A., Bamba, S., Tsujikawa, T., et al. (2010). The multidrug-resistance protein 4 polymorphism is a new factor accounting for thiopurine sensitivity in Japanese patients with inflammatory bowel disease. *J. Gastroenterol.* 45, 1014–1021. doi: 10.1007/s00535-010-0248-y

Beaugerie, L., Brousse, N., Bouvier, A. M., Colombel, J. F., Lémann, M., Cosnes, J., et al. (2009). Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. *Lancet* 374, 1617–1625. doi: 10.1016/S0140-6736(09)61302-7

Beigel, F., Steinborn, A., Schnitzler, F., Tillack, C., Breiteneicher, S., John, J. M., et al. (2014). Risk of malignancies in patients with inflammatory bowel disease treated with thiopurines or anti-TNF alpha antibodies. *Pharmacoepidemiol. Drug Saf.* 23, 735–744. doi: 10.1002/pds.3621

Bergan, S., Bentdal, O., Sødal, G., Brun, A., Rugstad, H. E., and Stokke, O. (1997). Patterns of azathioprine metabolites in neutrophils, lymphocytes, reticulocytes, and erythrocytes: relevance to toxicity and monitoring in recipients of renal allografts. *Ther. Drug Monit.* 19, 502–509.

Beswick, L., Dwyer, J. P., Friedman, A. B., Jakobovits, S. L., Paul, E., Headon, B., et al. (2014). P534 Co-prescription of allopurinol can overcome adverse events of thiopurine therapy and lead to remission in inflammatory bowel disease patients. *J. Crohns Colitis* 8, S290–S291. doi: 10.1016/S1873-9946(14)60654-1

Blaker, P. A., Arenas-Hernandez, M., Smith, M. A., Shobowale-Bakre, E. A., Fairbanks, L., Irving, P. M., et al. (2013). Mechanism of allopurinol induced TPMT inhibition. *Biochem. Pharmacol.* 86, 539–547. doi: 10.1016/j.bcp.2013.06.002

Blaker, P. A., Peters van Ton, A. M., Arenas Hernandez, M., Smith, M. A., Smith, C. H., Irving, P., et al. (2012). PWE-234 Optimising the response to thiopurine therapy: a search for novel explanations for thiopurine hypermethylation. *Gut* 61, A393–A393. doi: 10.1136/gutjnl-2012-302514d.234

Bloomfeld, R. S., and Onken, J. E. (2003). Mercaptopurine metabolite results in clinical gastroenterology practice. *Aliment. Pharmacol. Ther.* 17, 69–73. doi: 10.1046/j.1365-2036.2003.01392.x

Bradford, K., and Shih, D. Q. (2011). Optimizing 6-mercaptopurine and azathioprine therapy in the management of inflammatory bowel disease. *World J. Gastroenterol.* 17, 4166–4173. doi: 10.3748/wjg.v17.i37.4166

Calafat, M., Mañosa, M., Cañete, F., Panés, J., García Sánchez, V., Calvo, M., et al. (2018). OP034 The initiation of thiopurines in elderly patients with inflammatory bowel disease is associated with an increased risk of adverse effects: a case-control study of the ENEIDA registry. *J. Crohns Colitis* 12, S023–S025. doi: 10.1093/ecco-jcc/jjx180.033

Castelo-Branco, C., and Soveral, I. (2013). The immune system and aging: a review. *Gynecol. Endocrinol.* 30, 16–22. doi: 10.3109/09513590.2013.852531

Chadokufa, S., Lozinsky Rolnik, A., Sider, S., Acton, N., Huggett, B., Shah, N., et al. (2016). P331. Allopurinol and azathioprine co-therapy or thioguanine dose splitting: shifting the shunters in the mercaptopurine pathway in a paediatric inflammatory bowel disease population—a single-centre experience. *J. Crohns Colitis* 10, S260–S261. doi: 10.1093/ecco-jcc/jjw019.450

Chande, N., Patton, P. H., Tsoulis, D. J., Thomas, B. S., and MacDonald, J. K. (2015). Azathioprine or 6-mercaptopurine for maintenance of remission in Crohn's disease (Review). *Cochrane Database Syst. Rev.* 2015, 1–53. doi: 10.1002/14651858.CD000067.pub3

Chiengthong, K., Ittiwut, C., Muensri, S., Sophonphan, J., Sosothikul, D., Seksan, P., et al. (2016). NUDT15 c.415C > T increases risk of 6-mercaptopurine induced myelosuppression during maintenance therapy in children with acute lymphoblastic leukemia. *Haematologica* 101, e24–e26. doi: 10.3324/haematol.2015.134775

Choughule, K. V., Barnaba, C., Joswig-Jones, C. A., and Jones, J. P. (2014). In vitro oxidative metabolism of 6-mercaptopurine in human liver: insights into the role of the molybdoflavoenzymes aldehyde oxidase, xanthine oxidase, and xanthine dehydrogenase. *Drug Metab. Dispos.* 42, 1334–1340. doi: 10.1124/dmd.114.058107

Coelho, T., Andreoletti, G., Ashton, J. J., Batra, A., Afzal, N. A., Gao, Y., et al. (2016). Genes implicated in thiopurine-induced toxicity: comparing TPMT enzyme activity with clinical phenotype and exome data in a paediatric IBD cohort. *Sci. Rep.* 6:34658. doi: 10.1038/srep34658

Coenen, M. J., De Jong, D. J., Van Marrewijk, C. J., Derijks, L. J. J., Vermeulen, S. H., Wong, D. R., et al. (2015). Identification of patients with variants in TPMT and dose reduction reduces hematologic events during thiopurine treatment of inflammatory bowel disease. *Gastroenterology* 149, 907–917. doi: 10.1053/j.gastro.2015.06.002

Collie-Duguid, E. S., Pritchard, S. C., Powrie, R. H., Sludden, J., Collier, D. A., Li, T., et al. (1999). The frequency and distribution of thiopurine methyltransferase alleles in Caucasian and Asian populations. *Pharmacogenetics* 9, 37–42. doi: 10.1097/00008571-199902000-00006

Colombel, J. F., Sandborn, W. J., Reinisch, W., Mantzaris, G. J., Kornbluth, A., Rachmilewitz, D., et al. (2010). Infliximab, azathioprine, or combination therapy for Crohn's disease. *N. Engl. J. Med.* 362, 1383–1395. doi: 10.1056/NEJMoa0904492

Coulthard, S. A., Berry, P., McGarrity, S., Ansari, A., and Redfern, C. P. F. (2016). Liquid chromatography-mass spectrometry for measuring deoxythioguanosine in DNA from thiopurine-treated patients. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1028, 175–180. doi: 10.1016/j.jchromb.2016.06.017

Cuffari, C., Dassopoulos, T., Turnbough, L., Thompson, R. E., and Bayless, T. M. (2004). Thiopurine methyltransferase activity influences clinical response to azathioprine in inflammatory bowel disease. *Clin. Gastroenterol. Hepatol.* 2, 410–417.

Cuffari, C., Hunt, S., and Bayless, T. (2001). Utilisation of erythrocyte 6-thioguanine metabolite levels to optimise azathioprine therapy in patients with inflammatory bowel disease. *Gut* 48, 642–646. doi: 10.1136/gut.48.5.642

Curkovic, I., Rentsch, K. M., Frei, P., Fried, M., Rogler, G., Kullak-Ublick, G. A., et al. (2013). Low allopurinol doses are sufficient to optimize azathioprine therapy in inflammatory bowel disease patients with inadequate thiopurine metabolite concentrations. *Eur. J. Clin. Pharmacol.* 69, 1521–1531. doi: 10.1007/s00228-013-1500-1

Dassopoulos, T., Dubinsky, M. C., Bentsen, J. L., Martin, C. F., Galanko, J. A., Seidman, E. G., et al. (2014). Randomised clinical trial: individualised vs. weight-based dosing of azathioprine in Crohn's disease. *Aliment. Pharmacol. Ther.* 39, 163–175. doi: 10.1111/apt.12555

de Kreuk, B.-J., Nethe, M., Fernandez-Borja, M., Anthony, E. C., Hensbergen, P. J., Deelder, A. M., et al. (2011). The F-BAR domain protein PACSIN2

associates with Rac1 and regulates cell spreading and migration. *J. Cell Sci.* 124, 2375–2388. doi: 10.1242/jcs.080630

Dervieux, T., Meyer, G., Barham, R., Matsutani, M., Barry, M., Boulieu, R., et al. (2005). Liquid chromatography-tandem mass spectrometry analysis of erythrocyte thiopurine nucleotides and effect of thiopurine methyltransferase gene variants on these metabolites in patients receiving azathioprine/6-mercaptopurine therapy. *Clin. Chem.* 51, 2074–2084. doi: 10.1373/clinchem.2005.050831

Dewit, O., Vanheuverzwyn, R., Desager, J. P., and Horsmans, Y. (2002). Interaction between azathioprine and aminosalicylates: an *in vivo* study in patients with Crohn's disease. *Aliment. Pharmacol. Ther.* 16, 79–85.

D'Haens, G., Baert, F., van Assche, G., Caenepeel, P., Vergauwe, P., Tuynman, H., et al. (2008). Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 371, 660–667. doi: 10.1016/S0140-6736(08)60304-9

Ding, L., Zhang, F. B., Liu, H., Gao, X., Bi, H. C., Wang, X. D., et al. (2012). Hypoxanthine guanine phosphoribosyltransferase activity is related to 6-thioguanine nucleotide concentrations and thiopurine-induced leukopenia in the treatment of inflammatory bowel disease. *Inflamm. Bowel Dis.* 18, 63–73. doi: 10.1002/ibd.21676

Dubinsky, M. C., Lamothe, S., Yang, H. Y., Targan, S. R., Sinnett, D., Théorêt, Y., et al. (2000). Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology* 118, 705–713. doi: 10.1053/gg.2000.5925

Dubinsky, M. C., Yang, H., Hassard, P. V., Seidman, E. G., Kam, L. Y., Abreu, M. T., et al. (2002). 6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease. *Gastroenterology* 122, 904–915. doi: 10.1053/gast.2002.32420

Duley, J. A., Chocair, P. R., and Florin, T. H. (2005). Observations on the use of allopurinol in combination with azathioprine or mercaptopurine. *Aliment. Pharmacol. Ther.* 22, 1161–1162. doi: 10.1111/j.1365-2036.2005.02703.x

Duley, J. A., and Florin, T. H. (2005). Thiopurine therapies: problems, complexities, and progress with monitoring thioguanine nucleotides. *Ther. Drug Monit.* 27, 647–654. doi: 10.1097/01.ftd.0000169061.52715.3e

Duley, J. A., Harris, O., and Holmes, R. S. (1985). Analysis of human alcohol and aldehyde metabolizing isozymes by electrophoresis and isoelectric focusing. *Alcohol. Clin. Exp. Res.* 9, 263–271. doi: 10.1111/j.1530-0277.1985.tb05747.x

Economou, M., and Pappas, G. (2008). New global map of Crohn's disease: genetic, environmental, and socioeconomic correlations. *Inflamm. Bowel Dis.* 14, 709–720. doi: 10.1002/ibd.20352

Eklund, B. I., Moberg, M., Bergquist, J., and Mannervik, B. (2006). Divergent activities of human glutathione transferases in the bioactivation of azathioprine. *Mol. Pharmacol.* 70, 747–754. doi: 10.1124/mol.106.025288

Elion, G. B. (1989). The purine path to chemotherapy. *Biosci. Rep.* 9, 509–529. doi: 10.1007/BF01119794

Elsing, C., Placke, J., and Herrmann, T. (2007). Alcohol binging causes peliosis hepatis during azathioprine therapy in Crohn's disease. *World J. Gastroenterol.* 13, 4646–4648.

Fedeles, B. I., Singh, V., Delaney, J. C., Li, D., and Essigmann, J. M. (2015). The AlkB family of Fe(II)/α-ketoglutarate-dependent dioxygenases: repairing nucleic acid alkylation damage and beyond. *J. Biol. Chem.* 290, 20734–20742. doi: 10.1074/jbc.R115.656462

Feuerstein, J. D., Nguyen, G. C., Kupfer, S. S., Falck-Ytter, Y., Singh, S., Gerson, L., et al. (2017). American Gastroenterological Association institute guideline on therapeutic drug monitoring in inflammatory bowel disease. *Gastroenterology* 153, 827–834. doi: 10.1053/j.gastro.2017.07.032

Fiorino, G., and Danese, S. (2016). Adalimumab and azathioprine combination therapy for Crohn's disease: a shining diamond? *J. Crohns Colitis* 10, 1257–1258. doi: 10.1093/ecco-jcc/jjw119

Fong, S. C., Blaker, P. A., Arenas-Hernandez, M., Marinaki, A. M., and Sanderson, J. D. (2015). Getting the best out of thiopurine therapy: thiopurine S-methyltransferase and beyond. *Biomark. Med.* 9, 51–65. doi: 10.2217/bmm.14.97

Fotoohi, A. K., Coulthard, S. A., and Albertioni, F. (2010). Thiopurines: factors influencing toxicity and response. *Biochem. Pharmacol.* 79, 1211–1220. doi: 10.1016/j.bcp.2010.01.006

Fotoohi, A. K., Lindqvist, M., Peterson, C., and Albertioni, F. (2006). Involvement of the concentrative nucleoside transporter 3 and equilibrative nucleoside transporter 2 in the resistance of T-lymphoblastic cell lines to thiopurines. *Biochem. Biophys. Res. Commun.* 343, 208–215. doi: 10.1016/j.bbrc.2006.02.134

Fraser, A. G., Orchard, T. R., and Jewell, D. P. (2002). The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. *Gut* 50, 485–489. doi: 10.1136/gut.50.4.485

Gao, X., Zhang, F.-B., Ding, L., Liu, H., Wang, X.-D., Chen, B.-L., et al. (2012). The potential influence of 5-aminosalicylic acid on the induction of myelotoxicity during thiopurine therapy in inflammatory bowel disease patients. *Eur. J. Gastroenterol. Hepatol.* 24, 958–964. doi: 10.1097/MEG.0b013e3283545ae3

Gavazzi, G., and Krause, K. H. (2002). Ageing and infection. *Lancet Infect. Dis.* 2, 659–666. doi: 10.1016/S1473-3099(02)00437-1

Gearry, R. B., and Barclay, M. L. (2005). Azathioprine and 6-mercaptopurine pharmacogenetics and metabolite monitoring in inflammatory bowel disease. *J. Gastroenterol. Hepatol.* 20, 1149–1157. doi: 10.1111/j.1440-1746.2005.03832.x

Gilissen, L. P., Wong, D. R., Engels, L. G., Bierau, J., Bakker, J. A., Paulussen, A. D., et al. (2012). Therapeutic drug monitoring of thiopurine metabolites in adult thiopurine tolerant IBD patients on maintenance therapy. *J. Crohns Colitis* 6, 698–707. doi: 10.1016/j.crohns.2011.12.003

Gisbert, J. P., Gomollón, F., Cara, C., Luna, M., González-Lama, Y., Pajares, J. M., et al. (2007a). Thiopurine methyltransferase activity in Spain: a study of 14,545 patients. *Dig. Dis. Sci.* 52, 1262–1269. doi: 10.1007/s10620-006-9119-z

Gisbert, J. P., González-Lama, Y., and Maté, J. (2007b). Thiopurine-induced liver injury in patients with inflammatory bowel disease: a systematic review. *Am. J. Gastroenterol.* 102, 1518–1527. doi: 10.1111/j.1572-0241.2007.01187.x

Gisbert, J. P., González-Lama, Y., and Maté, J. (2006). Monitoring of thiopurine methyltransferase and thiopurine metabolites to optimize azathioprine therapy in inflammatory bowel disease. *Gastroenterol. Hepatol.* 29, 568–583. doi: 10.1157/13094355

Goel, R. M., Blaker, P., Mentzer, A., Fong, S. C. M., Marinaki, A. M., and Sanderson, J. D. (2015). Optimizing the use of thiopurines in inflammatory bowel disease. *Ther. Adv. Chronic Dis.* 6, 138–146. doi: 10.1177/2040622315579063

Gomollón, F., Dignass, A., Annese, V., Tilg, H., Van Assche, G., Lindsay, J. O., et al. (2017). 3rd European evidence-based consensus on the diagnosis and management of Crohn's disease 2016: part 1: diagnosis and medical management. *J. Crohns Colitis* 11, 3–25. doi: 10.1093/ecco-jcc/jjw168

González-Lama, Y., Bermejo, F., López-Sanromán, A., García-Sánchez, V., Esteve, M., Cabriada, J. L., et al. (2011). Thiopurine methyl-transferase activity and azathioprine metabolite concentrations do not predict clinical outcome in thiopurine-treated inflammatory bowel disease patients. *Aliment. Pharmacol. Ther.* 34, 544–554. doi: 10.1111/j.1365-2036.2011.04756.x

Govani, S. M., and Higgins, P. D. (2010). Combination of thiopurines and allopurinol: adverse events and clinical benefit in IBD. *J. Crohns Colitis* 4, 444–449. doi: 10.1016/j.crohns.2010.02.009

Hagen, J. W., and Pugliano-Mauro, M. A. (2018). Nonmelanoma skin cancer risk in patients with inflammatory bowel disease undergoing thiopurine therapy: a systematic review of the literature. *Dermatol. Surg.* 44, 469–480. doi: 10.1097/DSS.0000000000001455

Haglund, S., Almer, S., Peterson, C., and Söderman, J. (2013). Gene expression and thiopurine metabolite profiling in inflammatory bowel disease - novel clues to drug targets and disease mechanisms? *PLoS One* 8:e56989. doi: 10.1371/journal.pone.0056989

Haglund, S., Taipalensuu, J., Peterson, C., and Almer, S. (2008). IMPDH activity in thiopurine-treated patients with inflammatory bowel disease - relation to TPMT activity and metabolite concentrations. *Br. J. Clin. Pharmacol.* 65, 69–77. doi: 10.1111/j.1365-2125.2007.02985.x

Haglund, S., Vikingsson, S., Söderman, J., Hindorf, U., Grännö, C., Danelius, M., et al. (2011). The role of inosine-5′-monophosphate dehydrogenase in thiopurine metabolism in patients with inflammatory bowel disease. *Ther. Drug Monit.* 33, 200–208. doi: 10.1097/FTD.0b013e31820b42bb

Harbord, M., Eliakim, R., Bettenworth, D., Karmiris, K., Katsanos, K., Kopylov, U., et al. (2017). Third European evidence-based consensus on diagnosis and management of ulcerative colitis. Part 2: current management. *J. Crohns Colitis* 11, 769–784. doi: 10.1093/ecco-jcc/jjx009

Hareedy, M. S., El Desoky, E. S., Woillard, J.-B., Thabet, R. H., Ali, A. M., Marquet, P., et al. (2015). Genetic variants in 6-mercaptopurine pathway as potential factors of hematological toxicity in acute lymphoblastic leukemia patients. *Pharmacogenomics* 16, 1119–1134. doi: 10.2217/PGS.15.62

Hindorf, U., Lyrenäs, E., Nilsson, A., and Schmiegelow, K. (2004). Monitoring of long-term thiopurine therapy among adults with inflammatory bowel disease. *Scand. J. Gastroenterol.* 39, 1105–1112. doi: 10.1080/00365520410007980

Hon, Y. Y., Fessing, M. Y., Pui, C. H., Relling, M. V., Krynetski, E. Y., and Evans, W. E. (1999). Polymorphism of the thiopurine S-methyltransferase gene in African-Americans. *Hum. Mol. Genet.* 8, 371–376. doi: 10.1093/hmg/8.2.371

Janke, D., Mehralivand, S., Strand, D., Gödtel-Armbrust, U., Habermeier, A., Gradhand, U., et al. (2008). 6-Mercaptopurine and 9-(2-phosphonylmethoxyethyl) adenine (PMEA) transport altered by two missense mutations in the drug transporter gene *ABCC4*. *Hum. Mutat.* 29, 659–669. doi: 10.1002/humu.20694

Jharap, B., Seinen, M. L., De Boer, N. K., Van Ginkel, J. R., Linskens, R. K., Kneppelhout, J. C., et al. (2010). Thiopurine therapy in inflammatory bowel disease patients: analyses of two 8-year intercept cohorts. *Inflamm. Bowel Dis.* 16, 1541–1549. doi: 10.1002/ibd.21221

Johnson, H., Weaver, S., and McLaughlin, S. (2014). P419 Low dose azathioprine and allopurinol in azathioprine intolerant patients: is it tolerated and is it effective in IBD? *J. Crohns Colitis* 8, S240–S241. doi: 10.1016/S1873-9946(14)60539-0

Kakuta, Y., Naito, T., Onodera, M., Kuroha, M., Kimura, T., Shiga, H., et al. (2015). NUDT15 R139C causes thiopurine-induced early severe hair loss and leukopenia in Japanese patients with IBD. *Pharmacogenomics J.* 16, 1–6. doi: 10.1038/tpj.2015.43

Kandiel, A., Fraser, A. G., Korelitz, B. I., Brensinger, C., and Lewis, J. D. (2005). Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 54, 1121–1125. doi: 10.1136/gut.2004.049460

Karas-Kuzelicki, N., Milek, M., and Mlinaric-Rascan, I. (2010). MTHFR and TYMS genotypes influence TPMT activity and its differential modulation in males and females. *Clin. Biochem.* 43, 37–42. doi: 10.1016/j.clinbiochem.2009.09.003

Karas-Kuželički, N., Šmid, A., Tamm, R., Metspalu, A., and Mlinarič-Rašèan, I. (2014). From pharmacogenetics to pharmacometabolomics: SAM modulates TPMT activity. *Pharmacogenomics* 15, 1437–1449. doi: 10.2217/pgs.14.84

Karim, H., Ghalali, A., Lafolie, P., Vitols, S., and Fotoohi, A. K. (2013). Differential role of thiopurine methyltransferase in the cytotoxic effects of 6-mercaptopurine and 6-thioguanine on human leukemia cells. *Biochem. Biophys. Res. Commun.* 437, 280–286. doi: 10.1016/j.bbrc.2013.06.067

Karim, H., Hashemi, J., Larsson, C., Moshfegh, A., Fotoohi, A. K., and Albertioni, F. (2011). The pattern of gene expression and gene dose profiles of 6-mercaptopurine- and 6-thioguanine-resistant human leukemia cells. *Biochem. Biophys. Res. Commun.* 411, 156–161. doi: 10.1016/j.bbrc.2011.06.120

Karner, S., Shi, S., Fischer, C., Schaeffeler, E., Neurath, M. F., Herrlinger, K. R., et al. (2010). Determination of 6-thioguanosine diphosphate and triphosphate and nucleoside diphosphate kinase activity in erythrocytes: novel targets for thiopurine therapy? *Ther. Drug Monit.* 32, 119–128. doi: 10.1097/FTD.0b013e3181d12f19

Kelly, O., Trajkovski, A., Weizman, A., Nguyen, G., Steinhart, A. H., Silverberg, M., et al. (2016). Concentrations of 6-thioguanine nucleotide correlate with both infliximab and adalimumab levels in patients with inflammatory bowel disease on combination therapy. *J. Crohns Colitis* 10(Suppl. 1):S285. doi: 10.1093/ecco-jcc/jjw019

Kham, S. K., Soh, C. K., Liu, T. C., Chan, Y. H., Ariffin, H., Tan, P. L., et al. (2008). Thiopurine S-methyltransferase activity in three major Asian populations: a population-based study in Singapore. *Eur. J. Clin. Pharmacol.* 64, 373–379. doi: 10.1007/s00228-007-0426-x

Kim, H. S., Cheon, J. H., Jung, E. S., Park, J., Aum, S., Park, S. J., et al. (2016). A coding variant in FTO confers susceptibility to thiopurine-induced leukopenia in East Asian patients with IBD. *Gut* 66, 1926–1935. doi: 10.1136/gutjnl-2016-311921

Kim, J. H., Cheon, J. H., Kim, T. I., and Kim, W. H. (2009). A survey of actual clinical practice patterns in the treatment of inflammatory bowel disease in Korea. *Intest. Res.* 7, 79–85.

Komiyama, T., Yajima, T., Kubota, R., Iwao, Y., Sakuraba, A., Funakoshi, S., et al. (2008). Lower doses of 6-mercaptopurine/azathioprine bring enough clinical efficacy and therapeutic concentration of erythrocyte 6-mercaptopurine metabolite in Japanese IBD patients. *J. Crohns Colitis* 2, 315–321. doi: 10.1016/j.crohns.2008.05.002

Konidari, A., Anagnostopoulos, A., Bonnett, L. J., Pirmohamed, M., and El-Matary, W. (2014). Thiopurine monitoring in children with inflammatory bowel disease: a systematic review. *Br. J. Clin. Pharmacol.* 78, 467–476. doi: 10.1111/bcp.12365

Krishnamurthy, P., Schwab, M., Takenaka, K., Nachagari, D., Morgan, J., Leslie, M., et al. (2008). Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. *Cancer Res.* 68, 4983–4989. doi: 10.1158/0008-5472.CAN-07-6790

Kurzawski, M., Dziewanowski, K., Safranow, K., and Drozdzik, M. (2012). Polymorphism of genes involved in purine metabolism (XDH, AOX1, MOCOS) in kidney transplant recipients receiving azathioprine. *Ther. Drug Monit.* 34, 266–274. doi: 10.1097/FTD.0b013e31824aa681

Lee, H. J., Yang, S.-K., Kim, K.-J., Choe, J. W., Yoon, S. M., Ye, B. D., et al. (2009). The safety and efficacy of azathioprine and 6-mercaptopurine in the treatment of Korean patients with Crohn's disease. *Intest. Res.* 7, 22–31.

Lee, K.-M., Kim, Y. S., Seo, G. S., Kim, T. O., Yang, S.-K., and IBD Study Group of the Korean Association for the Study of Intestinal Diseases (2015). Use of thiopurines in inflammatory bowel disease: a consensus statement by the Korean Association for the Study of Intestinal Diseases (KASID). *Intest. Res.* 13, 193–207. doi: 10.5217/ir.2015.13.3.193

Lees, C. W., Maan, A. K., Hansoti, B., Satsangi, J., and Arnott, I. D. R. (2008). Tolerability and safety of mercaptopurine in azathioprine-intolerant patients with inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 27, 220–227. doi: 10.1111/j.1365-2036.2007.03570.x

Lennard, L. (2002). TPMT in the treatment of Crohn's disease with azathioprine. *Gut* 51, 143–146.

Lennard, L., Lilleyman, J. S., Van Loon, J., and Weinshilboum, R. M. (1990). Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. *Lancet* 336, 225–229. doi: 10.1016/0140-6736(90)91745-V

Lennard, L., and Singleton, H. J. (1992). High-performance liquid chromatographic assay of the methyl and nucleotide metabolites of 6-mercaptopurine: quantitation of red blood cell 6-thioguanine nucleotide, 6-thioinosinic acid and 6-methylmercaptopurine metabolites in a single sample. *J. Chromatogr. B Biomed. Sci. Appl.* 583, 83–90. doi: 10.1016/0378-4347(92)80347-S

Leong, R. W., Gearry, R. B., and Sparrow, M. P. (2008). Thiopurine hepatotoxicity in inflammatory bowel disease: the role for adding allopurinol. *Expert Opin. Drug Saf.* 7, 607–616. doi: 10.1517/14740338.7.5.607

Leung, Y., Sparrow, M. P., Schwartz, M., and Hanauer, S. B. (2009). Long term efficacy and safety of allopurinol and azathioprine or 6-mercaptopurine in patients with inflammatory bowel disease. *J. Crohns Colitis* 3, 162–167. doi: 10.1016/j.crohns.2009.02.003

Lewis, J. D., Schwartz, J. S., and Lichtenstein, G. R. (2000). Azathioprine for maintenance of remission in Crohn's disease: benefits outweigh the risk of lymphoma. *Gastroenterology* 118, 1018–1024. doi: 10.1053/gast.2000.7954

Li, B., Li, H., Bai, Y., Kirschner-Schwabe, R., Yang, J. J., Chen, Y., et al. (2015). Negative feedback-defective PRPS1 mutants drive thiopurine resistance in relapsed childhood ALL. *Nat. Med.* 21, 563–571. doi: 10.1038/nm.3840

Li, F., Fridley, B. L., Matimba, A., Kalari, K. R., Pelleymounter, L., Moon, I., et al. (2010). Ecto-5'-nucleotidase and thiopurine cellular circulation: association with cytotoxicity. *Drug Metab. Dispos.* 38, 2329–2338. doi: 10.1124/dmd.110.035220

Liu, C., Yang, W., Pei, D., Cheng, C., Smith, C., Landier, W., et al. (2017). Genomewide approach validates thiopurine methyltransferase activity is a monogenic pharmacogenomic trait. *Clin. Pharmacol. Ther.* 101, 373–381. doi: 10.1002/cpt.463

Lopez, A., Mounier, M., Bouvier, A.-M., Carrat, F., Maynadié, M., Beaugerie, L., et al. (2014). Increased risk of acute myeloid leukemias and myelodysplastic syndromes in patients who received thiopurine treatment for inflammatory bowel disease. *Clin. Gastroenterol. Hepatol.* 12, 1324–1329. doi: 10.1016/j.cgh.2014.02.026

Lysaa, R. A., Giverhaug, T., Libæk Wold, H., and Aarbakke, J. (1996). Inhibition of human thiopurine methyltransferase by furosemide, bendroflumethiazide and trichlormethiazide. *Eur. J. Clin. Pharmacol.* 49, 393–396. doi: 10.1007/s002280050038

Mao, R., Guo, J., Ben Horin, S., and Chen, M. (2017). P428 Optimizing thiopurines in Crohn's disease: low dose and low 6-TGN level are effective for maintenance

of remission in Asian population. *J. Crohns Colitis* 11, S294–S294. doi: 10.1093/ecco-jcc/jjx002.553

Marsh, S., King, C. R., Ahluwalia, R., and McLeod, H. L. (2004). Distribution of ITPA P32T alleles in multiple world populations. *J. Hum. Genet.* 49, 579–581. doi: 10.1007/s10038-004-0183-y

Martinez-Chantar, M. L., Garcia-Trevijano, E. R., Latasa, M. U., Perez-Mato, I., Sanchez del Pino, M. M., Corrales, F. J., et al. (2002). Importance of a deficiency in S-adenosyl-L-methionine synthesis in the pathogenesis of liver injury. *Am. J. Clin. Nutr.* 76, 1177S–1182S.

Matimba, A., Li, F., Livshits, A., Cartwright, C. S., Scully, S., Fridley, B. L., et al. (2014). Thiopurine pharmacogenomics: association of SNPs with clinical response and functional validation of candidate genes. *Pharmacogenomics* 15, 433–447. doi: 10.2217/pgs.13.226

Matricon, J., Barnich, N., and Ardid, D. (2010). Immunopathogenesis of inflammatory bowel disease. *Self Nonself* 1, 299–309. doi: 10.4161/self.1.4.13560

Matsumoto, T., Motoya, S., Watanabe, K., Hisamatsu, T., Nakase, H., Yoshimura, N., et al. (2016). Adalimumab monotherapy and a combination with azathioprine for Crohn's disease: a prospective, randomized trial. *J. Crohns Colitis* 10, 1259–1266. doi: 10.1093/ecco-jcc/jjw152

Milek, M., Smid, A., Tamm, R., Kuzelicki, N. K., Metspalu, A., and Mlinaric-Rascan, I. (2012). Post-translational stabilization of thiopurine S-methyltransferase by S-adenosyl-L-methionine reveals regulation of TPMT*1 and *3C allozymes. *Biochem. Pharmacol.* 83, 969–976. doi: 10.1016/j.bcp.2012.01.010

Moon, W., and Loftus, E. V. (2016). Review article: recent advances in pharmacogenetics and pharmacokinetics for safe and effective thiopurine therapy in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 43, 863–883. doi: 10.1111/apt.13559

Moreau, A. C., Paul, S., Del Tedesco, E., Rinaudo-Gaujous, M., Boukhadra, N., Genin, C., et al. (2014). Association between 6-thioguanine nucleotides levels and clinical remission in inflammatory disease. *Inflamm. Bowel Dis.* 20, 464–471. doi: 10.1097/01.MIB.0000439068.71126.00

Moriyama, T., Nishii, R., Perez-Andreu, V., Yang, W., Klussmann, F. A., Zhao, X., et al. (2016). *NUDT15* polymorphisms alter thiopurine metabolism and hematopoietic toxicity. *Nat. Genet.* 48, 367–373. doi: 10.1038/ng.3508

Neurath, M. F., Kiesslich, R., Teichgräber, U., Fischer, C., Hofmann, U., Eichelbaum, M., et al. (2005). 6-Thioguanosine diphosphate and triphosphate levels in red blood cells and response to azathioprine therapy in Crohn's disease. *Clin. Gastroenterol. Hepatol.* 3, 1007–1014. doi: 10.1016/S1542-3565(05)00697-X

Ochenrider, M. G., Patterson, D. J., and Aboulafia, D. M. (2010). Hepatosplenic T-cell lymphoma in a young man with Crohn's disease: case report and literature review. *Clin. Lymphoma Myeloma Leuk.* 10, 144–148. doi: 10.3816/CLML.2010.n.021

Oláh, T., Régely, K., and Mándi, Y. (1994). The inhibitory effects of allopurinol on the production and cytotoxicity of tumor necrosis factor. *Naunyn Schmiedebergs Arch. Pharmacol.* 350, 96–99.

Osterman, M. T., Kundu, R., Lichtenstein, G. R., and Lewis, J. D. (2006). Association of 6-thioguanine nucleotide levels and inflammatory bowel disease activity: a meta-analysis. *Gastroenterology* 130, 1047–1053. doi: 10.1053/j.gastro.2006.01.046

Panaccione, R., Ghosh, S., Middleton, S., Márquez, J. R., Scott, B. B., Flint, L., et al. (2014). Combination therapy with infliximab and azathioprine is superior to monotherapy with either agent in ulcerative colitis. *Gastroenterology* 146, 392–400.e3. doi: 10.1053/j.gastro.2013.10.052

Panaccione, R., Ghosh, S., Middleton, S., Velazquez, J. R. M., Khalif, I., Flint, L., et al. (2011). Infliximab, azathioprine, or infliximab + azathioprine for treatment of moderate to severe ulcerative colitis: the UC SUCCESS trial. *J. Crohns Colitis* 5, S8–S9. doi: 10.4292/wjgpt.v8.i2.103

Peng, X. X., Shi, Z., Damaraju, V. L., Huang, X. C., Kruh, G. D., Wu, H. C., et al. (2008). Up-regulation of MRP4 and down-regulation of influx transporters in human leukemic cells with acquired resistance to 6-mercaptopurine. *Leuk. Res.* 32, 799–809. doi: 10.1016/j.leukres.2007.09.015

Pieters, R., Huismans, D. R., Loonen, A. H., Peters, G. J., Hahlen, K., van der Does-van den Berg, A., et al. (1992). Hypoxanthine-guanine phosphoribosyltransferase in childhood leukemia: relation with immunophenotype, *in vitro* drug resistance and clinical prognosis. *Int. J. Cancer* 51, 213–217. doi: 10.1002/ijc.2910510208

Poppe, D., Tiede, I., Fritz, G., Becker, C., Bartsch, B., Wirtz, S., et al. (2006). Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. *J. Immunol.* 176, 640–651. doi: 10.4049/jimmunol.176.1.640

Prefontaine, E., Macdonald, J. K., and Sutherland, L. R. (2010). Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. *Cochrane Database Syst. Rev.* 30:CD000545. doi: 10.1002/14651858.CD000545.pub4

Rahier, J. F., Magro, F., Abreu, C., Armuzzi, A., Ben-Horin, S., Chowers, Y., et al. (2014). Second European evidence-based consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. *J. Crohns Colitis* 8, 443–468. doi: 10.1016/j.crohns.2013.12.013

Reinshagen, M., Schutz, E., Armstrong, V. W., Behrens, C., Von Tirpitz, C., Stallmach, A., et al. (2007). 6-Thioguanine nucleotide-adapted azathioprine therapy does not lead to higher remission rates than standard therapy in chronic active Crohn disease: results from a randomized, controlled, open trial. *Clin. Chem.* 53, 1306–1314. doi: 10.1373/clinchem.2007.086215

Roberts, R. L., Gearry, R. B., Barclay, M. L., and Kennedy, M. A. (2007). IMPDH1 promoter mutations in a patient exhibiting azathioprine resistance. *Pharmacogenomics J.* 7, 312–317. doi: 10.1038/sj.tpj.6500421

Salavaggione, O. E., Wang, L., Wiepert, M., Yee, V. C., and Weinshilboum, R. M. (2005). Thiopurine S-methyltransferase pharmacogenetics: variant allele functional and comparative genomics. *Pharmacogenet. Genomics* 15, 801–815. doi: 10.1097/01.fpc.0000174788.69991.6b

Sasaki, H., Tsuru, K., Nakamura, J., Konishi, R., and Shibasaki, J. (1987). Effect of allopurinol on the intestinal absorption of 6-mercaptopurine in rats. *J. Pharmacobiodyn.* 10, 697–702.

Seinen, M. L., van Asseldonk, D. P., de Boer, N. K., Losekoot, N., Smid, K., Mulder, C. J., et al. (2013). The effect of allopurinol and low-dose thiopurine combination therapy on the activity of three pivotal thiopurine metabolizing enzymes: results from a prospective pharmacological study. *J. Crohns Colitis* 7, 812–819. doi: 10.1016/j.crohns.2012.12.006

Shah, S. A. V., Paradkar, M., Desai, D., and Ashavaid, T. F. (2016). Nucleoside diphosphate-linked moiety X-type motif 15 C415T variant as a predictor for thiopurine-induced toxicity in Indian patients. *J. Gastroenterol. Hepatol.* 32, 620–624. doi: 10.1111/jgh.13494

Shi, H. Y., Chan, F. K., Leung, W. K., Li, M. K., Leung, C. M., Sze, S. F., et al. (2016). Low-dose azathioprine is effective in maintaining remission in steroid-dependent ulcerative colitis: results from a territory-wide Chinese population-based IBD registry. *Ther. Adv. Gastroenterol.* 9, 449–456. doi: 10.1177/1756283X16643509

Shih, D. Q., Nguyen, M., Zheng, L., Ibanez, P., Mei, L., Kwan, L. Y., et al. (2012). Split-dose administration of thiopurine drugs: a novel and effective strategy for managing preferential 6-MMP metabolism. *Aliment. Pharmacol. Ther.* 36, 449–458. doi: 10.1111/j.1365-2036.2012.05206.x

Shipkova, M., Armstrong, V. W., Wieland, E., and Oellerich, M. (2003). Differences in nucleotide hydrolysis contribute to the differences between erythrocyte 6-thioguanine nucleotide concentrations determined by two widely used methods. *Clin. Chem.* 49, 260–268. doi: 10.1373/49.2.260

Shipkova, M., Franz, J., Abe, M., Klett, C., Wieland, E., and Andus, T. (2011). Association between adverse effects under azathioprine therapy and inosine triphosphate pyrophosphatase activity in patients with chronic inflammatory bowel disease. *Ther. Drug Monit.* 33, 321–328. doi: 10.1097/FTD.0b013e31821a7c34

Simsek, M., Meijer, B., Mulder, C. J. J., van Bodegraven, A. A., and de Boer, N. K. H. (2017). Analytical pitfalls of therapeutic drug monitoring of thiopurines in patients with inflammatory bowel disease. *Ther. Drug. Monit.* 39, 584–588. doi: 10.1097/FTD.0000000000000455

Smith, M. A., Blaker, P., Marinaki, A. M., Anderson, S. H., Irving, P. M., and Sanderson, J. D. (2012). Optimising outcome on thiopurines in inflammatory bowel disease by co-prescription of allopurinol. *J. Crohns Colitis* 6, 905–912. doi: 10.1016/j.crohns.2012.02.007

Smith, M. A., Marinaki, A. M., Arenas, M., Shobowale-Bakre, M., Lewis, C. M., Ansari, A., et al. (2009). Novel pharmacogenetic markers for treatment outcome in azathioprine-treated inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 30, 375–384. doi: 10.1111/j.1365-2036.2009.04057.x

Smith, M. A., Marinaki, A. M., and Sanderson, J. D. (2010). Genetic polymorphism in the multi-drug resistance-5 gene is associated with non-response to

azathioprine treatment in inflammatory bowel disease. *Gastroenterology* 138, S87–S88. doi: 10.1016/S0016-5085(10)60400-3

Somerville, L., Krynetski, E. Y., Krynetskaia, N. F., Beger, R. D., Zhang, W., Marhefka, C. A., et al. (2003). Structure and dynamics of thioguanine-modified duplex DNA. *J. Biol. Chem.* 278, 1005–1011. doi: 10.1074/jbc.M204243200

Sparrow, M. P., Hande, S. A., Friedman, S., Cao, D., and Hanauer, S. B. (2007). Effect of allopurinol on clinical outcomes in inflammatory bowel disease nonresponders to azathioprine or 6-mercaptopurine. *Clin. Gastroenterol. Hepatol.* 5, 209–214. doi: 10.1016/j.cgh.2006.11.020

Sparrow, M. P., Hande, S. A., Friedman, S., Lim, W. C., Reddy, S. I., Cao, D., et al. (2005). Allopurinol safely and effectively optimizes tioguanine metabolites in inflammatory bowel disease patients not responding to azathioprine and mercaptopurine. *Aliment. Pharmacol. Ther.* 22, 441–446. doi: 10.1111/j.1365-2036.2005.02583.x

Stocco, G., Cheok, M. H., Crews, K. R., Dervieux, T., French, D., Pei, D., et al. (2009). Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.* 85, 164–172. doi: 10.1038/clpt.2008.154

Stocco, G., Cuzzoni, E., De Iudicibus, S., Franca, R., Favretto, D., Malusà, N., et al. (2014). Deletion of glutathione-S-transferase M1 reduces azathioprine metabolite concentrations in young patients with inflammatory bowel disease. *J. Clin. Gastroenterol.* 48, 43–51. doi: 10.1097/MCG.0b013e31828b2866

Stocco, G., Londero, M., Campanozzi, A., Martelossi, S., Marino, S., Malusa, N., et al. (2010). Usefulness of the measurement of azathioprine metabolites in the assessment of non-adherence. *J. Crohns Colitis* 4, 599–602. doi: 10.1016/j.crohns.2010.04.003

Stocco, G., Yang, W., Crews, K. R., Thierfelder, W. E., Decorti, G., Londero, M., et al. (2012). *PACSIN2* polymorphism influences TPMT activity and mercaptopurine-related gastrointestinal toxicity. *Hum. Mol. Genet.* 21, 4793–4804. doi: 10.1093/hmg/dds302

Sumi, S., Marinaki, A. M., Arenas, M., Fairbanks, L., Shobowale-Bakre, M., Rees, D. C., et al. (2002). Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency. *Hum. Genet.* 111, 360–367. doi: 10.1007/s00439-002-0798-z

Teml, A., Schaeffeler, E., Herrlinger, K. R., Klotz, U., and Schwab, M. (2007). Thiopurine treatment in inflammatory bowel disease. *Clin. Pharmacokinet.* 46, 187–208. doi: 10.2165/00003088-200746030-00001

Tiede, I., Fritz, G., Strand, S., Poppe, D., Dvorsky, R., Strand, D., et al. (2003). CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J. Clin. Invest.* 111, 1133–1145. doi: 10.1172/JCI200316432

Timmer, A., Patton, P. H., Chande, N., McDonald, J. W. D., and MacDonald, J. K. (2016). Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst. Rev.* 18:CD000478. doi: 10.1002/14651858.CD000478.pub4

Van Dieren, J. M., Hansen, B. E., Kuipers, E. J., Nieuwenhuis, E. E. S., and Van Der Woude, C. J. (2007). Meta-analysis: inosine triphosphate pyrophosphatase polymorphisms and thiopurine toxicity in the treatment of inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 26, 643–652. doi: 10.1111/j.1365-2036.2007.03412.x

Van Egmond, R., Chin, P., Zhang, M., Sies, C. W., and Barclay, M. L. (2012). High TPMT enzyme activity does not explain drug resistance due to preferential 6-methylmercaptopurine production in patients on thiopurine treatment. *Aliment. Pharmacol. Ther.* 35, 1181–1189. doi: 10.1111/j.1365-2036.2012.05084.x

Vande Casteele, N., Herfarth, H., Katz, J., Falck-Ytter, Y., and Singh, S. (2017). American Gastroenterological Association institute technical review on the role of therapeutic drug monitoring in the management of inflammatory bowel diseases. *Gastroenterology* 153, 835.e–857.e. doi: 10.1053/j.gastro.2017.07.031

Vikingsson, S., Almer, S., Peterson, C., Carlsson, B., and Josefsson, M. (2013). Monitoring of thiopurine metabolites - A high-performance liquid chromatography method for clinical use. *J. Pharm. Biomed. Anal.* 75, 145–152. doi: 10.1016/j.jpba.2012.11.027

Vikingsson, S., Carlsson, B., Almer, S. H., and Peterson, C. (2009). Monitoring of thiopurine metabolites in patients with inflammatory bowel disease-what is actually measured? *Ther. Drug Monit.* 31, 345–350. doi: 10.1097/FTD.0b013e3181a1ea58

Warner, B., Johnston, E., Arenas-HernandeZ, M., Marinaki, A., Irving, P., and Sanderson, J. (2018). A practical guide to thiopurine prescribing and monitoring in IBD. *Frontline Gastroenterol.* 9, 10–15. doi: 10.1136/flgastro-2016-100738

Warner, B., Johnston, E., Fong, S., Blaker, P., Arenas-Hernandez, M., Marinaki, A., et al. (2016). P349. The effects of smoking on thiopurine metabolism. *J. Crohns Colitis* 10, S271–S272. doi: 10.1093/ecco-jcc/jjw019.468

Weingarden, A. R., and Vaughn, B. P. (2017). Intestinal microbiota, fecal microbiota transplantation, and inflammatory bowel disease. *Gut Microbes* 8, 238–252. doi: 10.1080/19490976.2017.1290757

Weng, N. (2006). Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* 24, 495–499. doi: 10.1016/j.immuni.2006.05.001

Weyer, N., Kröplin, T., Fricke, L., and Iven, H. (2001). Human thiopurine S-methyltransferase activity in uremia and after renal transplantation. *Eur. J. Clin. Pharmacol.* 57, 129–136.

Wielinga, P. R., Reid, G., Challa, E. E., van der Heijden, I., van Deemter, L., de Haas, M., et al. (2002). Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. *Mol. Pharmacol.* 62, 1321–1331. doi: 10.1124/mol.62.6.1321

Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., et al. (2000). Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7476–7481. doi: 10.1073/pnas.120159197

Wright, S., Sanders, D. S., Lobo, A. J., and Lennard, L. (2004). Clinical significance of azathioprine active metabolite concentrations in inflammatory bowel disease. *Gut* 53, 1123–1128. doi: 10.1136/gut.2003.032896

Yang, J. J., Landier, W., Yang, W., Liu, C., Hageman, L., Cheng, C., et al. (2015). Inherited NUDT15 variant is a genetic determinant of mercaptopurine intolerance in children with acute lymphoblastic leukemia. *J. Clin. Oncol.* 33, 1235–1242. doi: 10.1200/JCO.2014.59.4671

Yang, S.-K., Hong, M., Baek, J., Choi, H., Zhao, W., Jung, Y., et al. (2014). A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leukopenia. *Nat. Genet.* 46, 1017–1020. doi: 10.1038/ng.3060

Yarur, A. J., Kubiliun, M. J., Czul, F., Sussman, D. A., Quintero, M. A., Jain, A., et al. (2015). Concentrations of 6-thioguanine nucleotide correlate with trough levels of infliximab in patients with inflammatory bowel disease on combination therapy. *Clin. Gastroenterol. Hepatol.* 13, 1118–1124.e3. doi: 10.1016/j.cgh.2014.12.026

Zaza, G., Cheok, M., Krynetskaia, N., Thorn, C., Stocco, G., Hebert, J. M., et al. (2010). Thiopurine pathway. *Pharmacogenet. Genomics* 20, 573–574. doi: 10.1097/FPC.0b013e328334338f

Zhang, A. L., Yang, J., Wang, H., Lu, J. L., Tang, S., and Zhang, X. J. (2017). Association of NUDT15 c.415C>T allele and thiopurine-induced leukocytopenia in Asians: a systematic review and meta-analysis. *Ir. J. Med. Sci.* 187, 145–153. doi: 10.1007/s11845-017-1608-x

Zhang, J. P., Guan, Y. Y., Wu, J. H., Xu, A. L., Zhou, S., and Huang, M. (2004). Phenotyping and genotyping study of thiopurine S-methyltransferase in healthy Chinese children: a comparison of Han and Yao ethnic groups. *Br. J. Clin. Pharmacol.* 58, 163–168. doi: 10.1111/j.1365-2125.2004.02113.x

Zhu, X., Wang, X.-D., Chao, K., Zhi, M., Zheng, H., Ruan, H.-L., et al. (2016). NUDT15 polymorphisms are better than thiopurine S-methyltransferase as predictor of risk for thiopurine-induced leukopenia in Chinese patients with Crohn's disease. *Aliment. Pharmacol. Ther.* 44, 967–975. doi: 10.1111/apt.13796

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 31 October 2018
doi: 10.3389/fphar.2018.01235

Qingchang Suppository Ameliorates Colonic Vascular Permeability in Dextran-Sulfate-Sodium-Induced Colitis

Boyun Sun[1†], Jianye Yuan[1†], Shiying Wang[1], Jiang Lin[1], Wanjun Zhang[1], Jiadong Shao[1], Ruiqing Wang[1], Bei Shi[1] and Hongyi Hu[1,2]*

[1] Department of Gastroenterology, China–Canada Center of Research for Digestive Diseases, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2] Shanghai University of Traditional Chinese Medicine, Shanghai, China

Ulcerative colitis (UC), with a long course and repeated attack, severely affects patient's life quality and increases economic burden all over the world. However, the concrete causes and mechanisms of UC are still unclear, but it is generally considered that many factors participate in this process. Qingchang Suppository (QCS) has been used in treating rectitis and colitis for about 30 years in Shanghai, China. Its satisfactory clinical effects have been proved. The aim of this study is to investigate the effect and mechanisms of QCS on colonic vascular endothelial barrier in dextran sulfate sodium (DSS)-induced colitis. The results indicated that increased vascular permeability (VP) appeared earlier than increased intestinal epithelial permeability (EP) in the process of DSS-induced colitis. QCS attenuated colonic tissue edema, vascular congestion and inflammatory cell infiltration. QCS inhibited the elevation of MPO, TNF-α, and IL-6 levels in colon tissues and alleviated the microvascular damage induced by DSS. QCS also improved colonic hypoxia and decreased the expression of VEGF, HIF-1α, and iNOS. These results revealed that QCS can reduce colonic VP and can improve vascular endothelial barrier function maybe by regulating the VEGF/HIF-1α signaling pathway.

Keywords: qingchang suppository, ulcerative colitis, epithelial permeability, vascular permeability, vascular endothelial barrier

OPEN ACCESS

Edited by:
Luca Antonioli,
Università degli Studi di Pisa, Italy

Reviewed by:
Rocchina Colucci,
Università degli Studi di Padova, Italy
Maria Grazia Zizzo,
Università degli Studi di Palermo, Italy

****Correspondence:***
Hongyi Hu
hongyihu2003@shutcm.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 July 2018*
Accepted: *11 October 2018*
Published: *31 October 2018*

Citation:
Sun B, Yuan J, Wang S, Lin J, Zhang W, Shao J, Wang R, Shi B and Hu H (2018) Qingchang Suppository Ameliorates Colonic Vascular Permeability in Dextran-Sulfate-Sodium-Induced Colitis. Front. Pharmacol. 9:1235. doi: 10.3389/fphar.2018.01235

INTRODUCTION

Ulcerative colitis (UC), a subtype of inflammatory bowel disease (IBD), is characterized by chronic inflammation of colonic mucosa and recurrent attack. Abdominal pain, diarrhea and mucopurulent bloody stools are the main clinical manifestations of UC (Floch, 2011). Its lesions mainly involve the mucous membrane and submucosa of the rectum, sigmoid, and descending colon, some parts of the transverse colon, or even the whole colon. Severe ulcers may invade the muscular layer and serosa, leading to perforation. UC with a wide range and >10 years is easy to develop into colorectal cancer (Masakazu, 2014). The World Health Organization has listed UC as one of the refractory diseases as it has a strong impact on quality of life due to its long course and repeated attacks. At present, the focus of UC treatment is to control mucosal inflammation and inhibit excessive immunoreactivity. Treatments with conventional drugs, including aminosalicylic acid, hormones, immunosuppressants, and biological agents, have been improved greatly in recent years.

The pathogenesis of UC is complicated, and so far, the exact etiology and pathogenesis remain unclear. UC is subject to immunological, mental, dietary, infectious, allergic, genetic and

environmental factors. Thus, it is regarded as a multi-factorial disease resulting from interaction between host reactions, which are affected by immunity and heredity, and exogenous stimulation. Although many studies have reported the factors that may be involved in UC, no consensus has been reached on the primary cause of cell and tissue damage. It is disputed whether inflammation and the immune response are part of the initial damage or are secondary reactions. Intestinal epithelial barrier dysfunction is characterized by increased epithelial permeability (EP), which causes intraluminal bacteria and other antigens to traverse the epithelium into the mucosa, enhancing the immune response, triggering or aggravating UC (Mankertz and Schulzke, 2007; Alsadi et al., 2009). Most studies on the role of the epithelial barrier in the pathogenesis of UC have focused on increased EP (Alsadi et al., 2009; Jump and Levine, 2010). In fact, EP is not only determined by the epithelium, but also by the mucosal blood vessels. The vascular endothelial barrier plays an important role in keeping the integrity of the epithelium as it can maintain blood flow and deliver oxygen and nutrients to the epithelium, and prevent infiltration of inflammatory cells or proteins (Thornton and Solomon, 2002). Increased mucosal vascular permeability (VP) and reduced blood flow can cause intestinal tissue hypoxia (Taylor and Colgan, 2007), which induces increased expression of oxyradical or other inflammatory mediators, leading to intestinal epithelial cell damage and destruction of adjacent tight junctions (Rezaie et al., 2007). EP is increased when the integrity of the epithelial barrier is destroyed. Therefore, increased VP may be the initial factor in UC occurrence and recurrence, earlier than increased EP (Tolstanova et al., 2012). So, it is important to study the mechanisms involved in the increase of VP in the early phase of UC, which may be helpful for prevention and treatment.

The traditional Chinese medicine (TCM) Xilei San (Hao et al., 2014) is widely used to treat mucosal inflammation in China. Qingchang Suppository (QCS) (Dai et al., 2010) is a pure TCM preparation, composed of Indigo Naturalis, Herba Portulacae, Radix Notoginseng, Gallnut and borneol. It was first prescribed by Professor Ma Guitong, a renowned TCM doctor in China, based on Xilei San theory accumulated clinical practice. QCS has shown satisfactory clinical results in treating UC in recent years. It can clear away heat and toxic materials, promote blood circulation and remove blood stasis, and eliminate turbidity to promote tissue regeneration and ulcer recovery. As a suppository, it has the advantages of convenient use and easy absorption. This also confirms the fact that UC lesions are often located in the distal colon, sigmoid colon and rectum.

The aim of this study was to investigate the effect of QCS on colonic microvascular permeability in the onset and progression of DSS-induced colitis and the mechanisms involved.

MATERIALS AND METHODS

Herbs and Reagents

QCS (Z05170722) was supplied by Longhua Hospital, Shanghai University of Traditional Chinese Medicine. It consisted of the following herbs: Radix Notoginseng, Indigo Naturalis, Gallnut, Herba Portulacae and borneol, in a ratio of 2:2:5:5:1. Sulfasalazine Suppository (SASP) is a yellow suppository which was made of 0.5 g sulfasalazine with fatty matrix. SASP was purchased from Shanxi Tongda Pharmaceutical Co. Ltd. (Shanxi, China). Dextran sulfate sodium salt (DSS) (MW 36,000–50,000) was purchased from MP Biologicals (Santa Ana, CA, USA). Indirubin, Notoginsenoside R1, Ginsenoside Rg1, Ginsenoside Rb1, Ginsenoside Re, and Luteolin were purchased from the National Institutes for Food and Drug Control (Shanghai, China). Kaempferol, Quercetin, Gallic acid, α-Linolenic acid, and Methyl gallate were purchased from Shanghai Macklin Biochemical Co. Ltd. HPLC grade acetonitrile was obtained from Fisher (Geel, Belgium) and HPLC grade formic acid from ANPEL Laboratory Technologies (Shanghai, China). Water was purified using a Milli-Q Academic System (Millipore, Billerica, MA, USA).

Animals

Male Sprague-Dawley rats (170–200 g) were obtained from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). All animals were housed in a specific pathogen-free laboratory in the Department of Laboratory Animal Science of Shanghai University of Traditional Chinese Medicine (Shanghai, China). Animals were maintained on a 12 h light/dark cycle under controlled temperature (22 ± 2°C) and humidity (50 ± 10%) with standard environmental conditions. All animals had unlimited access to standard diet and water. They were allowed to acclimatize for 7 d before use.

Experimental Design

This experiment includes two parts.

The first part was to confirm the sequence of VP and EP changes in experimental colitis by observing the dynamic changes of intestinal epithelial permeability and vascular permeability in consecutive 6 days (the day before oral administration of DSS, the first day, 2nd, 3th, 4th, 5th day with oral administration of DSS). In the first part, experimental rats were randomly divided into six groups: A: DSS treating 0 d; B: DSS treating 1d; C: DSS treating 2 d; D: DSS treating 3 d; E: DSS treating 4 d; F: DSS treating 5 d. Meanwhile, control rats were set up which only drink water instead of DSS, and rats were killed every day for DAI evaluation and for EP, VP observation. Group A had 10 rats and there were 8 rats in each other group. The rats were euthanized on day 1–5 after treatment with 5% DSS except that in the group A, which were given distilled water and were euthanized on the day before treatment with 5% DSS.

The second part was carried out to investigate the effect of Qingchang suppository (QCS) on the intestinal VP when it increased according to the results of the first part. In the second part, rats were randomly divided into six groups: control, model, QCS low dose (QCS-L), QCS medium dose (QCS-M), QCS high dose (QCS-H) and SASP. Each group had 8 rats. Rats were given 5% DSS water for 3 d except that in the control group, which were given distilled water. The rats in the QCS-L, QCS-M, QCS-H and SASP groups were treated with 0.36, 0.72 or 1.44 g/kg QCS or 0.135 g/kg SASP separately (The medium dose of QCS and the dose of SASP was defined according to human-rat equivalent dosage conversion. The low dose of QCS

was defined as the half of the medium dose of QCS and the high dose of QCS was defined as the 2 times of the medium dose of QCS.) from day 1 to day 3 by rectal administration. In briefly, We used a flexible catheter to deliver the QCS and SASP 2 ml into rectum of rats when they were heated up to about 37°C and were turned into liquid. The rats in the control and model groups were treated with the same volume of distilled water by rectal administration. DSS solution was prepared freshly every day. The volume of DSS solution ingested by each rat was measured daily. Two centimeters of distal colon was removed for morphological examination, VP evaluation, tissue hypoxia detection, ultrastructural observation, and western blotting analysis. All the animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (No. SZY201608003).

Identification of the Chemical Composition of QCS

Preparation of Sample Solutions

QCS (2.94 g) was mashed then extracted with 40 mL methanol by ultrasonication at room temperature for 2 h, and the supernatants were obtained by centrifugation at 5,000 rpm (Kubota 3740, Japan) for 20 min. Finally, the supernatants were filtered through a 0.22-μm membrane for LC–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) analysis.

HPLC

HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-30AD binary pump, DGU-14A degasser, SIL-30AC autosampler and CTO-30AC column oven. The mobile phase consisted of acetonitrile and water containing 0.1 (v/v) formic acid. The samples were analyzed on an Agilent ZORBAX SB-C18 column (1.8 m, 100 × 2.1 mm) with gradient elution system for 0–15 min, using 10–60% acetonitrile. The flow rate was 0.2 mL/min, and the column temperature was set at 45°C.

Each of the 11 reference compounds was accurately weighed and then dissolved in methanol to prepare the stock solutions of 1.0 mg/mL, and a diluted solution of each standard solution (100 ng/mL) was analyzed qualitatively and quantitatively. QCS (2.94 g) was mashed then extracted with 40 mL methanol by ultrasonication at room temperature for 2 h, and the supernatants were obtained by centrifugation at 5,000 rpm (Kubota 3740, Japan) for 20 min. Finally, the supernatants were filtered through a 0.22-μm filter membrane for LC-ESI-MS/MS.

LC-ESI-MS/MS

A triple quadrupole tandem mass spectrometer (Shimadzu) equipped with an ESI source interface was connected to the above Shimadzu HPLC system. Negative ion mode was preferred owing to its high sensitivity for analysis of most of the compounds. The optimized mass parameters were set as follows: collision energy, 35 V; nebulizing gas flow, 3.0 L/min; interface temperature, 300°C; drying gas flow, 10 L/min; DL temperature, 250°C; heat gas flow 10 L/min; heat block temperature, 400°C; Collision-Induced Dissociation gas, 230 kPa.

Evaluation of Disease Activity Index

Body weight, stool consistency, and occult blood or gross bleeding was scored each day during the experimental period. Disease activity index (DAI) was determined by calculating the mean of each score to evaluate the degree of colitis, as previously described (Murthy et al., 1993). DAI was calculated by grading on a scale of 0 to 4 using the following parameters: loss of body weight (0: normal; 1: 0–5%; 2: 5–10%; 3: 10–15%; 4: >15%); stool consistency (0: normal; 2: loose stools; 4: watery diarrhea); and occult blood (0: negative; 2: positive; 4: gross bleeding). The final result was expressed as the average of the three.

Histopathology

Distal colon sections were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. The samples were sliced into 4 μm thick sections, then deparaffinized in xylene and were rehydrated in a decreasing concentration gradient of ethanol and were stained with hematoxylin and eosin (H&E). Colonic morphology was visualized using light microscopy with Olympus image analysis software (Olympus America, Melville, NY, USA). Colonic damage was assessed as described previously (Xiao et al., 2013). Briefly, each colon was scored considering (1) the severity of inflammation (0, none; 1, mild; 2, moderate; 3, severe); (2) the extent of inflammation (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural); and (3) crypt damage (0, none; 1, 1/3 damaged; 2, 2/3 damaged; 3, crypt loss but surface epithelium present; 4, both crypt and surface epithelium lost). Scores were then added, resulting in a total histological score that ranged from 0 to 10.

Quantitative Evaluation of EP

We determined the plasma concentration of fluorescein isothiocyanate- conjugated (FITC)–dextran (MW 3.0–5.0 kDa; Sigma, St. Louis, MO, USA) after its intragastric administration to quantitatively evaluate the colonic EP. Before the experiments, rats were fasted for 18 h, but had unlimited access to water. Rats were given 20 mL/kg phosphate-buffered saline (PBS; pH 7.4) containing 22 mg/mL FITC–dextran by gavage 4 h before sacrifice. Blood samples were obtained by cardiac puncture and were centrifuged (3,000 rpm at 4°C for 20 min. Plasma (100 μL) concentration of fluorescein was measured using a Synergy H4 Hybrid Multi-Mode microplate reader (BioTeck, Winooski, VT, USA) with excitation wavelength of 485 nm and emission wavelength of 520 nm. Serially diluted samples of the marker were set as standards.

Quantitative Evaluation of VP

VP was determined as described previously (Tolstanova et al., 2009) with slight modification. Evans blue dye is a marker of albumin leakage since it binds tightly to albumin and it crosses the endothelial barrier as the complex Evans blue/albumin (MW 67 kDa). All rats were anesthetized with sodium pentobarbital and then received Evans blue (10 mg/kg) by tail vein injection 15 min before sacrifice. We measured EP and VP in the same animals. The distal colon was removed, cleaned, dried, and weighed. The colon samples were soaked in a centrifuge tube containing 2.0 mL formamide and the tube was placed

in a thermostatic water bath. After incubation for 24 h at 60°C, the formamide extract was collected and the Evans blue concentration was measured by spectrophotometry at 610 nm. Results were expressed as mg dye/g dry weight of colon.

Ultrastructural Observation

Samples of colonic tissue were cut into three strips of 1 mm width, and were fixed in 2.5% glutaraldehyde immediately for 2 h at 4°C. After fully washing three times in 0.1 mol/L PBS, they were embedded in epoxy resin. The sliced sections (0.5–1 mm) were stained with toluidine blue for 30 s and washed in 0.1 M PBS. Thin sections (75 nm) from the selected area were stained with uranyl acetate and lead citrate and viewed under a H-600 transmission electron microscope at 80 kV (Hitachi, Tokyo, Japan).

Detection of Tissue Hypoxia

Colonic tissue hypoxia was detected using the Hypoxyprobe-1 Omni Kit (Natural Pharmacia International, Burlington, MA, USA) as previously described (Tolstanova et al., 2012). Rats were anesthetized with sodium pentobarbital. Pimonidazole-HCl (60 mg/kg) was injected intravenously 90 min before autopsy after 5% DSS treatment, vehicle (water) ingestion or drug treatment. The removed distal colon (2 cm) was fixed in 4% formaldehyde for 24 h, dehydrated, and embedded in paraffin. The paraffin-embedded colon sections (5 mm thick) were deparaffinized, hydrated, blocked with 3% H_2O_2/water, and subjected to microwave antigen retrieval using a Dako target retrieval solution (Dako, Carpinteria, CA, USA). After overnight incubation with affinity-purified rabbit anti-pimonidazole antibody (PAb2627AP) at 4°C, they were incubated with anti-rabbit polymer horseradish peroxidase (Dako), with diaminobenzidine used as a peroxidase chromogen. We used hematoxylin for counterstaining and five fields from each slide were randomly selected, viewed and imaged under a fluorescence microscope (Nikon TE2000-U, Nikon, Japan), and analyzed using the Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD). The minimal pixel was set at 50 pixels, and the final result was expressed as integral optical density (IOD)/ area of effective statistical.

Western Blotting

Distal colon tissue was homogenized in RIPA buffer (P0013B; Beyotime, Hangzhou, China) with phosphatase inhibitors (S1873; Beyotime), and protein concentration was determined using the bicinchoninic acid assay method. 20 micrograms of total protein that was extracted from 100 mg colonic mucosa was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). After the membranes were blocked with 5% skimmed milk in Tris buffer saline–Tween 20 (TBST), membranes were immunoblotted with the primary antibody against vascular endothelial growth factor (VEGF) (ab46154; Abcam, Shanghai, China), hypoxia-inducible factor (HIF)-1α (ab2185; Abcam), inducible NO synthase (iNOS) (ab49999; Abcam) or β-actin (Huaan Biological Technology, Hangzhou, China). The primary antibodies were visualized with goat anti-rabbit peroxidase-conjugated antibody (Cell Signaling Technology, Danvers, MA, USA) using an enhanced chemiluminescence detection system (Millipore). The images of blots were acquired by the GBOX Chemi XT4 System (Syngene, Cambridge, UK) and GeneTools software (Syngene) was used for semi-quantitative analysis.

Statistical Analysis

SPSS version 16.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (La Jolla, CA, USA) were used for data analysis. Each value was expressed as mean ± *SD*. Differences between two groups were analyzed using the Student's *t*-test, and statistical comparisons among more than two groups were carried out by One-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization and Quantification of Main Biochemical Components in QCS

The chemical profiles of QCS were analyzed using LC-ESI-MS/MS. In the typical base peak chromatogram (BPC) of QCS samples (**Figure 1**), 21 major peaks were detected and quantified (**Supplementary Table 1**). Eleven compounds (3–10, 13, 15, and 18) were unambiguously assigned by comparing with the respective reference standards, and five compounds (1, 2, 11, 12, and 21) were tentatively identified according to the MS/MS fragmentation patterns as well as information from the literature (Xin et al., 2008; Xu et al., 2012).

Dynamic Pathological Changes of Distal Colon in DSS Models of UC

The paraffin sections of colon were stained with H&E. Rare obvious histological changes were observed in the distal colon on day 0 (**Figure 2A**), followed by extensive submucosal edema at 24 h (**Figure 2B**) after induction of DSS colitis, and focal lamina propria inflammation on day 4 (**Figure 2E**) when the surface epithelial cell layer was still intact (**Figures 2B–E**). The blood vessels in the lamina propria and submucosa were dilated on day 4 (**Figure 2E**) after DSS ingestion. On day 5 after initiation of DSS treatment, the partial mucosal epithelium disappeared, and inflammatory cells increased and accumulated in the submucosa as well as the lamina propria (**Figure 2F**). DSS-induced colitis is characterized as a marked decrease in colon length (Xiao et al., 2013). After DSS administration, the DAI increased gradually (**Figure 2G**). Starting from day 4 (14.13 ± 1.78), DSS induced a rapid decrease in colon length (on day 5, 12.46 ± 1.77) (**Figure 2H**) and serious clinical symptoms (diarrhea and fecal blood).

Increased Colonic VP and EP in DSS-Induced Colitis

The blue color was restricted to the distal colon. No apparent morphological changes were present (data not shown) on day 1 and there was no significant difference between the water and DSS-treated rats. On day 2, the concentration of Evans blue dye in tissue extracts of DSS-treated rats was significantly higher than that in the control rats, and it gradually increased from day 2

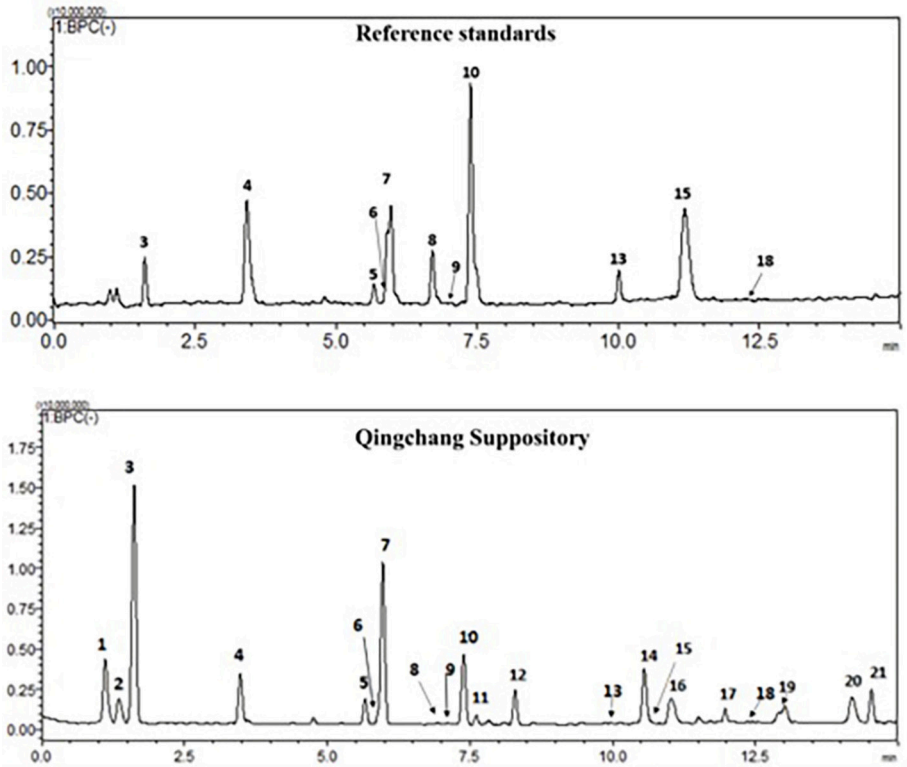

FIGURE 1 | Typical chromatogram of QCS in negative ion mode by LC-ESI-MS.

to day 5 (**Figure 3A**). In contrast to the increased content of Evans blue in colonic tissue, VP and plasma concentration of FITC–dextran were not obviously changed during the first 4 d but were significantly increased on day 5 after DSS treatment (**Figure 3B**). These results confirmed that increased colonic VP preceded increased EP after DSS treatment. In other words, the colonic vascular endothelium injury occurred earlier than colonic epithelial barrier disruption in DSS-induced colitis.

QCS Attenuated DSS Induced Colitis

To confirm the effect of QCS on colonic VP in rats, early colitis was induced by continuous oral administration of 5% DSS in drinking water for 3 d. At the same time, QCS and SASP were administered to rats each day. As expected, DSS induced colonic tissue damage, including the distorted arrangement of cells in crypts, extensive submucosal edema, dilated blood vessels in the submucosa, and little inflammatory cell infiltration (**Figures 4A,B**). On the contrary, the colonic damage in rats treated with QCS-H and SASP, but not QCS-L or QCS-M, were lessened (**Figures 4C–F**). The histological scores of the QCS-H group (2.76 ± 0.94) and the SASP group (2.50 ± 0.74) were significantly reduced, compared to the DSS model group (4.05 ± 1.04) (**Figure 4G**). But there was no significant difference in DAI scores among groups (**Figure 4H**). In addition, as another important symptomatic parameter in DSS induced colitis, colonic MPO activity, was increased in the DSS model group (654.07 ± 88.36) and was significantly suppressed by QCS (QCS-L group: 417.97 ± 62.27; QCS-M group: 273.03 ± 53.61; QCS-H group: 249.30 ± 24.69;) in a dose-dependent manner (**Figure 4I**).

QCS Attenuated DSS Induced VP Increase in Colon

To evaluate the effect of QCS on VP, Evans blue concentration in colon tissue in each group was determined. Evans blue concentration in the model group (137.49 ± 21.26) was significantly higher than that in the control group (56.94 ± 5.51) (**Figure 5**). Compared with the model group, the colonic concentration of Evans blue was decreased in the rats treated with QCS at different doses. Moreover, concentration of Evans blue in the colonic tissues of all the treatment groups (QCS-M group: 92.20 ± 13.74; QCS-H: 81.9 ± 23.31) was significantly lower than that in the model group, except for the QCS-L group (155.63 ± 27.35) and SASP group (115.91 ± 19.26). These results suggest that QCS restore VP to improve tissue hypoxia.

Effect of QCS on Ultrastructural Pathological Changes in Colonic Tissue

Transmission electron microscopy showed that the structure of the colonic vascular endothelium in the control group was intact, and the cells had normal morphology (**Figure 6A**). In the model group, the colonic vascular endothelium was

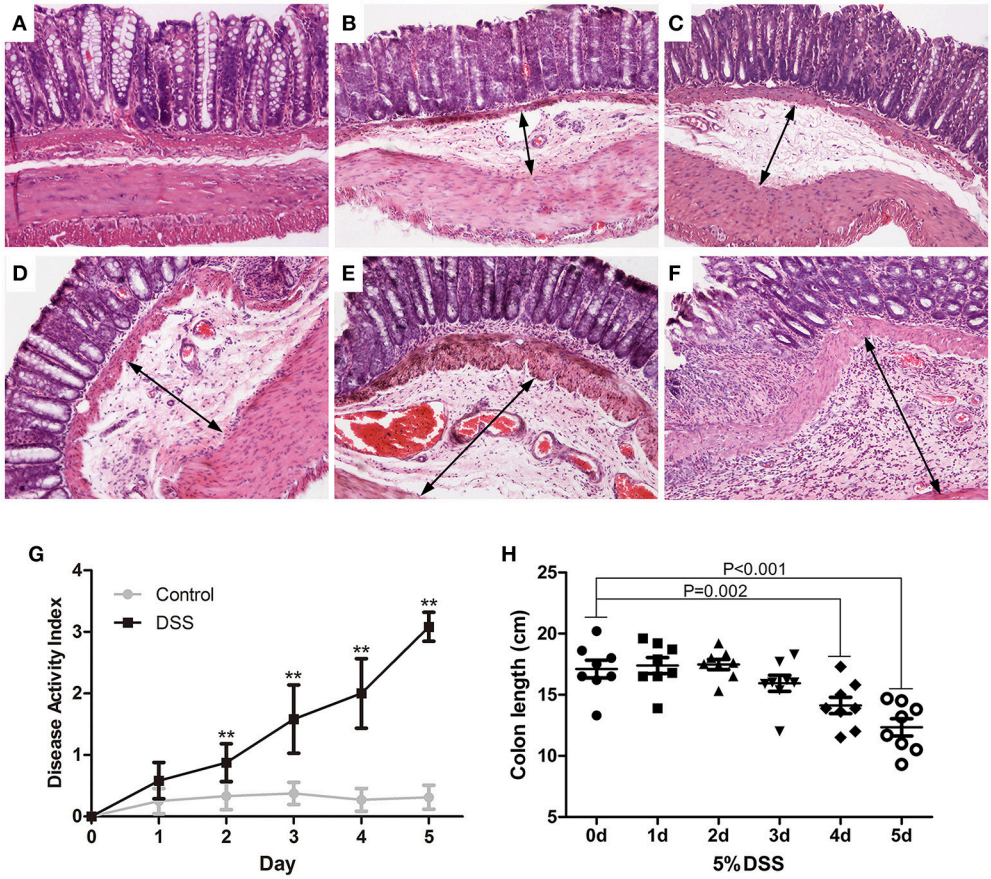

FIGURE 2 | Dynamic pathological changes of the distal colon in DSS models of UC. Paraffin sections of the distal colon were stained with HandE (**A**: DSS 0 d; **B**: DSS 1d; **C**: DSS 2 d; **D**: DSS 3 d; **E**: DSS 4 d; **F**: DSS 5 d) (HandE staining, ×100). DAI **(G)** was assessed daily and colon length was measured on day 5 **(H)**. Data were expressed as mean ± *SD* ($n = 8$–10), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group or vs. 0 d group.

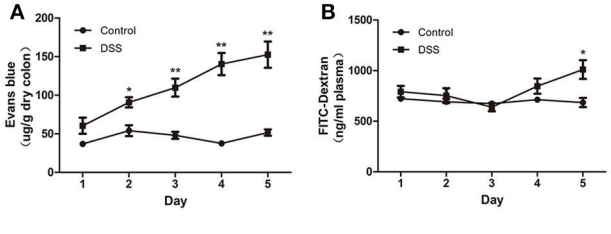

FIGURE 3 | DSS administration increased colonic VP that preceded increased colonic EP in rats. **(A)** Quantitative measurement of VP by extracting extravasated Evans blue in the colonic mucosa. **(B)** Quantitative measurement of colonic epithelial permeability by determining serum concentration of FITC–dextran. Both VP and EP in the same animal were measured. $n = 8$. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control group.

swollen; the intercellular spaces were widened and the cells were ruptured; the basement membrane was not intact; and a large number of platelets aggregated in the vascular endothelium, with signs of some leakage. There was also perivascular edema and a large amount of fibrin deposition that likely resulted from vascular rupture (**Figure 6B**). The QCS-L group showed small breaks in the endothelial lining and platelets adhering to endothelial cells, with some extravascular platelets, but a decrease in fibrin deposition (**Figure 6C**). QCS-M and QCS-H groups showed reduced edema around the blood vessels and intact vascular basement membrane with no obvious platelet aggregation (**Figures 6D,E**). However, in the SASP group, a large number of red blood cells were clustered in the colonic vasculature, and increased micropinocytotic vesicles, swelling of endothelial cells and apoptosis, and perivascular edema were found (**Figure 6F**).

Effect of QCS on Colonic Tissue Hypoxia

No significant hypoxia was observed in colonic tissue of the control group (**Figure 7A**). Conversely, colonic epithelium showed severe hypoxia, which extended to the lamina propria and submucosa in the model group (**Figure 7B**). Compared with the model group, the hypoxic state of the colonic tissue was significantly reduced in the QCS groups (**Figures 7C–E**), and was especially in the QCS-H group (**Figure 7E**). However, there was still severe hypoxia in the colonic mucosa of the SASP group (**Figure 7F**). Quantified data of hypoxia in colonic

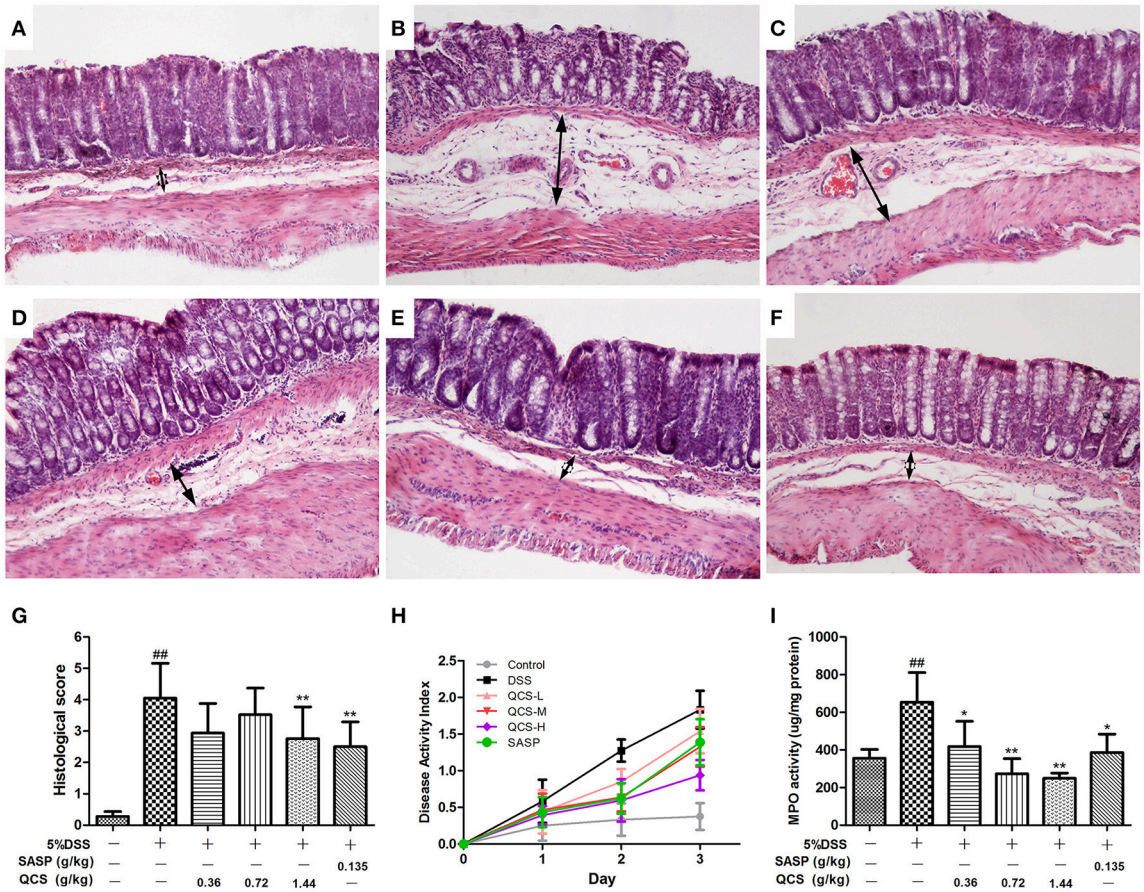

FIGURE 4 | Effects of QCS on histopathological changes, DAI and MPO activity in colon of rats with DSS-induced colitis. **(A)** Control; **(B)** DSS model; **(C)** QCS 0.36 g/kg; **(D)** QCS 0.72 g/kg; **(E)** QCS 1.44 g/kg; **(F)** SASP 0.135 g/kg; (HandE staining, ×100); **(G)** histological score; and **(H)** The disease activity index (DAI) was determined by combining scores of body weight loss, stool consistency, and occult blood, The final result was expressed as the average of the three. **(I)** MPO activity. DSS administration was performed in all groups except the control group. QCS and SASP were administered to rats each day after DSS treatment. All rats were killed on day 3 after DSS administration, three sections from each animal tissue were scored, colonic tissue damage was evaluated by histopathological analysis (HandE staining). MPO activity in colonic tissue was determined. Data were expressed as mean ± *SD* ($n = 8$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group; $^{\#\#}P < 0.01$, vs. control group.

mucosa were measured (**Figure 7G**). These results suggest that QCS can improve hypoxia in colonic tissue induced by DSS.

QCS Inhibited DSS Induced TNF-α and IL-6 Production and Expression of VEGF, HIF-1α and iNOS in Colonic Tissue

The levels of TNF-α and IL-6 in colonic tissue of the model rats (1182.6 ± 148.56, 131.63 ± 25.73) were significantly higher than those in the control rats (790.27 ± 30.06, 60.30 ± 10.18) (**Figures 8A,B**). However, treatment with different doses of QCS and SASP suppressed the increased levels of TNF-α (QCS-L group: 947.54 ± 23.63; QCS-M group: 874.51 ± 21.38; QCS-H group: 852.87 ± 22.44; SASP group: 854.51± 25.66) and IL-6 (QCS-M group: 79.01 ± 12.56; QCS-H group: 83.37 ± 10.17; SASP group: 68.29 ± 8.49) in DSS-induced colitis. These results suggest that QCS has anti-inflammatory effects. Expression of VEGF, HIF-1α, and iNOS in model group (88.30 ± 4.08; 103.16 ± 23.14; 111.78 ± 9.96) rats was substantially higher than that in the control group (50.31 ± 2.42; 17.96 ± 4.41; 27.69 ± 2.73) (**Figures 8C,D**). In contrast, expression of VEGF, HIF-1α and iNOS was inhibited in the QCS- and SASP-treated groups, especially in the QCS-H group (52.75 ± 13.06, 20.72 ± 4.46, 15.79 ±1.61). These results indicate that QCS maybe restores VP by suppressing the activation of the VEGF/HIF-1α signaling pathway.

DISCUSSION

Three major findings were extracted from this study: (1) vascular endothelial injury of UC occurs earlier than colonic mucosal dysfunction; (2) local tissue hypoxia may induce or aggravate intestinal epithelial barrier dysfunction; and (3) QCS may prevent the development of UC by improving damage to the

colonic vascular endothelium to ameliorate tissue hypoxia, and by anti-inflammatory activity.

The chronological changes of DSS-induced colitis were categorized into two phases (Saijo et al., 2015); namely the early phase (days 2–3) and late phase (days 4–5). Hematochezia, as a primary clinical symptom, presents throughout the development of UC. Studies on rats have shown that hematochezia and weight loss occur on days 3–4 after DSS administration, but DSS directly penetrates the lamina propria at 24 h after DSS administration. Vascular smooth muscle and endothelial cells are attacked by DSS directly or indirectly through histamine regulation (Johansson et al., 2010). The results of histomorphological observation and EP determination confirmed that no significant change in intestinal epithelial structure was found in the early stage of DSS-induced colitis. It is further suggested that vascular injury occurs earlier than destruction of intestinal epithelial structure in the early stage of UC. Moreover, the indirect effect of histamine may be attributed to the increased expression of mast cells in the lamina propria (Kurashima et al., 2012), which also provides evidence for the early colon tissue edema of DSS-induced colitis.

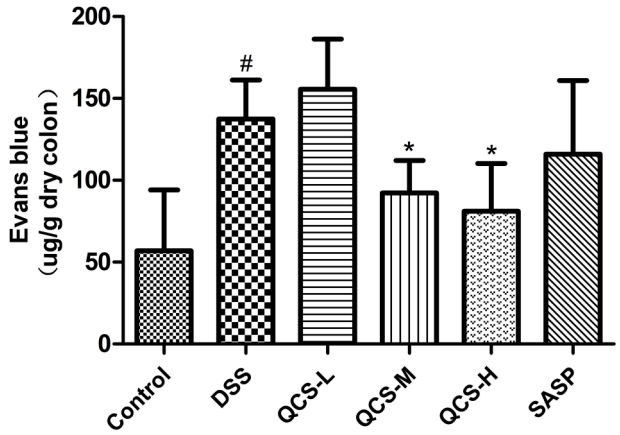

FIGURE 5 | Effects of QCS on VP in colon of rats with DSS-induced colitis. Quantitative measurement of VP. Data were expressed as mean ± *SD* ($n = 8$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group; $^{\#\#}P < 0.01$, vs. control group.

An ischemic colitis model in mice showed massive bleeding and intestinal epithelial cell exfoliation after ligation of the distal colon artery for 3 d (Irkorucu et al., 2008). Experimental vascular congestion induced by ligation of the inferior mesenteric vein may lead to dysfunction of the intestinal mucosa. This phenomenon is similar to that of DSS-induced colitis. One study has shown that chronic inflammation in patients with UC leads to a reduction in mucosal vasodilation due to strong oxidative stress (Hatoum et al., 2003). Angiographic studies have suggested that colon blood vessels are twisted, dilated and distributed irregularly in the early stage of UC, and there is a decrease in the diameter of the injured vessels, blood vessel density and blood flow with further development of UC (Chidlow et al., 2007). Furthermore, confocal endoscopy has also confirmed that VP increases in colon mucosa of patients with UC (Tarnawski et al., 2009). Disturbance of microcirculation in colon tissue may result in slowed blood flow and increased VP, which leads to local tissue hypoxia because blood oxygen cannot be transported to the

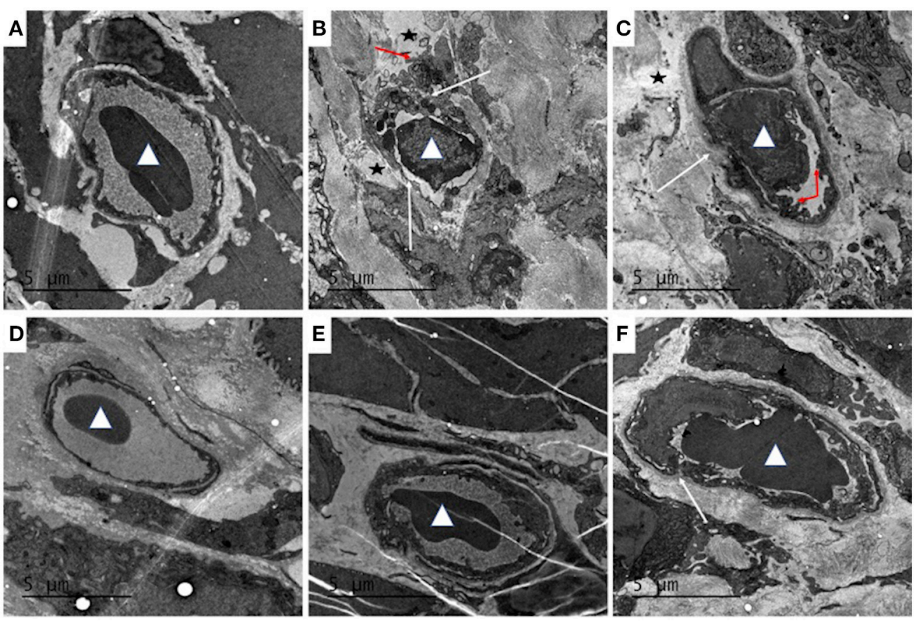

FIGURE 6 | Effects of QCS on DSS-induced ultrastructural pathology changes in colon tissue. Histopathological examination of colon tissues by TEM (4,200×), $n = 5$. **(A)** Control; **(B)** DSS model; **(C)** QCS 0.36 g/kg; **(D)** QCS 0.72 g/kg; **(E)** QCS 1.44 g/kg; **(F)** SASP 0.135 g/kg. Red blood cells (white triangles). Platelet aggregation (red arrow). The microvascular basement membrane and endothelium (white arrow).

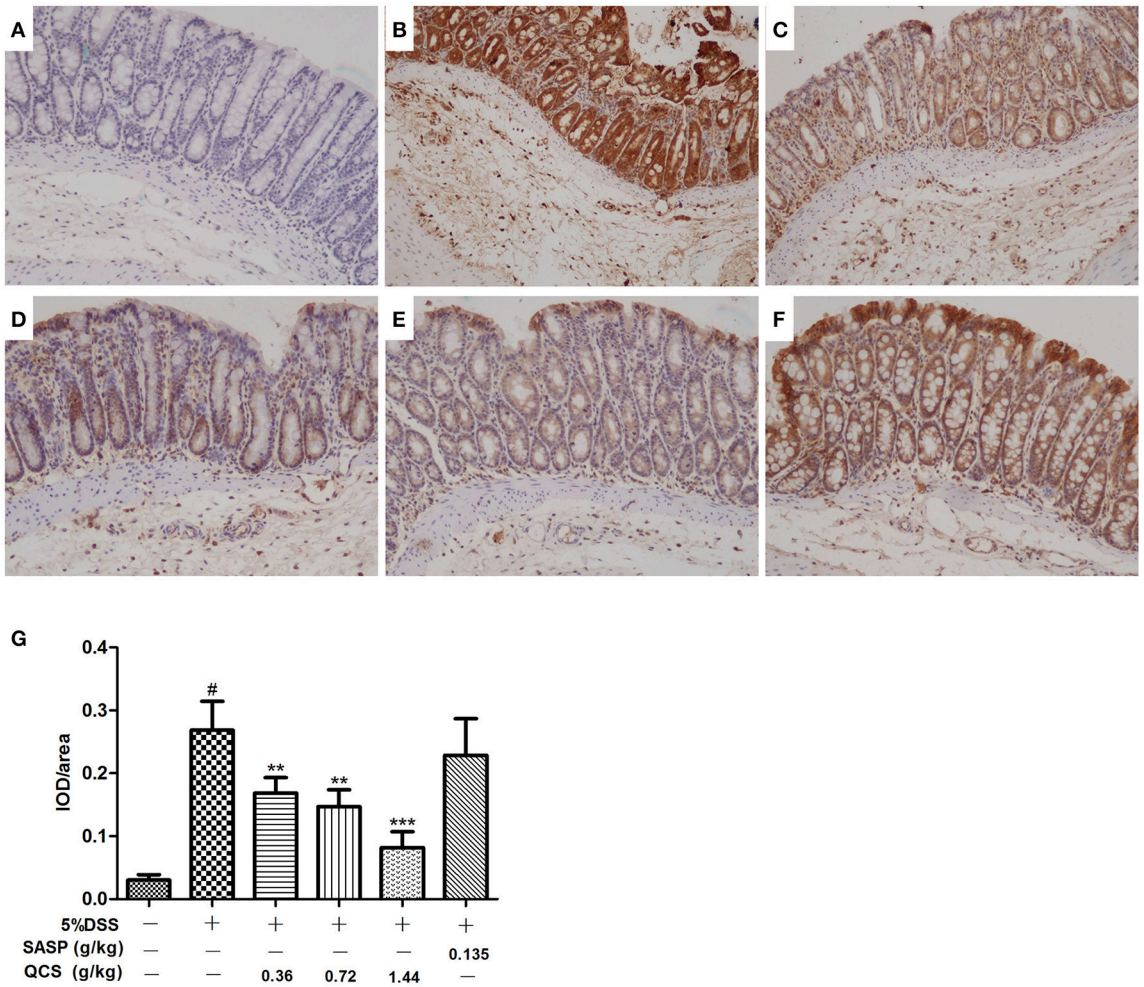

FIGURE 7 | Effects of QCS on DSS-induced colon tissues hypoxia. Visualization of hypoxia in colonic mucosa by Hypoxyprobe-1 staining. Images are representative of three tissue slices (brown staining, × 100). **(A)** Control; **(B)** DSS model; **(C)** QCS 0.36 g/kg; **(D)** QCS 0.72 g/kg; **(E)** QCS 1.44 g/kg; **(F)** SASP 0.135 g/kg. **(G)** Quantified data of hypoxia in colonic mucosa. The final result was expressed as integral optical density (IOD)/ area of effective statistical. ($n = 6$/group). $^{**}P < 0.01$; $^{***}P < 0.001$, vs. model group; $^{\#}P < 0.05$, vs. control group.

mucosal epithelial cells. So, increased VP may be an important factor in the pathogenesis of UC.

VEGF is one of the most potent angiogenic factors, which has specificity for vascular endothelial cells. VEGF is directly associated with angiogenesis and inflammation in human and experimental UC (Scaldaferri et al., 2009). It has been confirmed that VEGF induces an increase in VP in UC, especially in the early stage, and VEGF inhibition has been shown to decrease VP and prevent further development of UC (Tolstanova et al., 2009). It has been shown that lymphangiogenesis, a process also enhanced in IBD and driven by VEGF-C, plays a protective role in animal models of UC (D'Alessio et al., 2014). Another study (Koutroubakis et al., 2004) has shown that IBD patients with elevated levels of VEGF expression in blood, which also indicates that VEGF is involved in the angiogenesis and VP change in IBD. In the early stage of UC, the increased expression of VEGF in colonic tissue may be due to vascular destruction and increased HIF-1 expression (Trojanowska, 2010). These results support the idea that increased VP by VEGF precedes mucosal disorder in the early stage of UC.

HIF-1α is an intranuclear protein that is produced by a decreased level of intracellular oxygen. HIF-1α has been shown to be a major inducer of hypoxia-driven VEGF. Increased levels of HIF-1α in tissue specimens and high levels of VEGF in serum samples of UC patients have been reported (Tajdini et al., 2013). Likewise, expression of HIF-1α increased in colonic submucosal vascular endothelial cells, vascular smooth muscle cells and myenteric plexus neurons in the DSS-induced UC model. As one of the target genes regulated by HIF-1α, it has been shown that the expression of iNOS gene could be induced by HIF-1(Surh et al., 2001). Besides, many iNOS-positive cells also appear in the lamina propria of the colonic tissue of DSS induced UC (Saijo et al., 2015). The occurrence of iNOS-positive cells

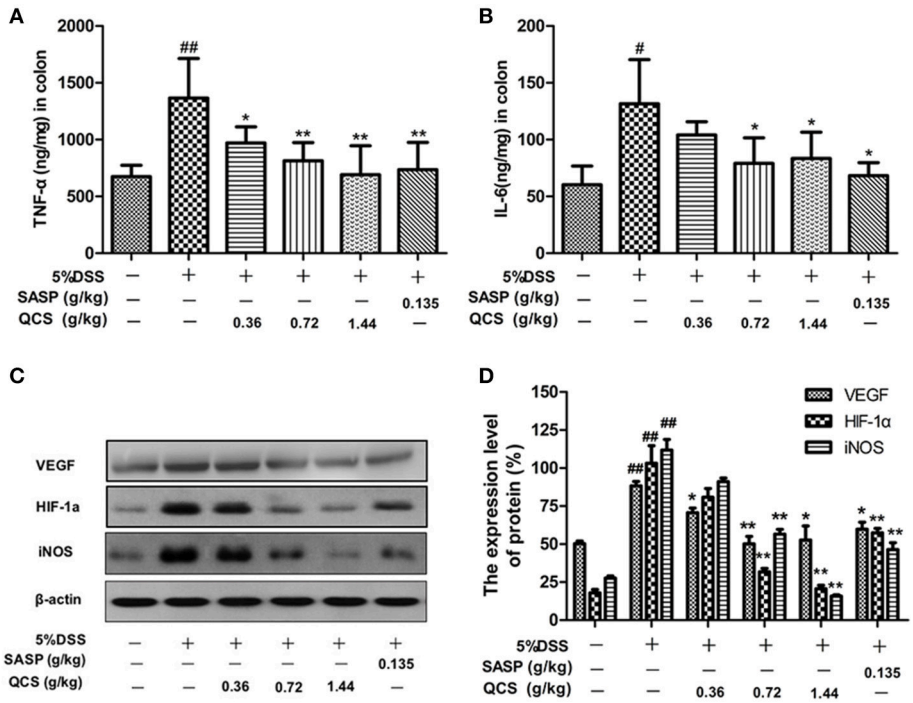

FIGURE 8 | Effects of QCS on DSS-induced production of TNF-α and IL-6 and expression of VEGF, HIF-1α and iNOS in colonic tissue. **(A)** Concentration of TNF-α in colonic tissue; **(B)** concentration of IL-6 in colonic tissue. **(C,D)** Protein expression of VEGF, HIF-1α, and iNOS. β-Actin levels were used as loading controls. Results are expressed as mean ± *SD*; $n = 5$, $^{*}P < 0.05$; $^{**}P < 0.01$, vs. model group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, vs. control group.

is associated with IBD activity (Beck et al., 2004). With the aggravation of inflammation, the expression of iNOS increases rapidly, which activates production of large amounts of NO and oxyradicals, causing tissue and cell damage. Beyond that, iNOS is the primary cause of increased synthesis of prostaglandins (PGs; mostly PGE2) when inflammation occurs. Excessive PGE2 and NO result in increased vasodilation and VP, thus leading to mucosal congestion and edema (Blouin et al., 2004), which contributes to the initiation and development of inflammation.

Most studies have shown that intestinal epithelial oxygen supply decreases in patients with IBD (Hatoum et al., 2005; Taylor and Colgan, 2007). Mice with mild colitis (early stage) are complicated with hypoxia and decreased hematocrit and vascular density. On the contrary, mice with severe colitis (late stage) have reduced hypoxia and increased hematocrit and vascular density (Harris et al., 2011). Hypoxia not only maintains or aggravates inflammation via stabilization of HIF-1α (Palazon et al., 2014), but also influences the local mucosal tissue pH (Mogi et al., 2009). Subsequently, an acidic environment is not only the result of inflammation, but also affects the degree and outcome of inflammation. In this study, severe hypoxia was observed in the early stage of DSS-induced colitis, which even extended to the lamina propria and submucosa. This is consistent with previous studies (Saijo et al., 2015). The pathological and microstructural observation of colon tissues revealed that submucosal vascular congestion, endothelial damaged, and platelet aggregation occurred before colonic epithelial destruction in the early stage of UC, which further demonstrated that colon tissue microcirculation disturbance co-existed with hypoxia and also occurred before damage to intestinal epithelial barrier function.

Expression of TNF-α and IL-6 in colonic tissue was significantly increased. This indicated the presence of hypoxia and cytokine imbalance in the early stage of UC, which can be explained as follows. (1) Vascular and lymphocyte hyperplasia and cell metabolic activities increase in the early stage of IBD (Neurath et al., 2001). (2) During the occurrence and development of IBD, many platelets are activated (Suzuki et al., 2001), and the types, number and bioactivities of glycoproteins on the platelet membranes change, which causes platelet adhesion and aggregation, thus increasing blood viscosity, vasoconstriction, and formation of micro-thrombi that may adversely influence intestinal mucosal microcirculation and aggravate intestinal mucosal hypoxia damage. (3) Obvious anoxia is observed in colonic mucosal tissues, which, with increased expression of HIF-1α and oxidative stress, could increase HIF-1α expression directly or indirectly by regulating matrix connective tissue cells releasing cytokines (Giatromanolaki et al., 2003). (4) Inflammatory factors like TNF-α and IL-6 increase significantly with enhanced activity in the early stage of UC (Fiocchi, 2004), which induces activation of HIF-1α (Albina et al., 2001). Tissue hypoxia induced by microcirculatory disturbance affects the occurrence, development or even prognosis of UC.

According to the results of LC-ESI-MS/MS, QCS contained multiple bioactive compounds, in which indirubin has significant anti-inflammatory activity (Gao et al., 2016). Notoginsenoside R1 and ginsenosides Rb1 and Rg1 are the primary active ingredients of Radix notoginseng. Notoginsenoside R1 down-regulates the increased expression of VEGF and matrix metalloproteinase-2, promotes regeneration of endothelial cells, and reduces the thickened extracellular matrix (Chen et al., 2004). Ginsenosides Rb1 and Rg1 activate and produce NO by regulating the PI3K/Akt/eNOS signal pathways and arginine transformation on endothelial cells, so as to increase the endothelium-dependent hemangiectasis in rats (Pan et al., 2012). Our recent study found that Panax notoginseng could promote repair of injuries of colonic mucosa and microvessels via downregulating VEGFA isoforms and inhibiting Rap1GAP/TSP1 signaling pathway (Wang et al., 2018). Gallic acid (Chang et al., 2015), a primary active ingredient of Galla chinensis, has multiple bioactivities like anti-inflammation, anti-mutation, anti-oxidation, and anti-free radicals. Portulaca oleracea (Zhou et al., 2015) possesses a wide spectrum of pharmacological properties such as neuroprotective, antimicrobial, antidiabetic, antioxidant, anti-inflammatory, antiulcerogenic, and anticancer activities. These may be why QCS has multi-pathway and multi-target therapeutic efficacy in the treatment of DSS-induced colitis.

Although this study proved that colonic VP increased earlier than EP in the early stage of colitis induced by DSS, damage of the vascular endothelial barrier occurred earlier than that of intestinal mucosa. The mechanisms of VP increase leading to EP increase in the early stage of colitis are still unclear and need to be investigated further. Meanwhile, a reasonable orthogonal design study on QCS ingredients is required to verify whether the clinical efficacy can be maximized by optimizing compatibility of prescription and regulating the dosage of a single drug.

In summary, QCS treatment could alleviate colonic tissue inflammatory, microvascular structural damage and local tissue hypoxia in DSS-induced colitis. All of these may be attributed to the improving effect of QCS on vascular endothelial barrier function by regulating the VEGF/HIF-1α signaling pathway, and therefore QCS could serve as alternative medicine for patients suffering from colitis.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

BYS and JY contributed equally to this work. HH designed the research and wrote the paper; BYS and JY performed the rat experiments; SW, WZ, RW, and BS performed biochemical analysis; JL and JS analyzed the data.

FUNDING

This work was supported by the National Natural Science Foundation of China [No. 81373616].

ACKNOWLEDGMENTS

We thank Dr. Yan-Hong Shi for technical support in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01235/full#supplementary-material

REFERENCES

Albina, J. E., Mastrofrancesco, B., Vessella, J. A., Louis, C. A., Henry, W. L., and Reichner, J. S. (2001). HIF-1 expression in healing wounds: HIF-1α induction in primary inflammatory cells by TNF-α. *Am. J. Physiol. Cell Physiol.* 281:C1971. doi: 10.1152/ajpcell.2001.281.6.C1971

Alsadi, R., Boivin, M., and Ma, T. (2009). Mechanism of cytokine modulation of epithelial tight junction barrier. *Front. Biosci.* 14, 2765–2778. doi: 10.2741/3413

Beck, P. L., Xavier, R., Wong, J., Ezedi, I., Mashimo, H., Mizoguchi, A., et al. (2004). Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *Am. J. Physiol. Gastrointest. Liver Physiol.* 286, 137–147. doi: 10.1152/ajpgi.00309.2003

Blouin, C. C., Soucy, G. M., and Richard, D. E. (2004). Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. *Blood* 103:1124–1187. doi: 10.1182/blood-2003-07-2427

Chang, Y. J., Hsu, S. L., Liu, Y. T., Lin, Y. H., Lin, M. H., Huang, S. J., et al. (2015). Gallic acid induces necroptosis via TNF-α signaling pathway in activated hepatic stellate cells. *PLoS ONE* 10:e0120713. doi: 10.1371/journal.pone.0120713

Chen, S. W., Li, X. H., Ye, K. H., Jiang, Z. F., and Ren, X. D. (2004). Total saponins of Panax notoginseng protected rabbit iliac artery against balloon endothelial denudation injury. *Acta Pharmacol. Sin.* 25, 1151–1156.

Chidlow, J. H., Shukla, D., Grisham, M. B., and Kevil, C. G. (2007). Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293:G5. doi: 10.1152/ajpgi.00107.2007

Dai, Y. C., Tang, Z. P., Ma, G. T., Gong, Y. P., Liu, W., and Zhang, Y. L. (2010). A review of Qingchang Shuan for treatment of ulcerative colitis. *J. Tradition. Chin. Med.* 30, 237–240. doi: 10.1016/S0254-6272(10)60049-0

D'Alessio, S., Correale, C., Tacconi, C., Gandelli, A., Pietrogrande, G., Vetrano, S., et al. (2014). VEGF-C–dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. *J. Clin. Invest.* 124, 3863–3878. doi: 10.1172/JCI72189

Fiocchi, C. (2004). Inflammatory bowel disease: autoimmune or immune-mediated pathogenesis? *Clin. Dev. Immunol.* 11:195. doi: 10.1080/17402520400004201

Floch, M. H. (2011). Inflammatory bowel disease. *J. Clin. Gastroenterol.* 45, 141–170. doi: 10.1097/MCG.0b013e31822be119

Gao, W., Guo, Y., Wang, C., Lin, Y., Li, Y., Sheng, T., et al. (2016). Indirubin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice through the inhibition of inflammation and the induction of Foxp3-expressing regulatory T cells. *Acta Histochem.* 118, 606–614. doi: 10.1016/j.acthis.2016.06.004

Giatromanolaki, A., Sivridis, E., Maltezos, E., Papazoglou, D., Simopoulos, C., Gatter, K. C., et al. (2003). Hypoxia inducible factor 1α and 2α overexpression in inflammatory bowel disease. *J. Clin. Pathol.* 56:209. doi: 10.1136/jcp.56.3.209

Hao, Y., Nagase, K., Hori, K., Wang, S., Kogure, Y., Fukunaga, K., et al. (2014). Xilei san ameliorates experimental colitis in rats by selectively degrading proinflammatory mediators and promoting mucosal repair. *Evid. Based Compl. Alternat. Med.* 2014:10. doi: 10.1155/2014/569587

Harris, N. R., Carter, P. R., Yadav, A. S., Watts, M. N., Zhang, S., Kosloskidavidson, M., et al. (2011). Relationship between inflammation and tissue hypoxia in a mouse model of chronic colitis. *Inflamm. Bowel Dis.* 17:742. doi: 10.1002/ibd.21423

Hatoum, O. A., Binion, D. G., and Gutterman, D. D. (2005). Paradox of simultaneous intestinal ischaemia and hyperaemia in inflammatory bowel disease. *Eur. J. Clin. Invest.* 35, 599–609. doi: 10.1111/j.1365-2362.2005.01567.x

Hatoum, O. A., Miura, H., and Binion, D. G. (2003). The vascular contribution in the pathogenesis of inflammatory bowel disease. *Am. J. Physiol. Heart Circ. Physiol.* 285:H1791. doi: 10.1152/ajpheart.00552.2003

Irkorucu, O., Tascilar, O., Karakaya, K., Emre, A., Ucan, B., Bahadir, B., et al. (2008). The effect of sildenafil on an animal model for ischemic colitis. *Dig. Dis. Sci.* 53, 1618–1623. doi: 10.1007/s10620-007-0033-9

Johansson, M. E., Gustafsson, J. K., Sjöberg, K. E., Petersson, J., Holm, L., Sjövall, H., et al. (2010). Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. *PLoS ONE* 5:e12238. doi: 10.1371/journal.pone.0012238

Jump, R. L., and Levine, A. D. (2010). Mechanisms of natural tolerance in the intestine: implications for inflammatory bowel disease. *Inflamm. Bowel Dis.* 10, 462–478. doi: 10.1097/00054725-200407000-00023

Koutroubakis, I. E., Xidakis, C., Karmiris, K., Sfiridaki, A., Kandidaki, E., and Kouroumalis, E. A. (2004). Serum angiogenin in inflammatory bowel disease. *Dig. Dis. Sci.* 49, 1758–1762. doi: 10.1007/s10620-004-9565-4

Kurashima, Y., Amiya, T., Nochi, T., Fujisawa, K., Haraguchi, T., Iba, H., et al. (2012). Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat. Commun.* 3:1034. doi: 10.1038/ncomms,2023

Mankertz, J., and Schulzke, J. (2007). Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications. *Curr. Opin. Gastroenterol.* 23:379. doi: 10.1097/MOG.0b013e32816aa392

Masakazu, Y. (2014). Ulcerative colitis-associated colorectal cancer. *World J. Gastroenterol.* 20:16389. doi: 10.3748/wjg.v20.i44.16389

Mogi, C., Tobo, M., Tomura, H., Murata, N., He, X. D., Sato, K., et al. (2009). Involvement of proton-sensing TDAG8 in extracellular acidification-induced inhibition of proinflammatory cytokine production in peritoneal macrophages. *J. Immunol.* 182:3243. doi: 10.4049/jimmunol.0803466

Murthy, S. N., Cooper, H. S., Shim, H., Shah, R. S., Ibrahim, S. A., and Sedergran, D. J. (1993). Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. *Dig. Dis. Sci.* 38, 1722–1734. doi: 10.1007/BF01303184

Neurath, M. F., Finotto, S., Fuss, I., Boirivant, M., Galle, P. R., and Strober, W. (2001). Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question. *Trends Immunol.* 22:21. doi: 10.1016/S1471-4906(00)01798-1

Palazon, A., Goldrath, A., Nizet, V., Johnson, R. S., et al. (2014). HIF transcription factors, inflammation, and immunity. *Immunity* 41, 518–528. doi: 10.1016/j.immuni.2014.09.008

Pan, C., Huo, Y., An, X., Singh, G., Chen, M., Yang, Z., et al. (2012). Panax notoginseng and its components decreased hypertension via stimulation of endothelial-dependent vessel dilatation. *Vascul. Pharmacol.* 56:150. doi: 10.1016/j.vph.2011.12.006

Rezaie, A., Parker, R. D., and Abdollahi, M. (2007). Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig. Dis. Sci.* 52, 2015–2021. doi: 10.1007/s10620-006-9622-2

Saijo, H., Tatsumi, N., Arihiro, S., Kato, T., Okabe, M., Tajiri, H., et al. (2015). Microangiopathy triggers, and inducible nitric oxide synthase exacerbates dextran sulfate sodium-induced colitis. *Lab. Invest.* 95, 728–748. doi: 10.1038/labinvest.2015.60

Scaldaferri, F., Vetrano, S., Sans, M., Arena, V., Straface, G., Stigliano, E., et al. (2009). VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology* 136, 585–595. doi: 10.1053/j.gastro.2008.09.064

Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum, S. Y., Park, K. K., et al. (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mut Res.* 480–481, 243–268. doi: 10.1016/S0027-5107(01)00183-X

Suzuki, K., Sugimura, K., Hasegawa, K., Yoshida, K., Suzuki, A., Ishizuka, K., et al. (2001). Activated platelets in ulcerative colitis enhance the production of reactive oxygen species by polymorphonuclear leukocytes. *Scand. J. Gastroenterol.* 36, 1301–1306. doi: 10.1080/003655201317097164

Tajdini, M., Mirbagheri, S. A., Nikooie, R., Ostovaneh, M. R., Ghoreyshi Hefzabad, S. M., Garg, S. K., et al. (2013). Tissue hypoxia in pathogenesis of ulcerative colitis: should we change all our beliefs? *Gastroenterology* 144:1487. doi: 10.3109/00365521.2013.845798

Tarnawski, A. S., Coron, E., Mosnier, J. F., Ahluwalia, A., Rhun, M. L., Galmiche, J. P., et al. (2009). *In vivo* detection by confocal endomicroscopy of two distinct structural abnormalities in angioarchitecture and increased vascular permeability in colonic mucosa of patients with IBD in remission: mechanistic implications. *Gastroenterology* 136, A-112. doi: 10.1016/S0016-5085(09)60503-5

Taylor, C. T., and Colgan, S. P. (2007). Hypoxia and gastrointestinal disease. *J. Mol. Med.* 85, 1295–1300. doi: 10.1007/s00109-007-0277-z

Thornton, M., and Solomon, M. J. (2002). Crohn's disease: in defense of a microvascular aetiology. *Int. J. Colorectal Dis.* 17, 287–297. doi: 10.1007/s00384-002-0408-5

Tolstanova, G., Deng, X., French, S. W., Lungo, W., Paunovic, B., Khomenko, T., et al. (2012). Early endothelial damage and increased colonic vascular permeability in the development of experimental ulcerative colitis in rats and mice. *Lab. Invest.* 92:9. doi: 10.1038/labinvest.2011.122

Tolstanova, G., Khomenko, T., Deng, X., Chen, L., Tarnawski, A., Ahluwalia, A., et al. (2009). Neutralizing anti-vascular endothelial growth factor (VEGF) antibody reduces severity of experimental ulcerative colitis in rats: direct evidence for the pathogenic role of VEGF. *J. Pharmacol. Exp. Ther.* 328:749. doi: 10.1124/jpet.108.145128

Trojanowska, M. (2010). Cellular and molecular aspects of vascular dysfunction in systemic sclerosis. *Nat. Rev. Rheumatol.* 6:453. doi: 10.1038/nrrheum.2010.102

Wang, S., Tao, P., Zhao, L., Zhang, W., Hu, H., and Lin, J. (2018). Panax notoginseng promotes repair of colonic microvascular injury in sprague-dawley rats with experimental colitis. *Evid. Based Compl. Alternat. Med.* 2018, 1–8. doi: 10.1155/2018/4386571

Xiao, H. T., Lin, C. Y., Ho, D. H., Peng, J., Chen, Y., Tsang, S. W., et al. (2013). Inhibitory effect of the gallotannin corilagin on dextran sulfate sodium-induced murine ulcerative colitis. *J. Nat. Prod.* 76, 2120–2125. doi: 10.1021/np4006772

Xin, H. L., Yan-Feng, X. U., Yue, X. Q., Hou, Y. H., Min, L. I., and Ling, C. Q. (2008). [Analysis of chemical constituents in extract from Portulaca oleracea L.with GC-MS method]. *Pharm. J. Chin. Peoples Liber. Army*. 24, 133–136. (Chinese)

Xu, W., Qiu, X., Zhang, J., Zhu, D., Yang, Y., and Lu, C. (2012). [Analysis of saponins in Panax notoginseng by UPLC-LTQ-Orbitrap MS/MS]. *Yao Xue Xue Bao.* 47, 773–778. (Chinese)

Zhou, Y. X., Xin, H. L., Rahman, K., Wang, S. J., Peng, C., and Zhang, H. (2015). *Portulaca oleracea* L.: a review of phytochemistry and pharmacological effects. *Biomed Res. Int.* 2015:925631. doi: 10.1155/2015/925631

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 03 December 2018
doi: 10.3389/fphar.2018.01405

A Comparative Study on the Efficacy of NLRP3 Inflammasome Signaling Inhibitors in a Pre-clinical Model of Bowel Inflammation

Carolina Pellegrini[1†], Matteo Fornai[2†], Rocchina Colucci[3], Laura Benvenuti[2], Vanessa D'Antongiovanni[2], Gianfranco Natale[4], Federica Fulceri[4], Marta Giorgis[5], Elisabetta Marini[5], Simone Gastaldi[5], Massimo Bertinaria[5], Corrado Blandizzi[2] and Luca Antonioli[2]*

[1] Department of Pharmacy, University of Pisa, Pisa, Italy, [2] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, [3] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padua, Italy, [4] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy, [5] Department of Drug Science and Technology, University of Turin, Turin, Italy

OPEN ACCESS

Edited by:
Raffaele Capasso,
Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Hesham N. Mustafa,
King Abdulaziz University, Saudi Arabia
Lorenzo Di Cesare Mannelli,
Università degli Studi di Firenze, Italy

***Correspondence:**
Rocchina Colucci
rok.colucci@gmail.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 07 September 2018
Accepted: 15 November 2018
Published: 03 December 2018

Citation:
Pellegrini C, Fornai M, Colucci R, Benvenuti L, D'Antongiovanni V, Natale G, Fulceri F, Giorgis M, Marini E, Gastaldi S, Bertinaria M, Blandizzi C and Antonioli L (2018) A Comparative Study on the Efficacy of NLRP3 Inflammasome Signaling Inhibitors in a Pre-clinical Model of Bowel Inflammation. Front. Pharmacol. 9:1405. doi: 10.3389/fphar.2018.01405

Nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome is pivotal in maintaining intestinal homeostasis and sustaining enteric immune responses in the setting of inflammatory bowel diseases. Drugs acting as NLRP3 blockers could represent innovative strategies for treatment of bowel inflammation. This study was performed in rats with dinitrobenzenesulfonic acid (DNBS)-induced colitis, to investigate how the direct blockade of NLRP3 inflammasome with an irreversible inhibitor (INF39) compares with Ac-YVAD-cmk (YVAD, caspase-1 inhibitor) and anakinra (IL-1β receptor antagonist), acting downstream on NLRP3 signaling. Animals with DNBS-colitis received YVAD (3 mg/kg) or anakinra (100 mg/Kg) intraperitoneally, and INF39 (25 mg/kg) or dexamethasone (DEX, 1 mg/kg) orally for 6 days, starting on the same day of colitis induction. Under colitis, there was a body weight decrease, which was attenuated by YVAD, anakinra or INF39, but not DEX. All test drugs counteracted the increase in spleen weight. The colonic shortening and morphological colonic alterations associated with colitis were counteracted by INF39, anakinra and DEX, while YVAD was without effects. Tissue increments of myeloperoxidase, tumor necrosis factor and interleukin-1β were more effectively counteracted by INF39 and DEX, than YVAD and anakinra. These findings indicate that: (1) direct inhibition of NLRP3 inflammasome with INF39 is more effective than caspase-1 inhibition or IL-1β receptor blockade in reducing systemic and bowel inflammatory alterations; (2) direct NLRP3 inhibition can be a suitable strategy for treatment of bowel inflammation.

Keywords: anakinra, caspase-1, colitis, colon, NLRP3 inflammasome, interleukin-1beta, bowel inflammation

Abbreviations: DEX, dexamethasone; DMSO, dimethyl sulfoxide; DNBS, 2,4-dinitrobenzenesulfonic acid; IL-1β, interleukin-1beta; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NLRP3, nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor.

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are chronic relapsing disorders characterized by inflammation and tissue damage in the digestive tract (Neurath, 2014). Such diseases are associated with marked morbidity and have a remarkable negative impact on patients' quality of life, which highlights the need for setting up novel anti-inflammatory therapeutic strategies (Blonski et al., 2011).

Recent studies have shown that the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing protein 3 (NLRP3) inflammasome cytosolic complex, besides acting as a key player in the maintenance of intestinal homeostasis, shapes innate immune responses against commensal bacteria. Indeed, NLRP3 over-activation during bowel inflammation is associated with a breakdown of enteric immune balance, suggesting an involvement of NLRP3 in the pathogenesis of bowel inflammation (Bauer et al., 2010; Kanneganti, 2017; Pellegrini et al., 2017b).

The activation of NLRP3 inflammasome requires two parallel and independent steps: transcription and oligomerization (Martín-Sanchez et al., 2016; Gaidt and Hornung, 2018; Mangan et al., 2018). The first step is regulated by innate immune signaling, mediated primarily by toll-like receptors (TLRs), myeloid differentiation primary response 88 (MyD88) and/or cytokine receptors, such as TNF receptor, which, in turn, activate pro-IL-1β and NLRP3 transcription *via* NF-κB activation. The second step results in NLRP3 inflammasome oligomerization, leading to caspase-1 activation and, in turn, IL-1β and IL-18 processing and release (Sutterwala et al., 2014).

Of note, clinical evidence has documented an increase in IL-1β release from colonic tissues and macrophages of IBD patients, these patterns being correlated with disease severity, thus suggesting IL-1β as a relevant pro-inflammatory cytokine involved in the pathophysiology of IBDs (Coccia et al., 2012). Given the involvement of the inflammasome pathway in the pathophysiology of intestinal inflammation, current research efforts are being focused on the potential therapeutic benefits resulting from the pharmacological modulation of NLRP3 inflammasome. In this respect, previous studies investigated the role of NLRP3 inflammasome in several experimental models of colitis, highlighting remarkable beneficial effects as a results of its pharmacological modulation (Pellegrini et al., 2017b). For instance, two recent studies showed that both the *in vivo* caspase-1 inhibition and the selective blockade of NLRP3 inflammasome complex significantly attenuated colonic inflammation in spontaneous colitis mice (Zhang et al., 2014; Perera et al., 2018). In addition, Stoffels et al. (2014) reported that anakinra reduced post-operative inflammation and ameliorated post-operative ileus in mice, thus suggesting that IL-1β receptor blockade exerts beneficial effects during intestinal inflammation (Stoffels et al., 2014). Moreover, in a recent paper, we observed that the *in vivo* direct irreversible inhibition of NLRP3 with INF39, a novel acrylate compound able to inhibit NLRP3 ATPase activity, exerts beneficial effects on bowel inflammation (Cocco et al., 2017).

These findings suggest that both upstream and downstream inhibition of NLRP3 inflammasome could represent suitable pharmacological approaches to the treatment of bowel inflammation. However, a direct comparative study on the efficacy of NLRP3 inflammasome signaling inhibitors in a pre-clinical model of bowel inflammation is lacking.

Based on this background, the present study was designed to investigate how the direct blockade of NLRP3 with INF39 in experimental colitis compares, in terms of efficacy, with other drugs acting downstream (caspase-1 inhibition or IL-1β receptor blockade) on NLRP3 signaling.

MATERIALS AND METHODS

Animals

Experimental procedures were performed on male Sprague-Dawley rats, 200–250 g body weight. The animals received standard laboratory chow and tap water without any restriction and were housed, three in a cage, in temperature- controlled rooms on a 12-h light cycle at 22–24°C and 50–60% humidity. Animal care and handling were in accordance with the provisions of the European Community Council Directive 2010/63/UE, recognized and adopted by the Italian Government. All experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n° 674/2016-PR). In the present study, animal data have been presented according to the ARRIVE guidelines.

Induction of Colitis and Drug Treatments

Colitis was induced in accordance with the method described previously (Antonioli et al., 2010). The subsequent experimental procedures were performed 6 days after DNBS administration to allow a full development of colonic inflammation. Rats were subjected to administration of the test drugs by intragastric or intraperitoneal (i.p.) route for 6 days, starting on the same day of DNBS injection. DNBS-untreated (controls) and DNBS-treated animals were treated as follows: INF39 (25 mg/kg/day, oral), Ac-YVAD-cmk (YVAD, caspase-1 inhibitor, 3 mg/Kg/day, i.p.), anakinra (IL-1β receptor antagonist, 100 mg/Kg/day i.p.) or DEX (active comparator, 1 mg/kg/day, oral). INF39 and DEX were suspended in olive oil and 1% methylcellulose, respectively. YVAD was dissolved in sterile DMSO, and further dilutions were made with sterile saline.

Subgroups of DNBS-untreated and DNBS-treated rats received drug vehicles to serve as controls. Body weight was assessed daily, starting from the onset of drug administrations. The doses of INF39 and DEX were selected on the basis of our previous study performed on the same rat model of colitis (Cocco et al., 2017).

The doses of YVAD and anakinra were selected on the basis of previous studies performed in rat models of diabetes induced by stress or in streptozotocin, respectively (Maslanik et al., 2013; Vallejo et al., 2014), and by preliminary experiments designed to test increasing doses of both compounds (YVAD, 0.75, 1.5, 3, and 6 mg/kg; anakinra, 25, 50, 100, and 200 mg/Kg) on colonic MPO levels in the model

of DNBS-induced colitis. Macroscopic and histological scores were assessed on the whole colon, while biochemical assays were performed on colonic segments collected from an inflamed region adjacent and distal to the gross necrotic damage.

Assessment of Colitis

Colonic tissues were scored for macroscopic and histological damage, as reported previously (Antonioli et al., 2010; Cocco et al., 2017). The criteria for macroscopic scoring of colonic damage were as follows: (1) presence of adhesions between colonic tissue and other organs (0, none; 1, minor; 2, major adhesions); (2) consistency of colonic fecal material (0, formed; 1, loose; 2, liquid stools); and (3) presence of ulceration (0, none; 1, hyperemia; 2, ulceration without hyperemia; 3, ulceration with inflammation at one side; 4, $\geq$ 2 sites of ulceration and inflammation; 5, major sites of damage; and 6, major sites of damage extending $>$ 2 cm). The score was then increased by one unit for each millimeter of colonic wall thickness. Microscopic damage and inflammation were assessed by light microscopy on hematoxylin/eosin-stained histological sections obtained from whole gut specimens. The histological criteria included mucosal architecture loss (0 –3), cellular infiltrate (0 –3), muscle thickening (0 –3), crypt abscess (0, absent; 1, present), and goblet cell depletion (0, absent; 1, present). All parameters of macroscopic and histological damages were recorded and scored for each rat by two observers blinded to the treatment. At the time of experiment, the weight of spleen and the colonic length were also measured.

Evaluation of Tissue Myeloperoxidase Levels

The evaluation of MPO levels, regarded as quantitative index for quantification of the degree of intestinal tissue infiltration by polymorphonuclear cells, was performed as described previously (Fornai et al., 2016). Colonic samples (30 mg) were put in 0.6 ml of ice-cold lysis buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 g/ml leupeptin and 28 g/ml aprotinin (pH 7.4) and homogenized on ice with a polytron homogenizer (QIAGEN, Milan, Italy). After centrifugation (2 times at 4°C for 15 min at 1,500*g*), the supernatant was diluted 1:5 and used for MPO concentration assessment by an enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, Netherlands). Data were expressed as nanograms of MPO per milligram of colonic tissue.

Evaluation of Tissue TNF and IL-1β Levels

The evaluation of TNF and IL-1β levels in colonic tissues was performed by an ELISA kit (Abcam), as described previously by Pellegrini et al. (2017a). Briefly, colonic samples, were weighed and homogenized in 0.4 ml of PBS, pH 7.2/20 mg of tissue at 4°C, and centrifuged at 10,000*g* for 5 min. Supernatants were employed for the assay. The concentrations of TNF and IL-1β were expressed as picograms and nanograms per milligram of tissue, respectively.

Drugs and Reagents

Dimethyl sulfoxide, DNBS, DEX, Ac-YVAD-cmk (caspase-1 inhibitor) and methylcellulose were purchased from Sigma-Aldrich (St. Louis, MO). The synthesis of INF39 was performed as previously reported (Cocco et al., 2014, 2016, 2017). Anakinra was purchased from Sobi, Swedish Orphan Biovitrum s.r.l. (Parma, Italy).

STATISTICAL ANALYSIS

The results are presented as mean ± S.E.M. unless otherwise stated. Data have been reported also synoptically in **Table 1** as percent changes against the values estimated in animals with colitis for the purpose of support to the discussion. The significance of differences was evaluated on raw data by one-way analysis of variance followed by *post hoc* analysis with Student-Newman-Keuls test. *P*-values $<$ 0.05 were considered significantly different. All statistical procedures were performed by a commercial software (GraphPad Prism, version 7.0 from GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Body Weight, Spleen Weight and Colonic Length

The administration of INF39, YVAD, anakinra and DEX to animals treated with DNBS vehicle did not elicit any significant change in both systemic and tissue parameters (data not shown). On this basis, we adopted the animals treated with DNBS vehicle rats as control group for all the evaluations on the drugs under investigation, collectively designed as test drugs.

Six days after administration of the DNBS vehicle, control rats showed a weight gain of 19.6 ± 0.9 g, while rats treated

TABLE 1 | Synoptic presentation of percent changes of tissue-related inflammatory parameters in DNBS-rats treated with YVAD, anakinra, INF39 or DEX against values estimated in animals with colitis.

	Colon length	Macroscopic damage score	Microscopic damage score	MPO	TNF	IL-1β
YVAD	+ 7.6	−38.1	−37.7	−61.5	+18.5	−32.4
Anakinra	+ 20.1	−55.1	−50.9	−66.7	+ 4.5	−12.6
INF39	+ 22.1	−62.8	−67.8	−83.4	−50.5	−65.5
DEX	+ 18.4	−71.8	−73.5	−90.1	−57.4	−76.3

DEX, Dexamethasone; DNBS, 2,4-dinitrobenzenesulfonic acid; TNF¸ tumor necrosis factor; IL-ß, interleukin-l beta; MPO:myeloperoxidase.

with DNBS displayed a decrease of 36.6 ± 4.4 g in their body weight. In rats with colitis, treatment with YVAD, anakinra and INF39 significantly attenuated the body weight loss, while DEX promoted a further, although not significant, decrease in body weight. In this respect, it is widely recognized that steroid therapy is associated with several systemic adverse effects, including muscular atrophy, with consequent loss of body weight (De Cassan et al., 2012).

Of note, INF39 was significantly more effective in blunting body weight loss, as compared with anakinra (**Figure 1**), suggesting that the upstream inhibition of NLRP3 activation is more effective than the downstream IL-1β receptor blockade.

Spleen weight was taken as an index of systemic inflammation (Chassaing et al., 2014) (Siegmund et al., 2001). Treatment with DNBS resulted in a significant increment of spleen weight (+44.3%), as compared with control animals. Such an increase was significantly counteracted to a similar extent by YVAD, anakinra and INF39 (**Figure 1B**).

Six days after DNBS administration, inflamed rats were characterized by a shortening of colonic length (−37.2%), as compared with control animals. Treatment of inflamed rats with anakinra, DEX or INF39 attenuated significantly the decrease in colonic length, while YVAD did not exert significant effects; (**Figure 2A** and **Table 1**), suggesting that both the upstream and downstream inhibition of NLRP3 signaling with INF39 and anakinra, respectively, is more effective than caspase-1 inhibition in counteracting the decrease in colonic length.

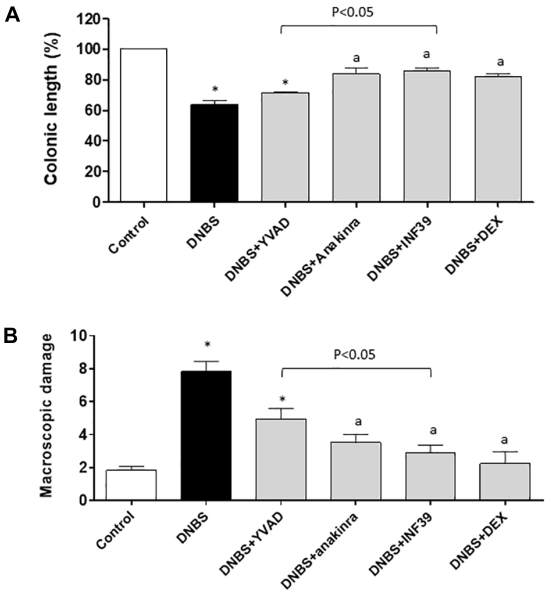

FIGURE 2 | Colonic length changes **(A)** and macroscopic damage score **(B)** in rats under normal conditions or with DNBS-induced colitis, either alone or after treatment with Ac-YVAD-cmk (YVAD, 3 mg/Kg), anakinra (100 mg/Kg), INF39 (25 mg/kg) or DEX (1 mg/kg). Each column represents the mean ± S.E.M. (n = 10). *P < 0.05, significant difference vs. control group; [a]P < 0.05, significant difference vs. DNBS group.

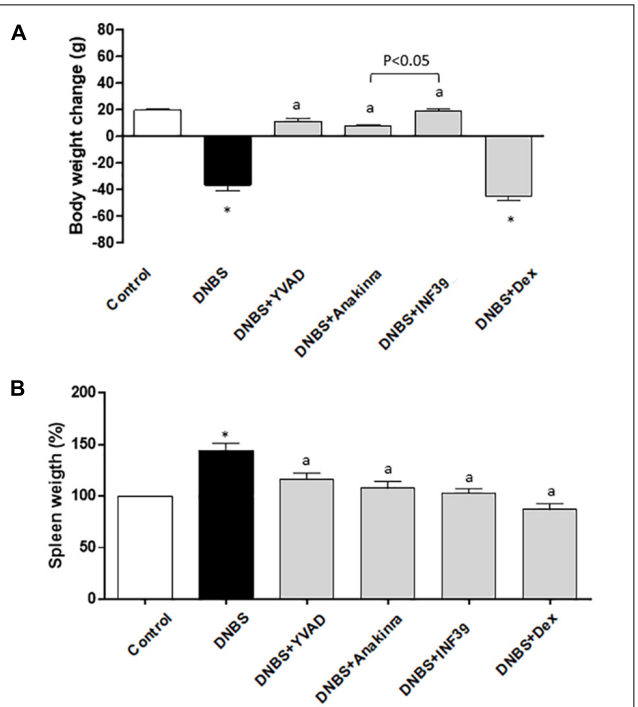

FIGURE 1 | Effects of Ac-YVAD-cmk (YVAD, 3 mg/Kg), anakinra (100 mg/Kg), INF39 (25 mg/kg) or DEX (1 mg/kg) on body weight gain/loss **(A)** and spleen weight changes **(B)**, at day 6 after induction of colitis with DNBS. Each column represents the mean ± S.E.M. (n = 10). *P < 0.05, significant difference vs. control group; [a]P < 0.05, significant difference vs. DNBS group.

Macroscopic Damage

The administration of DNBS was associated with colonic thickening and ulcerations, with marked areas of transmural inflammation. In addition, adhesions and bowel dilations were detected, with a macroscopic damage accounting for 7.8 ± 0.6. In this setting, the macroscopic damage was reduced to a similar extent and significantly by anakinra, INF39 and DEX, while the effect of YVAD did not achieve statistical significance; anakinra and INF39 acted with similar efficacy (**Figure 2B** and **Table 1**).

Microscopic Damage

Microscopic evaluation of colonic tissues revealed the presence of large areas of mucosal necrosis in animals treated with DNBS, as well as the destruction of glandular architecture.

The submucosa appeared thickened due to the presence of edema and inflammatory cell infiltration. The underlying muscular layer appeared also thickened and infiltrated with inflammatory cells following DNBS administration. The mucosa and submucosa surrounding the necrotic area displayed inflammation associated with marked cellular infiltration, as compared with tissues from control animals (**Figure 3A**).

In colonic tissues from inflamed rats, the microscopic score was significantly increased in comparison with control animals (5.3 ± 0.4 vs. 1.4 ± 0.1, respectively). When treated with anakinra, INF39 and DEX, animals with colitis displayed similar

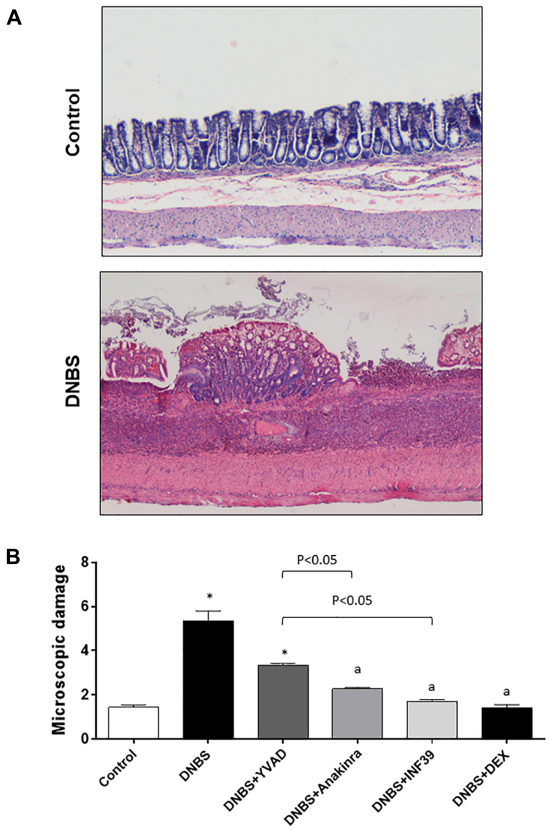

FIGURE 3 | (A) Hematoxylin and eosin-stained sections of rat colon. Representative microscopic images refer to control rats or animals with colitis induced by DNBS. **(B)** Microscopic damage score estimated for colon in rats under normal conditions or with DNBS-induced colitis, either alone or after treatment with Ac-YVAD-cmk (YVAD, 3 mg/Kg), anakinra (100 mg/Kg), INF39 (25 mg/kg) or DEX (1 mg/kg). Each column represents the mean ± S.E.M. (n = 10). *P < 0.05, significant difference vs. control group; [a]P < 0.05, significant difference vs. DNBS group.

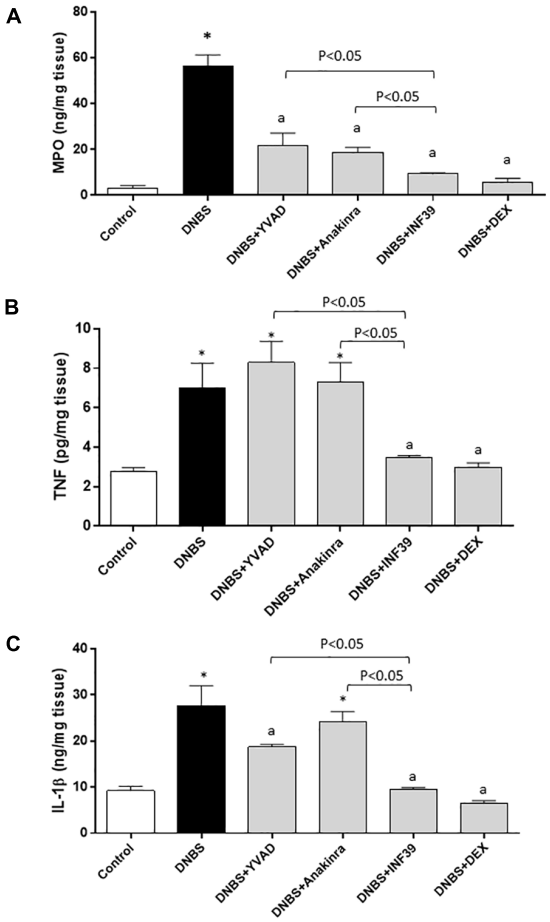

FIGURE 4 | Myeloperoxidase (MPO) **(A)**, tumor necrosis factor (TNF) **(B)** and interleukin-1β (IL-1β) **(C)** levels in colonic tissues from control rats or animals with DNBS-induced colitis, either alone or after treatment with Ac-YVAD-cmk (YVAD, 3 mg/Kg), anakinra (100 mg/Kg), INF39 (25 mg/kg) or DEX (1 mg/kg). Each column represents the mean ± S.E.M. (n = 10). *P < 0.05, significant difference vs. control group; [a]P < 0.05, significant difference vs. DNBS group.

and significant reductions of the microscopic damage, while the effect of YVAD did not achieve the level of statistical significance (**Figure 3B** and **Table 1**).

MPO Levels in Colonic Tissues

Rats with DNBS-induced colitis showed a marked increase in colonic MPO levels (56.3 ± 4.9 ng/mg tissue), as compared with control animals (3.2 ± 1.1 ng/mg tissue). Treatment with all test drugs prevented significantly the increments of colonic MPO levels associated with DNBS administration, with INF39 being more effective than YVAD and anakinra (**Figure 4A** and **Table 1**).

TNF Levels in Colonic Tissues

Colonic inflammation induced by DNBS was associated with a significant increase in tissue TNF levels (7.1 ± 1.2 pg/mg tissue), as compared with values obtained in control animals (2.7 ± 0.2 pg/mg tissue). Treatment with INF39 or DEX decreased significantly the concentration of this cytokine in colonic tissues, while YVAD and anakinra were without effects (**Figure 4B** and **Table 1**).

IL-1β Levels in Colonic Tissues

Rats with colitis displayed a significant increase in colonic IL-1β levels (27.7 ± 4.2 ng/mg tissue), as compared with values estimated in control animals (9.2 ± 0.9 ng/mg tissue). Treatment with YVAD, INF39 and DEX was associated with a significant decrease in IL-1β levels; INF39 was more effective than YVAD. Anakinra was without effects (**Figure 4C** and **Table 1**).

DISCUSSION

The involvement of inflammasome pathways in the pathophysiology of intestinal inflammation is fostering research about the potential therapeutic benefits, in terms of anti-inflammatory activity, resulting from the pharmacological

modulation of NLRP3 inflammasome. Nowadays, the majority of available studies have investigated the role of NLRP3 in several experimental models of colitis, highlighting remarkable beneficial effects associated with the pharmacological modulation of this enzymatic complex (Pellegrini et al., 2017b). In particular, the most investigated strategies, aimed at validating and developing novel pharmacological entities targeting NLRP3 signaling, have been: (i) inhibition of the activation of the transcription factor NF-κB; (ii) protection against mitochondrial damage; (iii) activation of the Keap-1/Nrf2 antioxidant pathway; (iv) inhibition of pro-caspase-1 cleavage through undetermined interactions with NLRP3 inflammasome; and (v) blockade of IL-1β receptor (Sutterwala et al., 2014; Cocco et al., 2017; Perera et al., 2017). However, at present, there is a lack of suitable drug candidates able to directly and selectively inhibit the NLRP3 ATPase activity. This represents an intriguing issue, since the development of drugs endowed with direct inhibitory effects on NLRP3 are expected to ensure a more efficient control of several NLRP3-dependent downstream signals, pivotally involved in the regulation of immune/inflammatory processes (Pellegrini et al., 2017b).

The above background was taken as a rationale for the development of INF39, an acrylate compound, able to block selectively and irreversibly the NLRP3 ATPase activity (Cocco et al., 2017). *In vitro* studies showed that INF39 counteracted significantly NLRP3 activation through a direct and irreversible interaction with the enzyme complex (Cocco et al., 2017). The impact of a direct NLRP3 blockade on bowel inflammation was then tested in a murine model of DNBS-induced colitis (Cocco et al., 2017). In this setting, INF39 exerted significant beneficial effects, alleviating both the systemic and tissue inflammatory outcomes of colitis, and showing also a satisfactory safety profile (Cocco et al., 2017).

In the present study, our specific purpose was to compare the efficacy of INF39 with other drugs, acting downstream on the NLRP3 signaling (i.e., caspase-1 inhibition or IL-1β receptor blockade), in an experimental model of bowel inflammation. To pursue this goal, we employed rats with DNBS-induced colitis, which match closely the patterns of Crohn's disease in humans, and are characterized by body weight loss, diarrhea, ulceration and bleeding, depletion of goblet cells and formation of granulomas within the colonic wall (Goyal et al., 2014). The model of DNBS-induced colitis is a suitable tool for the investigation of the anti-inflammatory activity of novel drugs with potential therapeutic efficacy in human bowel inflammatory disorders (Esposito et al., 2010). The appropriateness of this model was previously validated by our research group. Indeed, we confirmed DEX was able to ameliorate systemic and tissue inflammatory parameters, as also previously described by Antonioli et al. (2010).

Overall, our results provide convincing evidence that the pharmacological modulation of the NLRP3 inflammasome pathway attenuates bowel inflammation in DNBS-induced colitis, and that direct NLRP3 inhibition by INF39 is more effective than caspase-1 inhibition by YVAD or IL-1β receptor blockade by anakinra in controlling several parameters associated with intestinal inflammation. When considering the systemic indexes of inflammation, treatment with INF39, YVAD and anakinra counteracted the body weight loss and the increment of spleen weight in DNBS-rats. In addition, the inhibitors of NLRP3 signaling did not exert detrimental effects on body weight, at variance with DEX, which, being a systemically acting glucocorticoid derivative, is known to be associated with a variety of adverse effects, including muscular atrophy (De Cassan et al., 2012). When analyzing colonic length and bowel morphological parameters, INF39 and anakinra counteracted significantly the colonic shortening and improved both the macroscopic and histological features of colitis, as compared with YVAD, thus suggesting that the direct inhibition of NLRP3 or IL-1β receptor blockade can exert significant beneficial effects on tissue parameters related to inflammation than caspase-1 inhibition. Of note, when considering body weight and colonic length, we observed heterogeneous effects of the drugs under investigation. In this respect, it is noteworthy that body weight and colonic length in animals with colitis, despite being widely employed, are colonic coarse parameters, which can be associated also with high variability, as compared with the assessment of colonic microscopic damage or colonic MPO, TNF and IL-1β levels (see below). This is a relevant point since, when testing the effects of drugs on coarse parameters the net effect of individual drugs could be less evident. As a consequence, this circumstance might explain why anakinra was less effective than INF39 in attenuating body weight, or why YVAD did not exert a significant effect on colonic length.

Taken together, the present findings are in line with recent studies showing that treatment with MCC950, a potent and highly specific small molecule inhibitor of NLRP3 inflammasome counteracted bowel inflammation in a spontaneous colitis murine model (Winnie mice), and, that anakinra reduced post-operative inflammation and ameliorated post-operative ileus in mice (Stoffels et al., 2014; Perera et al., 2018). In addition, two recent studies showed that a synthetic benzimidazole compound and fumigaclavine C, a fungal metabolite exerted anti-inflammatory effects in mice with colitis induced by dextran sulfate sodium (DSS), through the inhibition of caspase-1 activation (Liu et al., 2013; Guo et al., 2015). However, although both compounds were able to inhibit caspase-1 activation, they influenced also other intracellular pathways, including MAPK, STAT1 and NF-κB signaling. Therefore, their anti-inflammatory effects on colitis may not result merely from the inhibition of caspase-1.

A variety of inflammatory mediators have been shown to take a significant part in the pathogenesis of bowel inflammation (Neurath, 2014). In particular, it is recognized that TNF plays a pivotal role, both in humans and experimental colitis models in the production of chemoattractants for neutrophils and their activation. Activated neutrophils infiltrate the mucosa and submucosa, and contribute to intestinal injury through a number of mechanisms (Neurath, 2014). This body of knowledge explains why anti-cytokine therapies based on TNF-specific blocking agents represent an important cornerstone of medical therapy in both Crohn's disease and ulcerative colitis (Danese and Fiocchi, 2011; Baumgart and Sandborn, 2012). Taking this rationale into account, we examined the effects of all test drugs on the colonic

levels of TNF and MPO (a quantitative index reflecting the degree of colonic infiltration by polymorphonuclear cells) in DNBS-rats. Interestingly, our results showed that INF39, but not YVAD and anakinra, counteracted significantly the increments of TNF, and that INF39 blunted the increased MPO levels with greater efficacy than the caspase-1 inhibitor and IL-1β receptor antagonist in rats with colitis, thus corroborating the concept that the direct blockade of NLRP3 could represent a better pharmacological strategy for treatment of bowel inflammation, than caspase-1 inhibition and IL-1β receptor blockade (Pellegrini et al., 2017b).

The above results are in line with a recent study showing that treatment of animals with spontaneous chronic colitis (Winnie mice) with a specific NLRP3 inhibitor (MCC950) counteracted bowel inflammation, improving both the macroscopic and histological features of colitis and reducing significantly the release of pro-inflammatory cytokines, including TNF levels (Perera et al., 2018).

Of note, the peculiar effect of INF39 in downregulating TNF tissue levels depends very likely on its ability to modulate TNF gene expression. Indeed, as observed in our previous study, the exposure of bone marrow-derived macrophages, stimulated with lipopolysaccharide, to INF39 was followed by a significant decrease in TNF gene expression, as compared with INF39-untreated cells (Cocco et al., 2017).

Several lines of pre-clinical and clinical evidence point out a key role of IL-1β in the pathophysiology of IBDs (Neurath, 2014). Indeed, colonic tissues and *lamina propria* macrophages from IBD patients showed an increased IL-1β secretion, this activity being well correlated with disease severity (Gustot et al., 2005; Coccia et al., 2012). A significant activation of NLRP3 inflammasome, with a consequent massive release of IL-1β, has been detected previously in monocytes infiltrating the lamina propria and M1 pro-inflammatory macrophages isolated from intestinal specimens of IBD patients (Lissner et al., 2015). For this reason in the present study, sets of experiments were devoted to evaluate the efficacy of test drugs in counteracting IL-1β release. In this setting, treatment with INF39 was more effective than anakinra and YVAD in counteracting the increase in IL-1β levels in colonic tissues from DNBS-rats. Anakinra, being a receptor blocker, was not expected indeed to modify IL-1β release. Furthermore, even though anakinra is currently employed for treatment of some immune-mediated inflammatory diseases (i.e., rheumatoid arthritis, ankylosing spondylitis and gout) (Dinarello et al., 2012), scarce beneficial effects of this drug were observed in patients with IBDs, thus indicating the selective blockade of IL-1β receptor as a loosing strategy for the management of such diseases (Carter et al., 2003; Hügle et al., 2017). Indeed, although several pre-clinical studies in animal models of colitis showed that the inhibition of IL-1β decreased tissue inflammation and necrosis, others found few or no beneficial effects following IL-1β inhibition (de Mooij et al., 2017). In addition, in a previous study, Carter et al. (2003) reported that the administration of anakinra to patients with Crohn's disease was associated with a worsening of clinical conditions, including fever, increased diarrhea and abdominal pain.

When considering the caspase-1 blocker YVAD, recent studies have shown that, besides caspase-1, a non-canonical NLRP3 inflammasome activation, which depends on caspase-11, plays a significant role also both in the maintenance of intestinal immune homeostasis and in sustaining the pathophysiological events that underlie bowel inflammation (Kayagaki et al., 2011; Vigano and Mortellaro, 2013). In particular, caspase-11 overactivation during bowel inflammation contributes to the massive release of IL-1β through activation of the NLRP3-ASC-caspase-1 pathway, and the inhibition of both canonical and non-canonical caspase-11-dependent NLRP3 activation has been found to exert anti-inflammatory effects on colitis in mice (Márquez-Flores et al., 2016). Other studies on murine bone marrow-derived dendritic cells (BMDCs) have shown that, besides canonical and non-canonical caspase-11-dependent NLRP3 activation, a caspase-8-dependent NLRP3 inflammasome activation can be called into play also to promote the processing and release of IL-1β. Indeed, IL-1β processing and caspase-8 activation were not evident in *Nlrp3*$^{-/-}$or *Asc*$^{-/-}$ BMDCs, thus indicating that caspase-8 can act as a direct IL-1β-converting enzyme (Antonopoulos et al., 2015;Chung et al., 2016). Moreover, Gringhuis et al. (2012) showed that the release of IL-1β from dendritic cells, stimulated with a fungal infection, occurred independently of caspase-1, but required an association of the inflammasome protein ASC with caspase-8. Based on these observations, it is conceivable that, in our experiments, INF39, through the specific inhibition of NLRP3 ATPase activity, ensured a blockade of both canonical and non-canonical caspase-8- and caspase-11-dependent NLRP3 activation, thus leading to a more effective blockade of IL-1β release, as compared with YVAD. Of interest, our results are consistent with the findings of a recent study, showing that the direct and selective blockade of inflammasome with MCC950, the

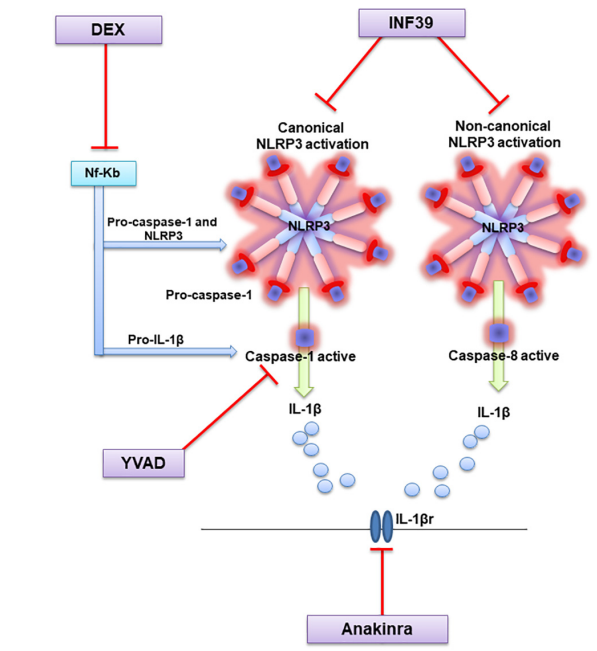

FIGURE 5 | Diagram showing the molecular mechanisms through which test drugs may inhibit NLRP3 signaling and counteract intestinal inflammation.

most specific and well characterized NLRP3 inhibitor available so far, attenuated colonic inflammation in mice with spontaneous colitis, likely through the inhibition of both canonical and non-canonical NLRP3 activation (Perera et al., 2018). However, the possible role and respective significance of non-canonical caspase-8- and caspase-11-dependent NLRP3 activation in the pathophysiology of bowel inflammation requires confirmation by means of specific experimental approaches. In addition, further studies are needed to evaluate the effects of *in vivo* selective blockade of non-canonical caspase-8- and caspase-11-dependent NLRP3 activation in animal models of colitis.

Of note, given the pivotal role of NLRP3 in regulating the integrity of intestinal homeostasis, the possibility that its pharmacological blockade during bowel inflammation could interfere with healing processes should be taken into account. In this respect, several lines of evidence have shown that, in the early acute phases of inflammation, NLRP3 inflammasome activation contributes to tissue repair and maintenance of epithelial barrier integrity, while in the later chronic phase of colitis, the overactivation of NLRP3 promotes the differentiation of T cells into effector Th1 and Th17 phenotypes, which contribute to sustain the inflammatory response. In line with this concept, the pharmacological inhibition of NLRP3 inflammasome signaling has been shown to exert beneficial effects in several experimental models of colitis (Pellegrini et al., 2017b). However, the possibility that long-term treatments with NLRP3 inhibitors could interfere with mucosal healing cannot be ruled out and deserves further investigations.

CONCLUSION

In conclusion, our results expand current knowledge on the beneficial effects arising from the pharmacological modulation of NLRP3 inflammasome in experimental colitis (Pellegrini et al., 2017b), suggesting that the direct and irreversible inhibition of NLRP3 inflammasome complex could represent a more viable approach to the medical management of bowel inflammation than IL-1β receptor blockade or caspase-1 inhibition (see **Figure 5**). In keeping with this perspective, INF39 might represent a lead compound for the identification of novel NLRP3 inhibitors, characterized by high degrees of efficacy and concomitant favorable safety profiles. Therefore, the present observations might pave the way to the design and clinical development of novel NLRP3 selective inhibitors for the therapeutic management of patients with IBDs.

AUTHOR CONTRIBUTIONS

CP, LA, MB, CB, and MF participated in research design. MF, LA, RC, CP, VD'A, LB, and FF conducted the experiments. MB, GN, FF, SG, EM, and MG contributed new reagents or analytic tools. LA, CP, and RC performed the data analysis. CP, MF, RC, LA, and CB wrote or contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

We are grateful to Dr. Gloria Lopez-Castejon from the Manchester Collaborative Center of Inflammation Research, Division of Infection, Immunity and Respiratory Medicine, School of Biology, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, United Kingdom for the research support.

REFERENCES

Antonioli, L., Fornai, M., Colucci, R., Awwad, O., Ghisu, N., Tuccori, M., et al. (2010). The blockade of adenosine deaminase ameliorates chronic experimental colitis through the recruitment of adenosine A_2A and A_3 receptors. *J. Pharmacol. Exp. Ther.* 335, 434–442. doi: 10.1124/jpet.110.171223

Antonopoulos, C., Russo, H. M., El Sanadi, C., Martin, B. N., Li, X., Kaiser, W. J., et al. (2015). Caspase-8 as an effector and regulator of NLRP3 inflammasome signaling. *J. Biol. Chem.* 290, 20167–20184. doi: 10.1074/jbc.M115.652321

Bauer, C., Duewell, P., Mayer, C., Lehr, H. A., Fitzgerald, K. A., Dauer, M., et al. (2010). Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* 59, 1192–1199. doi: 10.1136/gut.2009.197822

Baumgart, D. C., and Sandborn, W. J. (2012). Crohn's disease. *Lancet* 380, 1590–1605. doi: 10.1016/S0140-6736(12)60026-9

Blonski, W., Buchner, A. M., and Lichtenstein, G. R. (2011). Inflammatory bowel disease therapy: current state-of-the-art. *Curr. Opin. Gastroenterol.* 27, 346–357. doi: 10.1097/MOG.0b013e328347aef3

Carter, J. D., Valeriano, J., and Vasey, F. B. (2003). Crohn disease worsened by anakinra administration. *J. Clin. Rheumatol.* 9, 276–277. doi: 10.1097/01.RHU.0000081265.06408.e4

Chassaing, B., Aitken, J. D., Malleshappa, M., and Vijay-Kumar, M. (2014). Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr. Protoc. Immunol.* 4, 15.25.1–15.25.14. doi: 10.1002/0471142735.im1525s104

Chung, H., Vilaysane, A., Lau, A., Stahl, M., Morampudi, V., Bondzi-Simpson, A., et al. (2016). NLRP3 regulates a non-canonical platform for caspase-8 activation during epithelial cell apoptosis. *Cell Death Differ.* 23, 1331–1346. doi: 10.1038/cdd.2016.14

Coccia, M., Harrison, O. J., Schiering, C., Asquith, M. J., Becher, B., Powrie, F., et al. (2012). IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and $CD4^+$ Th17 cells. *J. Exp. Med.* 209, 1595–1609. doi: 10.1084/jem.20111453

Cocco, M., Garella, D., Di Stilo, A., Borretto, E., Stevanato, L., Giorgis, M., et al. (2014). Electrophilic warhead-based design of compounds preventing NLRP3 inflammasome-dependent pyroptosis. *J. Med. Chem.* 57, 10366–10382. doi: 10.1021/jm501072b

Cocco, M., Miglio, G., Giorgis, M., Garella, D., Marini, E., Costale, A., et al. (2016). Design, synthesis, and evaluation of acrylamide derivatives as direct NLRP3 inflammasome inhibitors. *ChemMedChem* 11, 1790–1803. doi: 10.1002/cmdc.201600055

Cocco, M., Pellegrini, C., Martinez-Banaclocha, H., Giorgis, M., Marini, E., Costale, A., et al. (2017). Development of an acrylate derivative targeting the NLRP3 inflammasome for the treatment of inflammatory bowel disease. *J. Med. Chem.* 60, 3656–3671. doi: 10.1021/acs.jmedchem.6b01624

Danese, S., and Fiocchi, C. (2011). Ulcerative colitis. *N. Engl. J. Med.* 365, 1713–1725. doi: 10.1056/NEJMra1102942

De Cassan, C., Fiorino, G., and Danese, S. (2012). Second-generation corticosteroids for the treatment of Crohn's disease and ulcerative colitis: more effective and less side effects? *Dig. Dis.* 30, 368–375. doi: 10.1159/000338128

de Mooij, C. E. M., Netea, M. G., van der Velden, W. J. F. M., and Blijlevens, N. M. A. (2017). Targeting theinterleukin-1 pathway in patients with hematological disorders. *Blood* 15, 3155–3164. doi: 10.1182/blood-2016-12-754994

Dinarello, C. A., Simon, A., and Van Der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11, 633–652. doi: 10.1038/nrd3800

Esposito, E., Mazzon, E., Paterniti, I., Dal Toso, R., Pressi, G., Caminiti, R., et al. (2010). PPAR-alpha contributes to the anti-inflammatory activity of verbascoside in a model of inflammatory bowel disease in mice. *PPAR Res.* 2010:917312. doi: 10.1155/2010/917312

Fornai, M., Antonioli, L., Pellegrini, C., Colucci, R., Sacco, D., Tirotta, E., et al. (2016). Small bowel protection against NSAID-injury in rats: effect of rifaximin, a poorly absorbed. GI targeted, antibiotic. *Pharmacol. Res.* 104, 186–196. doi: 10.1016/j.phrs.2015.12.031

Gaidt, M. M., and Hornung, V. (2018). The NLRP3 inflammasome renders cell death pro-inflammatory. *J. Mol. Biol.* 30, 133–141. doi: 10.1016/j.jmb.2017.11.013

Goyal, N., Rana, A., Ahlawat, A., Bijjem, K. R., and Kumar, P. (2014). Animal models of inflammatory bowel disease: a review. *Inflammopharmacology* 22, 219–233. doi: 10.1007/s10787-014-0207-y

Gringhuis, S. I., Kaptein, T. M., Wevers, B. A., Theelen, B., van der Vlist, M., Boekhout, T., et al. (2012). Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. *Nat. Immunol.* 2012, 246–254. doi: 10.1038/ni.2222

Guo, W., Hu, S., Elgehama, A., Shao, F., Ren, R., Liu, W., et al. (2015). Fumigaclavine C ameliorates dextran sulfate sodium-induced murine experimental colitis via NLRP3 inflammasome inhibition. *J. Pharmacol. Sci.* 129, 101–106. doi: 10.1016/j.jphs.2015.05.003

Gustot, T., Lemmers, A., Louis, E., Nicaise, C., Quertinmont, E., Belaiche, J., et al. (2005). Profile of soluble cytokine receptors in Crohn's disease. *Gut* 54, 488–495. doi: 10.1136/gut.2004.043554

Hügle, B., Speth, F., and Haas, J. P. (2017). Inflammatory bowel disease following anti-interleukin-1-treatment in systemic juvenile idiopathic arthritis. *Pediatr. Rheumatol. Online J.* 15:16. doi: 10.1186/s12969-017-0147-3

Kanneganti, T. D. (2017). Inflammatory Bowel Disease and the NLRP3 Inflammasome. *N. Engl. J. Med.* 377, 694–696. doi: 10.1056/NEJMcibr1706536

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., et al. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature* 479, 117–121. doi: 10.1038/nature10558

Lissner, D., Schumann, M., Batra, A., Kredel, L. I., Kuhl, A. A., Erben, U., et al. (2015). Monocyte and M1 macrophage-induced barrier defect contributes to chronic intestinal inflammation in IBD. *Inflamm. Bowel Dis.* 21, 1297–1305. doi: 10.1097/MIB.0000000000000384

Liu, W., Guo, W., Wu, J., Luo, Q., Tao, F., Gu, Y., et al. (2013). A novel benzo[d]imidazole derivate prevents the development of dextran sulfate sodium-induced murine experimental colitis via inhibition of NLRP3 inflammasome. *Biochem. Pharmacol.* 85, 1504–1512. doi: 10.1016/j.bcp.2013.03.008

Mangan, M. S. J., Olthava, E. J., Roush, W. R., Seidel, H. M., Glick, G. D., and Latz, E. (2018). Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat. Rev. Drug Dis.* 17, 588–606. doi: 10.1038/nrd.2018.97

Márquez-Flores, Y. K., Villegas, I., Cárdeno, A., Rosillo, M. Á, and Alarcón-de-la-Lastra, C. (2016). Apigenin supplementation protects the development of dextran sulfate sodium-induced murine experimental colitis by inhibiting canonical and non-canonical inflammasome signaling pathways. *J. Nutr. Biochem.* 30, 143–152. doi: 10.1016/j.jnutbio.2015.12.002

Martín-Sanchez, F., Diamond, C., Zeitler, M., Gomez, A. J., Baroja-Mazo, A., Bagnall, J., et al. (2016). Inflammasome-dependent IL-1β release depends upon membrane permeabilisation. *Cell Death Differ.* 23, 1219–1231. doi: 10.1038/cdd.2015.176

Maslanik, T., Mahaffey, L., Tannura, K., Beninson, L., Greenwood, B. N., and Fleshner, M. (2013). The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure. *Brain Behav. Immun.* 28, 54–62. doi: 10.1016/j.bbi.2012.10.014

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* 14, 329–342. doi: 10.1038/nri3661

Pellegrini, C., Antonioli, L., Colucci, R., Tirotta, E., Gentile, D., Ippolito, C., et al. (2017a). Effects of L-DOPA/benserazide co-treatment on colonic excitatory cholinergic motility and enteric inflammation following dopaminergic nigrostriatal neurodegeneration. *Neuropharmacology* 123, 22–33. doi: 10.1016/j.neuropharm.2017.05.016

Pellegrini, C., Antonioli, L., Lopez-Castejon, G., Blandizzi, C., and Fornai, M. (2017b). Canonical and non-canonical activation of NLRP3 inflammasome at the crossroad between immune tolerance and intestinal inflammation. *Front. Immunol.* 8:36. doi: 10.3389/fimmu.2017.00036

Perera, A. P., Fernando, R., Shinde, T., Gundamaraju, R., Southam, B., Sohal, S. S., et al. (2018). MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. *Sci. Rep.* 8:8618. doi: 10.1038/s41598-018-26775-w

Perera, A. P., Kunde, D., and Eri, R. (2017). NLRP3 inhibitors as potential therapeutic agents for treatment of inflammatory bowel disease. *Curr. Pharm. Des.* 23, 1–7. doi: 10.2174/1381612823666170201162414

Stoffels, B., Hupa, K. J., Snoek, S. A., van Bree, S., Stein, K., Schwandt, T., et al. (2014). Postoperative ileus involves interleukin-1 receptor signaling in enteric glia. *Gastroenterology* 146, 176.e1–87.e1. doi: 10.1053/j.gastro.2013.09.030

Sutterwala, F. S., Haasken, S., and Cassel, S. L. (2014). Mechanism of NLRP3 inflammasome activation. *Ann. N. Y. Acad. Sci.* 1319, 82–95. doi: 10.1111/nyas.12458

Vallejo, S., Palacios, E., Romacho, T., Villalobos, L., Peiro, C., and Sanchez-Ferrer, C. F. (2014). The interleukin-1 receptor antagonist anakinra improves endothelial dysfunction in streptozotocin-induced diabetic rats. *Cardiovasc. Diabetol.* 13:158. doi: 10.1186/s12933-014-0158-z

Viganò, E., and Mortellaro, A. (2013). Caspase-11: The driving factor for noncanon-ical inflammasomes. *Eur. J. Immunol.* 43, 2240–2245. doi: 10.1002/eji.201343800

Zhang, J., Fu, S., Sun, S., Li, Z., and Guo, B. (2014). Inflammasome activation has an important role in the development of spontaneous colitis. *Mucosal Immunol.* 7, 1139–1150. doi: 10.1038/mi.2014.1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 10 January 2019
doi: 10.3389/fphar.2018.01571

Microbial-Based Therapies in the Treatment of Inflammatory Bowel Disease – An Overview of Human Studies

*Paulo José Basso[1†], Niels Olsen Saraiva Câmara[1‡] and Helioswilton Sales-Campos[2]**

[1] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [2] Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Brazil

Inflammatory bowel disease (IBD) is a group of multifactorial and inflammatory infirmities comprised of two main entities: Ulcerative colitis (UC) and Crohn's disease (CD). Classic strategies to treat IBD are focused on decreasing inflammation besides inducing and extending disease remission. However, these approaches have several limitations such as low responsiveness, excessive immunosuppression, and refractoriness. Despite the multifactorial causality of IBD, immune disturbances and intestinal dysbiosis have been suggested as the central players in disease pathogenesis. Hence, therapies aiming at modulating intestinal microbial composition may represent a promising strategy in IBD control. Fecal microbiota transplantation (FMT) and probiotics have been explored as promising candidates to reestablish microbial balance in several immune-mediated diseases such as IBD. These microbial-based therapies have demonstrated the ability to reduce both the dysbiotic environment and production of inflammatory mediators, thus inducing remission, especially in UC. Despite these promising results, there is still no consensus on the relevance of such treatments in IBD as a potential clinical strategy. Thus, this review aims to critically review and describe the use of FMT and probiotics to treat patients with IBD.

Keywords: fecal microbiota transplantation, probiotics, Crohn's disease, Ulcerative colitis, dysbiosis

OPEN ACCESS

Edited by:
Luca Antonioli,
University of Pisa, Italy

Reviewed by:
Carolina Pellegrini,
University of Pisa, Italy
Dapeng Chen,
Dalian Medical University, China

****Correspondence:***
Helioswilton Sales-Campos
tonsales@ufg.br;
tonsales@hotmail.com
orcid.org/0000-0003-3252-2834

†orcid.org/0000-0003-4856-9207
‡orcid.org/0000-0001-5436-1248

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *10 October 2018*
Accepted: *24 December 2018*
Published: *10 January 2019*

Citation:
Basso PJ, Câmara NOS and Sales-Campos H (2019) Microbial-Based Therapies in the Treatment of Inflammatory Bowel Disease – An Overview of Human Studies. Front. Pharmacol. 9:1571. doi: 10.3389/fphar.2018.01571

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a group of immune-mediated diseases mainly represented by Ulcerative colitis (UC) and Crohn's disease (CD) (Mao et al., 2018). IBD presents a multifactorial etiology driven by immunological disturbances, genetic alterations and the influence of environmental factors such as diet, lifestyle, socioeconomic development, intestinal dysbiotic microbiota, among other aspects (Basso et al., 2014). Current therapies are based on pharmacological approaches using traditional medicines such as aminosalicylates, corticosteroids, thiopurines, folic acid antagonists, or biological therapies, aiming at controlling inflammation besides reducing disease relapse (Sales-Campos et al., 2015). However, these approaches are not curative, and patients may become refractory or intolerant to them. In this context, therapies aiming at modulating the microbes inhabiting the human body, especially the intestine, have been suggested as one of the most promising strategies to treat immune-mediated diseases such as IBD

(Ott et al., 2004; Alipour et al., 2016). This is of particular interest because recent investigations demonstrate that conventional treatments fail to completely restore the normal microbiota of patients with IBD, even if associated with special diets (Lewis et al., 2015). Though we understand the importance of other microbial interventions using symbiotics and prebiotics, for example, this review will focus on the human studies using fecal microbiota transplantation (FMT) and probiotics as strategies to restore the normal microbiota in IBD patients.

INTESTINAL MICROBIOTA

Before addressing the role of FMT and probiotics in IBD, it is important to introduce how the intestinal microbiota is able to interact with the vertebrate host, thus influencing health and disease status. Despite the great distribution of microorganisms in different sites of the human body, the most diverse microbial species is found in the gastrointestinal tract (GIT) (Hooper and Gordon, 2001; Hooper et al., 2001). More than 1000 microbial species, including bacteria, virus, and fungal, were identified in the human GIT (Turnbaugh et al., 2007). These commensal and symbiotic communities of microorganisms, also known as microbiota, are able to directly or indirectly influence local and systemic physiology of the human body, including but not limited to the immunologic, endocrine, and nervous systems (Lei et al., 2015). The composition of gut microbiota, in turn, can be influenced by different aspects such as diet, xenobiotics, lifestyle, and genetics (Goodrich et al., 2014; Wen and Duffy, 2017). Thus, it is reasonable to assume the great impact that perturbations in the complex bidirectional relationship between vertebrate hosts and gut microbes may have on host physiology. Further, this complex interaction can also lead to the onset and maintenance of several diseases, including IBD (Eck et al., 2017). Though gut microbiota is colonized by different microorganisms (bacteria, fungi, archaea, and viruses), the term "microbiota" is often used to refer to bacterial species within the GIT, which represents more than 96% of the total microbial population (Turnbaugh et al., 2007). However, fungal and viral dysbiosis have also been implicated in IBD development (Lewis et al., 2015; Duerkop et al., 2018).

To limit inappropriate activation in surfaces with great contact with microbes, like GIT, the human body has developed chemical and physical barriers to anatomically separate the microbiota from immune cells (Hooper et al., 2012). However, this interface is not insurmountable and some commensal microorganisms are able to interact with the immune, endocrine and nervous systems (Cani and Knauf, 2016). So far, two hypotheses were proposed to clarify the mechanisms concerning this interplay: the presence of pattern recognition receptors (PRR) in host cells sensing microbial associated molecular patterns (MAMPs)/danger associated molecular patterns (DAMPS), and the activity of microbial metabolites over different mammalian biological systems (Castro et al., 2015; Rangan et al., 2016). In this context, it is possible to highlight the beneficial role of the polysaccharide A of *Bacteroides fragilis*, which is able to stimulate the differentiation and activity of regulatory T cells (Treg) in the gut (Donaldson et al., 2016). The presence of Tregs in intestine is of great contribution to the maintenance of a tolerant environment, thus avoiding unnecessary inflammation (Hoeppli et al., 2018). Further, the production of immunoglobulin A (IgA) by intestinal plasma cells, which is crucial for the protection against pathobionts in intestine, is positively influenced by epithelium-associated bacteria such as *Mucispirillum* and segmented filamentous bacteria (SFB) (Bunker et al., 2015). One of the most studied groups of microbiota-derived metabolites with protective effects toward the mammalian host is the short chain fatty acids (SCFAs) that are mainly derived from fermentation of dietary fibers (Rios-Covian et al., 2016). SCFAs are primarily represented by three compounds acetate, propionate and butyrate, which contribute to the integrity of intestinal epithelium besides directly influencing host metabolic and immune functions (van de Wouw et al., 2018).

INTESTINAL DYSBIOSIS IN THE PATHOGENESIS OF IBD

Dysbiosis has been explored as a causative agent of several systemic and local diseases affecting GIT, including UC and CD (Kostic et al., 2014). The gut microbial changes in IBD are summarized in **Table 1**. In comparison to healthy subjects, IBD patients have reduced microbial composition (up to 25%), diversity, and richness with increased numbers of pathogenic/pathobionts microorganisms (e.g., Proteobacteria, Fusobacteria species, and *Ruminococcus gnavus* – Firmicutes) (Frank et al., 2007), and decreased numbers of beneficial microorganisms such as *Lachnospiraceae*

TABLE 1 | Changes in gut microbiota composition in inflammatory bowel disease patients.

Microorganism (s)	Commensal (C) or pathogenic (P) microorganisms*	UC	DC
Verrucomicrobia	C	↓	↓
Bifidobacterium	C	↓	↓
Roseburia species	C	↓	?
Bacteroides	C	↓↑	↑
Firmicutes	C	↓	↓
Clostridium species (clusters IV and XIVa	C	↓	↓↑
Saccharomyces cerevisiae	C	↓	↓
Pseudomonas	P	↓	↓
Proteobacteria	P	↑	↑
Fusobacterium	P	↑	↑
Ruminococcus gnavus	P	↑	↑
Candida albicans	P	↑	↑

*CD, Crohn's disease; UC, Ulcerative colitis. *Most of the species. References (Gophna et al., 2006; Frank et al., 2007; Kaakoush et al., 2012; Machiels et al., 2014; Lewis et al., 2015; Tahara et al., 2015; Shah et al., 2016; Sokol et al., 2017; Vrakas et al., 2017).*

(Firmicutes), *Bifidobacterium* species (Actinobacteria), *Roseburia* (Firmicutes), *Sutterella* (Proteobacteria) (Gilbert et al., 2016), and *Faecalibacterium prausnitzii* (Firmicutes), which are at least 10-fold reduced in IBD (Xiao et al., 2015). To note, *F. prausnitzii* has been suggested as one of the major microbial components of human healthy intestinal microbiota representing almost 5% of the total bacterial population (Louis and Flint, 2009). This bacterium contributes to the maintenance of a regulatory environment in intestine through the production of butyrate, besides providing energy to colonocytes (Sokol et al., 2008). The observation that intestinal microbes cooperate to the maintenance of epithelial integrity in intestine is of great importance since these mechanisms are frequently disrupted in IBD. One of the theories to explain the occurrence of dysbiosis in IBD relies on the inflammation. Results from both experimental and clinical investigations associate inflammatory responses and perturbations in microbial composition in ileum and other intestinal areas to the development of dysbiosis (Gevers et al., 2014; Forbes et al., 2016). On the other hand, a less dysbiotic environment is observed in non-affected areas of diseased subjects (Forbes et al., 2016).

There is evidence suggesting dysbiosis as a cause of IBD. Environmental factors, which directly affects intestinal microbiota composition, have been pointed out as one of the key players in the pathogenesis of IBD. In this regard, early life exposure to breastfeeding and maternal smoking during pregnancy, have been inversely and positively correlated to disease outcome in CD, respectively (Lindoso et al., 2018). Accordingly, patients with UC (Elinav et al., 2011) tend to have a better outcome when treated with microbial-based therapies (i.e., antibiotics, FMT, and probiotics). The mechanisms concerning the influence of dysbiosis in IBD outcome are still a matter of debate and investigation. Some studies suggest the association between the development of inflammation and the presence of some specific bacteria species. The reduction in strict anaerobes (e.g., *Clostridium* groups IV and XIVa), along with the expansion of facultative aerobic or aerobic bacteria, may increase the local concentration of oxygen, thus leading to augmented vascular and mucosal permeability, and promoting intestinal inflammation (Albenberg et al., 2014). Different strains of *Clostridium* species (e.g., IV, XIVa, and XVIII), which lack toxins and virulence factors, have their immunosuppressive activity demonstrated by inducing Treg cells in intestine in a TGF-β-, IL-10- or butyrate-dependent manner (Atarashi et al., 2013; Furusawa et al., 2013). These data suggest that microbial imbalance in IBD favors the development of inflammation by reducing crucial anti-inflammatory players, besides favoring the onset of pro-inflammatory mechanisms. On the other hand, inflammation *per se* also contributes to the onset of a dysbiotic environment. Regardless if inflammation leads to dysbiosis or vice-versa, they have a strong synergistic interaction that must be targeted to develop improved therapeutic strategies. For this reason, therapies aiming at reestablishing the microbial balance may represent the next frontier to treat inflammatory disorders, such as IBD, in which the dysbiosis plays a central role in disease pathogenesis.

FECAL MICROBIOTA TRANSPLANTATION (FMT)

Fecal microbiota transplantation has long been used to treat recurrent *Clostridium difficile* infection (CDI) presenting great effectiveness and significant safeness, with cure rates reaching 90% (Khan et al., 2018). One of the main mechanisms proposed to explain the ability of FMT to treat CDI is attributed to its capacity to restore intestinal microbial balance (Gagliardi et al., 2018). This characteristic has expanded the use of FMT to treat both local and systemic illnesses associated with gut dysbiosis, such as irritable bowel syndrome (IBS) (Mizuno et al., 2017), IBD (Angelberger et al., 2013; Kunde et al., 2013) and metabolic syndrome (Vrieze et al., 2012).

Because of the importance of elucidating how microbiota donors are selected and how FMT is delivered to recipients, these aspects will be clarified first. Then, we are going to present and discuss the most important scientific studies regarding the therapeutic use of FMT in IBD (**Table 2**).

FMT Donor Screening and Routes of Administration

Several aspects must be considered in the search for microbiota donors. Prior to the gut microbial sequencing *per se*, a putative donor must be screened for the presence of infectious agents in feces, including *C. difficile*, intestinal parasites and virus (e.g., Norovirus) (Paramsothy et al., 2015). In blood, aside from the complete blood count, electrolytes, liver, and kidney function tests, the presence of inflammatory markers, and transmissible infectious agents such as HIV, Hepatitis, HTLV, among others, must be performed (Paramsothy et al., 2015). Further, as inclusion criteria, the donor must have no history of suggestive GIT disease, no other major active comorbidities, and preferably, no use of medications, especially, antimicrobials (Paramsothy et al., 2015; Holleran et al., 2018). To ensure that only healthy donors will be selected, additional criteria of exclusion must be used as follows: any family history of colorectal cancer affecting first-degree relatives; use of probiotics 3 months prior the donation period; household members with active GIT infections; any personal or familial history of malignancies, malnutrition, obesity, neurological, or developmental disorders (Paramsothy et al., 2015; Holleran et al., 2018). The difficulties to select FMT donors that fulfills all the stringency criteria along with the costs involved in the screening process have created some important barriers for the broader utilization of this microbial therapeutic approach. Unfortunately, this scenario has stimulated patients to perform FMT in a "homemade" fashion, using inappropriate screened donors, without medical supervision, which often result in serious complications (Hohmann et al., 2014).

For a long time, retention enema was the most used technique for FMT. However, alternative approaches have been used in this regard, including nasogastric tube, capsules, colonoscopy, and self-administered enemas, as previously reviewed (Allegretti et al., 2017). Colonoscopy and retention enema are by far, the most frequently used routes of FMT administration (Gough et al., 2011).

TABLE 2 | Clinical trials of fecal microbiota transplantation for inflammatory bowel disease.

Authors	Diagnosis	Number of patients (P) or studies (S)[*#]	FMT route	Therapeutic regimen[&]	Outcome
Paramsothy et al. (2017b)	UC	*n* = 41 (S)	N.A	N.A	33% of clinical remission
	CD	*n* = 11 (S)	N.A	N.A	52% of clinical remission
Moayyedi et al. (2015)	UC	*n* = 70 (P)	Enema	50 g offeces/300 mL of water; once weekly for 6 weeks	24% of clinical remission
Paramsothy et al. (2017a)	UC	*n* = 85 (P)	Enema	150 mL[$]; once a day, 5 days per week for 8 weeks	27% of clinical and endoscopic remission or response
Rossen et al. (2015)	UC	*n* = 50 (P)	Naso-duodenal tube	60 g of feces/500 mL of saline; two doses (days 0 and 21)	No statistical difference between control and treated patients
Vaughn et al. (2016)	CD	*n* = 19 (P)	Colonoscopy	50 g of feces/250 mL of saline; one dose	58% of clinical response (control group not included)
Cui et al. (2015)	CD	*n* = 30 (P)	Endoscopy	150–200 mL[$]; one dose	86.7 and 76.7% of clinical improvement and remission, respectively at week 4
Suskind et al. (2015)	CD	*n* = 9 (P)	Nasogastric tube	30 g of feces/100 or 200 mL of saline; one dose	77.77% of clinical remission at week 2 55.55% of clinical remission at weeks 6 and 12

CD, Crohn's disease; N.A, Not applicable; UC, Ulcerative colitis. [*]Both total number of patients for clinical trials and number of studies for systematic analysis or meta-analysis were included. [#]Includes the number of control patients. [&]Feces may have undergone additional steps for FMT samples preparation. [$]Initial solution concentration is not available.

FMT in IBD

Ulcerative colitis and Crohn's disease are the major entities represented by IBD. The role of FMT has been more explored in the former. From the 307 adult patients pooled in a meta-analysis from 24 UC cohort studies, FMT induced remission in 33%. In 6 pediatric cohort studies, totalizing 34 UC patients, clinical remission was slightly reduced to 23% (Paramsothy et al., 2017b). Three randomized controlled trials also presented promising results regarding the use of FMT to treat UC. From a total of 70 UC patients with active disease without infectious diarrhea enrolled in the study, 36 were treated with FMT, and 34 with placebo, once a week for a total of 6 weeks, and remission was induced in 24% of those treated with FMT compared to 5% in the placebo group (Moayyedi et al., 2015). It is important to mention that both placebo and FMT groups were under concomitant anti-inflammatory/immunosuppressive therapy (e.g,. corticosteroids, mesalamine, and anti-TNF therapy) while enrolled in the study (Moayyedi et al., 2015). Similar results were observed using enemas 5 days per week for 8 weeks, in a study in Australia that observed a remission rate of 27% in UC patients with active UC treated with FMT when compared to 8% in patients treated with placebo only (Paramsothy et al., 2017a). Regardless if patients had received FMT or not, they were also treated with immunosuppressive drugs such as 5-aminosalicylates, thiopurines, methotrexate, and/or oral prednisone, in a stable dose (Paramsothy et al., 2017a). On the other hand, the remission rates observed in UC patients treated with FMT from healthy donors were similar to those observed in UC patients receiving their own fecal microbiota (Rossen et al., 2015).

Unfortunately, data supporting the role of FMT in CD are scarcer than in UC, and so far, no results from randomized clinical trials are available. The evidence of the beneficial effects of FMT in CD are all derived from small and uncontrolled studies. A single dose of FMT performed by colonoscopy showed an improvement in clinical outcome of 58% of patients treated with FMT (Vaughn et al., 2016). This observation was followed by increased levels of Tregs in recipients' lamina propria followed by higher microbial diversity (Vaughn et al., 2016), which suggests a reestablishment of microbial balance and a less prominent inflammation. Similarly, a single treatment with FMT induced clinical improvement and remission based on clinical activity in CD patients (Cui et al., 2015). This amelioration was followed by increased patient's body weight after FMT (Cui et al., 2015). For all CD-patients enrolled in the study a 12-week washout period was required for those exposed to immunosuppressive therapies such as cyclosporine, tacrolimus, or infliximab. Antibiotics and probiotics were withdrawn 60 and 30 days before FMT, respectively (Vaughn et al., 2016). The beneficial role of FMT was also addressed in young patients with CD. Nine individuals, aged 12–19 years, presenting mild-to-moderate symptoms received FMT by nasogastric tube once and were followed by 12 weeks (Suskind et al., 2015). Based on the clinical score, 2 weeks after FMT, 7 of 9 patients were in remission, and 5 of 9 patients were in remission at 6 and 12 weeks after FMT. All patients enrolled in the study were allowed to receive immunomodulators during the FMT or placebo treatment (Suskind et al., 2015).

Limitations in FMT Studies

The studies presented here showed promising results regarding the use of FMT to induce remission in UC and to a less extent in CD patients. The differences in the route and

interval of administration, besides of the composition and bacterial load in FMT, may explain the dissimilarities observed among studies. Another important drawback is the lack of comprehensive guidelines to be used globally in the screening and standardization of putative microbiota donors (age, gender, and health status) along with strategies of production, dosage regimen and to evaluate the transplant engraftment. Further, probably because of economic reasons, clinical trials do not deeply investigate the microbial composition of fecal donors using 16S rRNA sequencing and their similarities to the recipients' microbiota. Thus, the observation of similarities between the intestinal microbiota composition of donors and recipients may dictate the successfulness of FMT engraftment. Without the proper identification of the microbial community and the total bacterial load transplanted from a healthy donor to a diseased subject, it is difficult to predict the impact of FMT in IBD or other disorders. Further, as the majority of clinical trials were conducted with concomitant use of immunomodulatory drugs, it is reasonable to assume that FMT may work better as an adjuvant therapy rather than an isolated strategy. To confirm the role of FMT in IBD, more controlled clinical trials with a great number of patients and more standardized fecal samples must be conducted. Additionally, strategies aiming at providing an intestinal microbiota rebalance using well-defined microbial species may represent an improved alternative to total FMT.

FMT Adverse Effects

In general, up to 10% of FMT recipients present minor to mild self-limited adverse effects. The majority of them are related to disturbances in GIT such as diarrhea and abdominal discomfort/pain (Hohmann et al., 2014; Baxter and Colville, 2016). Though less frequently observed, severe side effects can include IBD flares, CDI and other infections, colectomy, small bowel obstruction, pancreatitis, and even death, as recently reviewed (Qazi et al., 2017; Jeon et al., 2018). However, some evidences have shown no differences between FMT and control groups concerning the occurrence of undesirable effects (Narula et al., 2017). Despite the possibility of occurrence of adverse effects, FMT is considered to be safe in IBD. An in-depth screening of donors along with a broader comprehension of the physiopathology in IBD may facilitate the development of strategies to avoid the occurrence of such undesirable effects.

PROBIOTICS

Probiotics are used as safe food additives, pharmaceutical formulations or nutritional supplements defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" by the World Health Organization (WHO) (Hill et al., 2014). Nevertheless, studies have pointed out that dead microorganisms or their biologically active compounds *per se* can also play protective functions, inferring that the "probiotic" definition should be revisited or other classifications implemented (Rachmilewitz et al., 2004).

The underlying mechanisms of probiotics are dependent on microbial strain. Moreover, the effects of probiotic mixtures may be complementary (also referred to additive) or synergistic (Ruiz et al., 2009). In general, probiotic strains produce growth factors that strengthen the gut epithelium and antimicrobial substances (e.g., SCFAs, bacteriocins, hydroperoxides, bile acids, and lactic acids) that kill harmful microorganisms (Konieczna et al., 2012a). As a consequence, cellular components (e.g., cellular wall, DNA) are released in the gut environment, which activate immune responses by enhancing the pro-inflammatory cytokines production and immunoglobulin synthesis, besides of improving macrophage and lymphocytes activity (Markowiak and Slizewska, 2017). In this regard, the use of *Bifidobacterium infantis* 35624 in human volunteers increased the amount of IL-10 and FoxP3^{+} cells (Treg) in the circulation (Haskard et al., 2001; Konieczna et al., 2012b). Although immune tolerance is a putative consequence of these enhancements, there is still no consensus on this matter (Castellazzi et al., 2013).

Non-immunological benefits associated to probiotics include the digestion and absorption processes, competition with potential pathogens for nutrients and intestinal adhesion sites, pH alterations, agglutination of pathogenic microorganisms, and sequestration of metabolic toxins (Gagliardi et al., 2018). Animal models and *in vitro* assays describe that probiotics also decrease the apoptosis, increase the mucus synthesis, tissue repair, redistribution and production of tight junctions in gut epithelial cells, thus reducing the intestinal permeability and enhancing the barrier protection and function (Caballero-Franco et al., 2007; Zyrek et al., 2007).

Lactobacillus (e.g., *reuteri*, *rhamnosus*, *casei*, *acidophilus*, *plantarum*, *gasseri, paracasei, johnsonii, ghallinarum*, and *crispatus*) and Bifidobacterium (e.g., *bifidum, infantis, longum, animalis, breve*, *lactis*, and *adolescentis*) are the most used strains in probiotics formulations, but multispecies approach has been increasingly applied (Holzapfel et al., 2001). Others strains commonly used include *Streptococcus* spp., *Lactococcus* spp., *Enterococcus* spp., non-pathogenic *Escherichia coli* (strain Nissle), and *Clostridium* ssp (Kechagia et al., 2013).

New bacteria genera and species have also been investigated showing good perspectives in preclinical trials. These bacteria are described as new-generation probiotics bringing more complexity to common probiotics in attempt to simulate FMT treatments. The new-generation probiotics comprise *Clostridium* clusters *IV*, *XIVa*, and *XVIII*, *F. prausnitzii, Akkermansia muciniphila, Bacteroides uniformis, B. fragilis*, and *Eubacterium hallii* (El Hage et al., 2017). Technological limitations are current challenges for using these bacteria as probiotics. Importantly, *Clostridium* clusters XIVa and IV are described as promoters of Treg differentiation, critical for immune tolerance as described earlier (Atarashi et al., 2011). Indeed, these bacteria are decreased in the gut of IBD patients (Sokol et al., 2006; Kang et al., 2010; Machiels et al., 2014). Although the number of Tregs is increased in the gut of IBD patients, the expansion is not sufficient to restrain the inflammatory development.

Since gut microbiota is not composed only by bacteria, some formulations and studies use yeasts as probiotics in association with bacteria strains, or even in single-drug formulations. In this context, *Saccharomyces boulardii* is the most commonly used yeast strain and has several anti-inflammatory properties (Pothoulakis, 2009).

Criteria for New Probiotic Development

As pharmaceutical or nutraceutical products, probiotics must meet some criteria to be commercially available. Beyond efficacy, the safety properties of a given drug are the main concern of scientists and regulatory agencies (Doron and Snydman, 2015). Bacterial and yeast strains or their derived-products have distinct levels of regulations according to their purposes and must meet the requirements outlined in published and frequently updated guidelines designed by regulatory agencies (Doron and Snydman, 2015). They can be considered as food (food ingredient, medical food, and dietary supplement), drug (new drugs) or biological product (Degnan, 2008).

As in FMT, safety is a priority, since some inflammatory conditions or patients under immunosuppressive therapy increase the susceptibility to infectious complications, including sepsis (Farina et al., 2001; Riquelme et al., 2003). Probiotics must have human origin, scientifically proven positive effects, be safe even in high-risk populations, cannot cause allergies and must present good technology properties (e.g., feasible culture and large-scale production) (Markowiak and Slizewska, 2017). Several *in vitro* assays may be employed at the first glance to evaluate probiotic potential, epithelium adherence, microbicide activity, ability in reducing the number of pathogenic bacteria and resistance to antibiotic use, bile salts, stomach acids, digestive enzymes and pH (Saarela et al., 2000).

Although not mandatory, studies should also evaluate the adverse effects and drug interactions of probiotics since they have been used as adjuvant therapy in various diseases (Thomsen et al., 2018). For instance, the probiotic *E. coli* strain Nissle 1917 influences the pharmacokinetics of concomitantly taken antiarrhythmic drug amiodarone by increasing the drug bioavailability (Matuskova et al., 2014). Therefore, their presumed safety should be avoided, and the potential risks not neglected.

Probiotics in IBD

In general, probiotics have been effectively used in treating IBD to prevent dysbiosis in patients undergoing prolonged antibiotic or immunosuppressive therapies (Zuo and Ng, 2018). Further, these microorganisms have been used as adjuvant therapy on the attempt to reverse the dysbiotic environment associated with IBD onset and worsening (Yoshimatsu et al., 2015; Tamaki et al., 2016). Although the number of clinical and experimental studies using probiotics in IBD is substantially high, lack of standard practices in therapeutic regimens, low number of samples and poor disease characterization, have limited the relevant conclusions about the efficacy of probiotics in this scenario.

Probiotics have been described as an alternative to induce and maintain the remission in UC, while low or no effects are observed in CD. The adjuvant use of multispecies probiotic VSL#3, which contains four strains of Lactobacillus (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* subsp. *Bulgaricus*), three of Bifidobacterium (*B. longum*, *B. breve*, and *B. infantis*), and one of Streptococcus (*S. salivarius* subsp. *Thermophilus*), improved the clinical symptoms in patients with mild to moderately active UC after receiving the daily dose of 3.6×10^{12} CFU (Tursi et al., 2010; Mardini and Grigorian, 2014). Corroborating results were observed after treating mild-to-moderate UC patients with VSL#3 alone, twice a day at the same dose described earlier (Sood et al., 2009). The maintenance of remission rates in UC was also similar in patients under single drug treatment of either non-pathogenic *E. coli* Nissle 1917 ($5–50 \times 10^9$/day) or mesalazine (1500 mg/day) (Kruis et al., 2004).

However, the systematic review using rigorous statistical methods showed that the beneficial effects of both VSL#3 or *E. coli* Nissle on UC are weak or inconclusive, while there is no positive association in CD (Jonkers et al., 2012), confirming the need for further new randomized controlled trials to increase the significance level of these findings.

The use of Bifidobacterium-fermented milk (containing *B. breve*, *B. bifidum*, and *L. acidophilus*) as adjuvant therapy to treat 20 patients (including placebo control) with mild to moderately active UC, showed significant improvement in both clinical and endoscopic activity indexes after 12 weeks (10 billion bacteria/day) (Kato et al., 2004). Interestingly, the SCFAs concentration in feces was higher in the probiotic-treated group compared to the placebo group. However, a recent study using a similar therapeutic strategy (*B. breve*- and *L. acidophilus*-containing fermented milk) showed no efficacy to treat or maintain the remission of UC in 195 patients (Matsuoka et al., 2018). In fact, the use of *B. bifidum* as single strain-containing probiotic was sufficient to increase the levels of fecal SCFAs in healthy volunteers (Gargari et al., 2016), however, the protective role in UC or CD remains unknown. Despite some discrepancies regarding the number of patients used in the studies mentioned above, the first was the only one to confirm the increased number of Bifidobacteria in the feces of probiotics-treated patients and to perform endoscopic analysis.

The treatment with *Lactobacillus* GG (18×10^9 viable bacteria/day) alone or associated with mesalazine, prolonged the relapse-free period in UC patients compared to the group treated with immunosuppressant drug alone in a 12-month treatment regimen (Zocco et al., 2006). Similarly, a systematic review of randomized clinical trials showed the use of different lactic acid bacteria and Bifidobacteria as adjuvant therapy improved the course of disease and maintenance of clinical remission in UC (Saez-Lara et al., 2015).

As stated above, probiotics have poor or no effects on CD. However, studies have yielded positive results to induce remission by associating probiotics and prebiotics (defined as symbiotics) (Fujimori et al., 2007; Saez-Lara et al., 2015). Additionally, one open-label pilot study containing four children with mildly to moderately active CD had a significant improvement on clinical

TABLE 3 | Effective clinical trials using probiotics for treating inflammatory bowel disease.

Authors	Diagnosis	Number of patients (P) or studies (S)[*#]	Probiotic	Therapeutic regimen	Outcome
Mardini and Grigorian, 2014	UC	*n* = 5 (S) *n* = 441 (P)	VSL#3[&]	Oral; 3.6×10^{12} CFU/day[$]	53.4% of clinical responsiveness and 43.8% of clinical remission
Tursi et al., 2010	UC	*n* = 144 (P)	VSL#3[&]	Oral; 3.6×10^{12} CFU/day; once a day for 8 weeks	53.4% of clinical improvement and 47.3% of clinical remission
Sood et al., 2009	UC	*n* = 147 (P)	VSL#3[&]	Oral; 3.6×10^{12} CFU/dose; twice a day for 12 weeks	51.9% of clinical improvement and 42.9% of clinical remission at 12 weeks
Kruis et al., 2004	UC	*n* = 327 (P)	*Escheria coli* Nissle 1917	Oral; $5–50 \times 10^9$ viables bacteria; once a day for 12 months	No differences between probiotic- and mesalazine-treated groups
Kato et al., 2004	UC	*n* = 20 (P)	Fermented milk (*B. breve*, *B. bifidum* and *L. acidophilus*)	Oral; 10^9 bacteria/day; once a day for 12 weeks	70% of clinical responsiveness and 40% of clinical remission
Zocco et al., 2006	UC	*n* = 187 (P)	*Lactobacillus* GG	Oral; 9×10^9 viable bacteria/dose; twice a day for 12 months	No differences between probiotic- and mesalazine-treated groups
Fujimori et al., 2007	CD	*n* = 10 (P)	*B. breve*, *L. asei* and *B. longum*	Oral; 75×10^9 bacteria/day; once a day for 13 (±4.5) months	70% of clinical responsiveness and 60% of clinical remission
Gupta et al., 2000	CD	*n* = 4 (P)	*Lactobacillus* GG	Oral; 10^{10} CFU/dose; twice a day for 6 months	75% of clinical improvement at weeks 4 and 12

CD, Crohn's disease; UC: Ulcerative colitis. [*]Both total number of patients for clinical trials and number of studies for systematic analysis or meta-analysis were included. [#]Includes the number of control patients. [&]VSL#3 is composed by L. casei, L. plantarum, L. acidophilus, L. delbrueckii subsp. bulgaricus, B. longum, B. breve, B. infantis and Streptococcus sulivarius subsp. thermophiles. [$]Length of treatments not available.

aspects after treatment with *Lactobacillus* GG (10^{10} CFU/tablet, twice a day for 6 months) (Gupta et al., 2000). However, the low number of samples and the absence of appropriate control (placebo-treated patients or under regular therapy) undermine the rigor of study. Probiotics have no effects in maintaining the remission of CD (Bousvaros et al., 2005; Bourreille et al., 2013).

In conclusion, probiotics are potential options in inducing and maintaining remission of mild to moderately UC, however, seem to be ineffective in DC (**Table 3**). The results must be considered as preliminary evidence and further randomized double-blind placebo-controlled multicenter trials must be performed to increase the reliability of results.

Limitations on Probiotics Studies

Different therapeutic regimen (including dose and frequency of administration) is an important problem to design treatment protocols. Although doses vary according to bacterial strains, studies have shown that 10^8–10^{10} CFU/day are ingested after consuming 100 mL or 100 g of probiotic-containing product (Atarashi et al., 2011). As a consequence, meta-analysis studies have several biases to compare related clinical trials and to draw relevant conclusions.

Unlike FMT therapy, the route of administration is not a potential problem, since the majority of studies use the oral route as the main one, although enemas are also a potential method of probiotic delivery (Oliva et al., 2012).

Another important issue regarding probiotics formulations is the quality control. Several inconsistent data have been described between label information and product content, contamination, poor quality of strains, among others, as previously reviewed (Kolacek et al., 2017). Moreover, the same strain may show different efficacy in distinct batches as a result of a lack of standardization in bacterial culture procedures used throughout the studies and manufactures. Thus, both guidelines and improvements on supervision are highly encouraged to provide sufficient information on the design of new studies and to prevent unwanted and conflicting outcomes.

The immunosuppressive therapy is also a current challenge for clinicians and researchers. Since long-term use of immunosuppressants causes dysbiosis, it is important to determine whether this factor is a premise for the patient's responsiveness to probiotic treatment (Bhat et al., 2017).

Altogether, these factors represent important limitations in studies setup and the conflicting clinical results found in the literature may derive from poorly designed and standardized studies.

CONCLUSION AND FURTHER DIRECTIONS

Both FMT and probiotics are therapies with good prospects in the medical field, especially in IBD. However, like other newly developed therapies, the challenges encountered for increasing the reliability, safety, and standardization of FMT and probiotics are considerable. Thus, more multicenter studies must be performed to increase the number of samples and variables (features of IBD, phenotypic and genotypic characteristics of

the patients, standardizations in the therapeutic regimen, etc.), generating more significant conclusions.

AUTHOR CONTRIBUTIONS

PB wrote the manuscript, edited, generated the tables, and performed the literature review. NC contributed to review the manuscript and approval of the final version. HS-C designed the aim of the review, wrote the manuscript, performed the literature review, edited and contributed to final approval for the version to be published.

FUNDING

The authors would like to thank FAPESP for financial support under projects 2015/26682-6 and 2017/05264-7. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Albenberg, L., Esipova, T. V., Judge, C. P., Bittinger, K., Chen, J., Laughlin, A., et al. (2014). Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. *Gastroenterology* 147, 1055–1063.e8. doi: 10.1053/j.gastro.2014.07.020

Alipour, M., Zaidi, D., Valcheva, R., Jovel, J., Martinez, I., Sergi, C., et al. (2016). Mucosal barrier depletion and loss of bacterial diversity are primary abnormalities in paediatric ulcerative colitis. *J. Crohns Colitis* 10, 462–471. doi: 10.1093/ecco-jcc/jjv223

Allegretti, J., Eysenbach, L. M., El-Nachef, N., Fischer, M., Kelly, C., and Kassam, Z. (2017). The current landscape and lessons from fecal microbiota transplantation for inflammatory bowel disease: past, present, and future. *Inflamm. Bowel Dis.* 23, 1710–1717. doi: 10.1097/MIB.0000000000001247

Angelberger, S., Reinisch, W., Makristathis, A., Lichtenberger, C., Dejaco, C., Papay, P., et al. (2013). Temporal bacterial community dynamics vary among ulcerative colitis patients after fecal microbiota transplantation. *Am. J. Gastroenterol.* 108, 1620–1630. doi: 10.1038/ajg.2013.257

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500, 232–236. doi: 10.1038/nature12331

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331, 337–341. doi: 10.1126/science.1198469

Basso, P. J., Fonseca, M. T., Bonfa, G., Alves, V. B., Sales-Campos, H., Nardini, V., et al. (2014). Association among genetic predisposition, gut microbiota, and host immune response in the etiopathogenesis of inflammatory bowel disease. *Braz. J. Med. Biol. Res.* 47, 727–737. doi: 10.1590/1414-431X20143932

Baxter, M., and Colville, A. (2016). Adverse events in faecal microbiota transplant: a review of the literature. *J. Hosp. Infect.* 92, 117–127. doi: 10.1016/j.jhin.2015.10.024

Bhat, M., Pasini, E., Copeland, J., Angeli, M., Husain, S., Kumar, D., et al. (2017). Impact of immunosuppression on the metagenomic composition of the intestinal microbiome: a systems biology approach to post-transplant diabetes. *Sci. Rep.* 7:10277. doi: 10.1038/s41598-017-10471-2

Bourreille, A., Cadiot, G., Le Dreau, G., Laharie, D., Beaugerie, L., Dupas, J. L., et al. (2013). *Saccharomyces boulardii* does not prevent relapse of Crohn's disease. *Clin. Gastroenterol. Hepatol.* 11, 982–987. doi: 10.1016/j.cgh.2013.02.021

Bousvaros, A., Guandalini, S., Baldassano, R. N., Botelho, C., Evans, J., Ferry, G. D., et al. (2005). A randomized, double-blind trial of Lactobacillus GG versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflamm. Bowel Dis.* 11, 833–839. doi: 10.1097/01.MIB.0000175905.00212.2c

Bunker, J. J., Flynn, T. M., Koval, J. C., Shaw, D. G., Meisel, M., McDonald, B. D., et al. (2015). Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. *Immunity* 43, 541–553. doi: 10.1016/j.immuni.2015.08.007

Caballero-Franco, C., Keller, K., De Simone, C., and Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am. J. Physiol. Gastrointest Liver Physiol.* 292, G315–G322. doi: 10.1152/ajpgi.00265.2006

Cani, P. D., and Knauf, C. (2016). How gut microbes talk to organs: the role of endocrine and nervous routes. *Mol. Metab.* 5, 743–752. doi: 10.1016/j.molmet.2016.05.011

Castellazzi, A. M., Valsecchi, C., Caimmi, S., Licari, A., Marseglia, A., Leoni, M. C., et al. (2013). Probiotics and food allergy. *Ital. J. Pediatr.* 39:47. doi: 10.1186/1824-7288-39-47

Castro, C. N., Freitag, J., Berod, L., Lochner, M., and Sparwasser, T. (2015). Microbe-associated immunomodulatory metabolites: influence on T cell fate and function. *Mol. Immunol.* 68(2 Pt C), 575–584. doi: 10.1016/j.molimm.2015.07.025

Cui, B., Feng, Q., Wang, H., Wang, M., Peng, Z., Li, P., et al. (2015). Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: safety, feasibility, and efficacy trial results. *J. Gastroenterol. Hepatol.* 30, 51–58. doi: 10.1111/jgh.12727

Degnan, F. H. (2008). The US Food and Drug Administration and probiotics: regulatory categorization. *Clin. Infect. Dis.* 46(Suppl. 2), S133–S136; discussion S144–S151. doi: 10.1086/523324

Donaldson, G. P., Lee, S. M., and Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* 14, 20–32. doi: 10.1038/nrmicro3552

Doron, S., and Snydman, D. R. (2015). Risk and safety of probiotics. *Clin. Infect. Dis.* 60(Suppl. 2), S129–S134. doi: 10.1093/cid/civ085

Duerkop, B. A., Kleiner, M., Paez-Espino, D., Zhu, W., Bushnell, B., Hassell, B., et al. (2018). Murine colitis reveals a disease-associated bacteriophage community. *Nat. Microbiol.* 3, 1023–1031. doi: 10.1038/s41564-018-0210-y

Eck, A., de Groot, E. F. J., de Meij, T. G. J., Welling, M., Savelkoul, P. H. M., and Budding, A. E. (2017). Robust microbiota-based diagnostics for inflammatory bowel disease. *J. Clin. Microbiol.* 55, 1720–1732. doi: 10.1128/JCM.00162-17

El Hage, R., Hernandez-Sanabria, E., and Van de Wiele, T. (2017). Emerging trends in "smart probiotics": functional consideration for the development of novel health and industrial applications. *Front. Microbiol.* 8:1889. doi: 10.3389/fmicb.2017.01889

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., et al. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145, 745–757. doi: 10.1016/j.cell.2011.04.022

Farina, C., Arosio, M., Mangia, M., and Moioli, F. (2001). *Lactobacillus casei* subsp. rhamnosus sepsis in a patient with ulcerative colitis. *J. Clin. Gastroenterol.* 33, 251–252. doi: 10.1097/00004836-200109000-00019

Forbes, J. D., Van Domselaar, G., and Bernstein, C. N. (2016). Microbiome survey of the inflamed and noninflamed gut at different compartments within the gastrointestinal tract of inflammatory bowel disease patients. *Inflamm. Bowel Dis.* 22, 817–825. doi: 10.1097/MIB.0000000000000684

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., and Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13780–13785. doi: 10.1073/pnas.0706625104

Fujimori, S., Tatsuguchi, A., Gudis, K., Kishida, T., Mitsui, K., Ehara, A., et al. (2007). High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. *J. Gastroenterol. Hepatol.* 22, 1199–1204. doi: 10.1111/j.1440-1746.2006.04535.x

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504, 446–450. doi: 10.1038/nature12721

Gagliardi, A., Totino, V., Cacciotti, F., Iebba, V., Neroni, B., Bonfiglio, G., et al. (2018). Rebuilding the gut microbiota ecosystem. *Int. J. Environ. Res. Public Health* 15:E1679. doi: 10.3390/ijerph15081679

Gargari, G., Taverniti, V., Balzaretti, S., Ferrario, C., Gardana, C., Simonetti, P., et al. (2016). Consumption of a bifidobacterium bifidum strain for

4 weeks modulates dominant intestinal bacterial taxa and fecal butyrate in healthy adults. *Appl. Environ. Microbiol.* 82, 5850–5859. doi: 10.1128/AEM.01753-16

Gevers, D., Kugathasan, S., Denson, L. A., Vazquez-Baeza, Y., Van Treuren, W., Ren, B., et al. (2014). The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* 15, 382–392. doi: 10.1016/j.chom.2014.02.005

Gilbert, J. A., Quinn, R. A., Debelius, J., Xu, Z. Z., Morton, J., Garg, N., et al. (2016). Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature* 535, 94–103. doi: 10.1038/nature18850

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cell* 159, 789–799. doi: 10.1016/j.cell.2014.09.053

Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F., and Veldhuyzen van Zanten, S. J. (2006). Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *J. Clin. Microbiol.* 44, 4136–4141. doi: 10.1128/JCM.01004-06

Gough, E., Shaikh, H., and Manges, A. R. (2011). Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. *Clin. Infect. Dis.* 53, 994–1002. doi: 10.1093/cid/cir632

Gupta, P., Andrew, H., Kirschner, B. S., and Guandalini, S. (2000). Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J. Pediatr. Gastroenterol. Nutr.* 31, 453–457. doi: 10.1097/00005176-200010000-00024

Haskard, C. A., El-Nezami, H. S., Kankaanpaa, P. E., Salminen, S., and Ahokas, J. T. (2001). Surface binding of aflatoxin B(1) by lactic acid bacteria. *Appl. Environ. Microbiol.* 67, 3086–3091. doi: 10.1128/AEM.67.7.3086-3091.2001

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hoeppli, R. E., MacDonald, K. N., Leclair, P., Fung, V. C. W., Mojibian, M., Gillies, J., et al. (2018). Tailoring the homing capacity of human Tregs for directed migration to sites of Th1-inflammation or intestinal regions. *Am. J. Transplant.* 19, 62–76. doi: 10.1111/ajt.14936

Hohmann, E. L., Ananthakrishnan, A. N., and Deshpande, V. (2014). case records of the massachusetts general hospital. Case 25-2014. A 37-year-old man with ulcerative colitis and bloody diarrhea. *N. Engl. J. Med.* 371, 668–675. doi: 10.1056/NEJMcpc1400842

Holleran, G., Scaldaferri, F., Ianiro, G., Lopetuso, L., Mc Namara, D., Mele, M. C., et al. (2018). Fecal microbiota transplantation for the treatment of patients with ulcerative colitis and other gastrointestinal conditions beyond Clostridium difficile infection: an update. *Drugs Today* 54, 123–136. doi: 10.1358/dot.2018.54.2.2760765

Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J., and Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am. J. Clin. Nutr.* 73(2 Suppl.), 365S–373S. doi: 10.1093/ajcn/73.2.365s

Hooper, L. V., and Gordon, J. I. (2001). Commensal host-bacterial relationships in the gut. *Science* 292, 1115–1118. doi: 10.1126/science.1058709

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi: 10.1126/science.291.5505.881

Jeon, S. R., Chai, J., Kim, C., and Lee, C. H. (2018). Current evidence for the management of inflammatory bowel diseases using fecal microbiota transplantation. *Curr. Infect. Dis. Rep.* 20:21. doi: 10.1007/s11908-018-0627-8

Jonkers, D., Penders, J., Masclee, A., and Pierik, M. (2012). Probiotics in the management of inflammatory bowel disease: a systematic review of intervention studies in adult patients. *Drugs* 72, 803–823. doi: 10.2165/11632710-000000000-00000

Kaakoush, N. O., Day, A. S., Huinao, K. D., Leach, S. T., Lemberg, D. A., Dowd, S. E., et al. (2012). Microbial dysbiosis in pediatric patients with Crohn's disease. *J. Clin. Microbiol.* 50, 3258–3266. doi: 10.1128/JCM.01396-12

Kang, S., Denman, S. E., Morrison, M., Yu, Z., Dore, J., Leclerc, M., et al. (2010). Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm. Bowel Dis.* 16, 2034–2042. doi: 10.1002/ibd.21319

Kato, K., Mizuno, S., Umesaki, Y., Ishii, Y., Sugitani, M., Imaoka, A., et al. (2004). Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. *Aliment Pharmacol. Ther.* 20, 1133–1141. doi: 10.1111/j.1365-2036.2004.02268.x

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., et al. (2013). Health benefits of probiotics: a review. *ISRN Nutr.* 2013:481651. doi: 10.5402/2013/481651

Khan, M. Y., Dirweesh, A., Khurshid, T., and Siddiqui, W. J. (2018). Comparing fecal microbiota transplantation to standard-of-care treatment for recurrent Clostridium difficile infection: a systematic review and meta-analysis. *Eur. J. Gastroenterol. Hepatol.* 30, 1309–1317. doi: 10.1097/MEG.0000000000001243

Kolacek, S., Hojsak, I., Berni Canani, R., Guarino, A., Indrio, F., Orel, R., et al. (2017). Commercial probiotic products: a call for improved quality control. a position paper by the ESPGHAN working group for probiotics and prebiotics. *J. Pediatr. Gastroenterol. Nutr.* 65, 117–124. doi: 10.1097/MPG.0000000000001603

Konieczna, P., Akdis, C. A., Quigley, E. M., Shanahan, F., and O'Mahony, L. (2012a). Portrait of an immunoregulatory Bifidobacterium. *Gut Microbes* 3, 261–266. doi: 10.4161/gmic.20358

Konieczna, P., Groeger, D., Ziegler, M., Frei, R., Ferstl, R., Shanahan, F., et al. (2012b). Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. *Gut* 61, 354–366. doi: 10.1136/gutjnl-2011-300936

Kostic, A. D., Xavier, R. J., and Gevers, D. (2014). The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 146, 1489–1499. doi: 10.1053/j.gastro.2014.02.009

Kruis, W., Fric, P., Pokrotnieks, J., Lukas, M., Fixa, B., Kascak, M., et al. (2004). Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. *Gut* 53, 1617–1623. doi: 10.1136/gut.2003.037747

Kunde, S., Pham, A., Bonczyk, S., Crumb, T., Duba, M., Conrad, H. Jr., et al. (2013). Safety, tolerability, and clinical response after fecal transplantation in children and young adults with ulcerative colitis. *J. Pediatr. Gastroenterol. Nutr.* 56, 597–601. doi: 10.1097/MPG.0b013e318292fa0d

Lei, Y. M., Nair, L., and Alegre, M. L. (2015). The interplay between the intestinal microbiota and the immune system. *Clin. Res. Hepatol. Gastroenterol.* 39, 9–19. doi: 10.1016/j.clinre.2014.10.008

Lewis, J. D., Chen, E. Z., Baldassano, R. N., Otley, A. R., Griffiths, A. M., Lee, D., et al. (2015). Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric crohn's disease. *Cell Host Microbe* 18, 489–500. doi: 10.1016/j.chom.2015.09.008

Lindoso, L., Mondal, K., Venkateswaran, S., Somineni, H. K., Ballengee, C., Walters, T. D., et al. (2018). The effect of early-life environmental exposures on disease phenotype and clinical course of crohn's disease in children. *Am. J. Gastroenterol.* 113, 1524–1529. doi: 10.1038/s41395-018-0239-9

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., et al. (2014). A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 63, 1275–1283. doi: 10.1136/gutjnl-2013-304833

Mao, L., Kitani, A., Strober, W., and Fuss, I. J. (2018). The role of NLRP3 and IL-1beta in the pathogenesis of inflammatory bowel disease. *Front. Immunol.* 9:2566. doi: 10.3389/fimmu.2018.02566

Mardini, H. E., and Grigorian, A. Y. (2014). Probiotic mix VSL#3 is effective adjunctive therapy for mild to moderately active ulcerative colitis: a meta-analysis. *Inflamm. Bowel Dis.* 20, 1562–1567. doi: 10.1097/MIB.0000000000000084

Markowiak, P., and Slizewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients* 9:E1021. doi: 10.3390/nu9091021

Matsuoka, K., Uemura, Y., Kanai, T., Kunisaki, R., Suzuki, Y., Yokoyama, K., et al. (2018). Efficacy of bifidobacterium breve fermented milk in maintaining remission of ulcerative colitis. *Dig. Dis. Sci.* 63, 1910–1919. doi: 10.1007/s10620-018-4946-2

Matuskova, Z., Anzenbacherova, E., Vecera, R., Tlaskalova-Hogenova, H., Kolar, M., and Anzenbacher, P. (2014). Administration of a probiotic can change drug pharmacokinetics: effect of *E. coli* Nissle 1917 on amidarone absorption in rats. *PLoS One* 9:e87150. doi: 10.1371/journal.pone.0087150

Mizuno, S., Masaoka, T., Naganuma, M., Kishimoto, T., Kitazawa, M., Kurokawa, S., et al. (2017). Bifidobacterium-rich fecal donor may be a positive predictor for successful fecal microbiota transplantation in patients with irritable bowel syndrome. *Digestion* 96, 29–38. doi: 10.1159/000471919

Moayyedi, P., Surette, M. G., Kim, P. T., Libertucci, J., Wolfe, M., Onischi, C., et al. (2015). Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* 149, 102–109.e6. doi: 10.1053/j.gastro.2015.04.001

Narula, N., Kassam, Z., Yuan, Y., Colombel, J. F., Ponsioen, C., Reinisch, W., et al. (2017). Systematic review and meta-analysis: fecal microbiota transplantation for treatment of active ulcerative colitis. *Inflamm. Bowel Dis.* 23, 1702–1709. doi: 10.1097/MIB.0000000000001228

Oliva, S., Di Nardo, G., Ferrari, F., Mallardo, S., Rossi, P., Patrizi, G., et al. (2012). Randomised clinical trial: the effectiveness of *Lactobacillus reuteri* ATCC 55730 rectal enema in children with active distal ulcerative colitis. *Aliment Pharmacol. Ther.* 35, 327–334. doi: 10.1111/j.1365-2036.2011.04939.x

Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Folsch, U. R., et al. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* 53, 685–693. doi: 10.1136/gut.2003.025403

Paramsothy, S., Borody, T. J., Lin, E., Finlayson, S., Walsh, A. J., Samuel, D., et al. (2015). Donor recruitment for fecal microbiota transplantation. *Inflamm. Bowel Dis.* 21, 1600–1606. doi: 10.1097/MIB.0000000000000405

Paramsothy, S., Kamm, M. A., Kaakoush, N. O., Walsh, A. J., van den Bogaerde, J., Samuel, D., et al. (2017a). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 389, 1218–1228. doi: 10.1016/S0140-6736(17)30182-4

Paramsothy, S., Paramsothy, R., Rubin, D. T., Kamm, M. A., Kaakoush, N. O., Mitchell, H. M., et al. (2017b). Faecal microbiota transplantation for inflammatory bowel disease: a systematic review and meta-analysis. *J. Crohns Colitis* 11, 1180–1199. doi: 10.1093/ecco-jcc/jjx063

Pothoulakis, C. (2009). Review article: anti-inflammatory mechanisms of action of *Saccharomyces boulardii*. *Aliment Pharmacol. Ther.* 30, 826–833. doi: 10.1111/j.1365-2036.2009.04102.x

Qazi, T., Amaratunga, T., Barnes, E. L., Fischer, M., Kassam, Z., and Allegretti, J. R. (2017). The risk of inflammatory bowel disease flares after fecal microbiota transplantation: systematic review and meta-analysis. *Gut Microbes* 8, 574–588. doi: 10.1080/19490976.2017.1353848

Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., et al. (2004). Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 126, 520–528. doi: 10.1053/j.gastro.2003.11.019

Rangan, K. J., Pedicord, V. A., Wang, Y. C., Kim, B., Lu, Y., Shaham, S., et al. (2016). A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens. *Science* 353, 1434–1437. doi: 10.1126/science.aaf3552

Rios-Covian, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilan, C. G., and Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* 7:185. doi: 10.3389/fmicb.2016.00185

Riquelme, A. J., Calvo, M. A., Guzman, A. M., Depix, M. S., Garcia, P., Perez, C., et al. (2003). *Saccharomyces cerevisiae* fungemia after *Saccharomyces boulardii* treatment in immunocompromised patients. *J. Clin. Gastroenterol.* 36, 41–43. doi: 10.1097/00004836-200301000-00013

Rossen, N. G., Fuentes, S., van der Spek, M. J., Tijssen, J. G., Hartman, J. H., Duflou, A., et al. (2015). Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology* 149, 110–118.e4. doi: 10.1053/j.gastro.2015.03.045

Ruiz, F. O., Gerbaldo, G., Asurmendi, P., Pascual, L. M., Giordano, W., and Barberis, I. L. (2009). Antimicrobial activity, inhibition of urogenital pathogens, and synergistic interactions between lactobacillus strains. *Curr. Microbiol.* 59, 497–501. doi: 10.1007/s00284-009-9465-0

Saarela, M., Mogensen, G., Fonden, R., Matto, J., and Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* 84, 197–215. doi: 10.1016/S0168-1656(00)00375-8

Saez-Lara, M. J., Gomez-Llorente, C., Plaza-Diaz, J., and Gil, A. (2015). The role of probiotic lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. *Biomed. Res. Int.* 2015:505878. doi: 10.1155/2015/505878

Sales-Campos, H., Basso, P. J., Alves, V. B., Fonseca, M. T., Bonfa, G., Nardini, V., et al. (2015). Classical and recent advances in the treatment of inflammatory bowel diseases. *Braz. J. Med. Biol. Res.* 48, 96–107. doi: 10.1590/1414-431X20143774

Shah, R., Cope, J. L., Nagy-Szakal, D., Dowd, S., Versalovic, J., Hollister, E. B., et al. (2016). Composition and function of the pediatric colonic mucosal microbiome in untreated patients with ulcerative colitis. *Gut Microbes* 7, 384–396. doi: 10.1080/19490976.2016.1190073

Sokol, H., Leducq, V., Aschard, H., Pham, H. P., Jegou, S., Landman, C., et al. (2017). Fungal microbiota dysbiosis in IBD. *Gut* 66, 1039–1048. doi: 10.1136/gutjnl-2015-310746

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J. J., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16731–16736. doi: 10.1073/pnas.0804812105

Sokol, H., Seksik, P., Rigottier-Gois, L., Lay, C., Lepage, P., Podglajen, I., et al. (2006). Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm. Bowel Dis.* 12, 106–111. doi: 10.1097/01.MIB.0000200323.38139.c6

Sood, A., Midha, V., Makharia, G. K., Ahuja, V., Singal, D., Goswami, P., et al. (2009). The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin. Gastroenterol. Hepatol.* 7, 1202–1209.e1. doi: 10.1016/j.cgh.2009.07.016

Suskind, D. L., Brittnacher, M. J., Wahbeh, G., Shaffer, M. L., Hayden, H. S., Qin, X., et al. (2015). Fecal microbial transplant effect on clinical outcomes and fecal microbiome in active Crohn's disease. *Inflamm. Bowel Dis.* 21, 556–563. doi: 10.1097/MIB.0000000000000307

Tahara, T., Shibata, T., Kawamura, T., Okubo, M., Ichikawa, Y., Sumi, K., et al. (2015). Fusobacterium detected in colonic biopsy and clinicopathological features of ulcerative colitis in Japan. *Dig. Dis. Sci.* 60, 205–210. doi: 10.1007/s10620-014-3316-y

Tamaki, H., Nakase, H., Inoue, S., Kawanami, C., Itani, T., Ohana, M., et al. (2016). Efficacy of probiotic treatment with *Bifidobacterium longum* 536 for induction of remission in active ulcerative colitis: a randomized, double-blinded, placebo-controlled multicenter trial. *Dig. Endosc.* 28, 67–74. doi: 10.1111/den.12553

Thomsen, M., Clarke, S., and Vitetta, L. (2018). The role of adjuvant probiotics to attenuate intestinal inflammatory responses due to cancer treatments. *Benef. Microbes* doi: 10.3920/BM2017.0172 [Epub ahead of print].

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. (2007). The human microbiome project. *Nature* 449, 804–810. doi: 10.1038/nature06244

Tursi, A., Brandimarte, G., Papa, A., Giglio, A., Elisei, W., Giorgetti, G. M., et al. (2010). Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am. J. Gastroenterol.* 105, 2218–2227. doi: 10.1038/ajg.2010.218

van de Wouw, M., Boehme, M., Lyte, J. M., Wiley, N., Strain, C., O'Sullivan, O., et al. (2018). Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. *J. Physiol.* 596, 4923–4944. doi: 10.1113/JP276431

Vaughn, B. P., Vatanen, T., Allegretti, J. R., Bai, A., Xavier, R. J., Korzenik, J., et al. (2016). Increased intestinal microbial diversity following fecal microbiota transplant for active crohn's disease. *Inflamm. Bowel Dis.* 22, 2182–2190. doi: 10.1097/MIB.0000000000000893

Vrakas, S., Mountzouris, K. C., Michalopoulos, G., Karamanolis, G., Papatheodoridis, G., Tzathas, C., et al. (2017). Intestinal bacteria composition and translocation of bacteria in inflammatory bowel disease. *PLoS One* 12:e0170034. doi: 10.1371/journal.pone.0170034

Vrieze, A., Van Nood, E., Holleman, F., Salojarvi, J., Kootte, R. S., Bartelsman, J. F., et al. (2012). Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 143, 913–916.e7. doi: 10.1053/j.gastro.2012.06.031

Wen, L., and Duffy, A. (2017). Factors influencing the gut microbiota, inflammation, and type 2 diabetes. *J. Nutr.* 147, 1468S–1475S. doi: 10.3945/jn.116.240754

Xiao, L., Feng, Q., Liang, S., Sonne, S. B., Xia, Z., Qiu, X., et al. (2015). A catalog of the mouse gut metagenome. *Nat. Biotechnol.* 33, 1103–1108. doi: 10.1038/nbt.3353

Yoshimatsu, Y., Yamada, A., Furukawa, R., Sono, K., Osamura, A., Nakamura, K., et al. (2015). Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. *World J. Gastroenterol.* 21, 5985–5994. doi: 10.3748/wjg.v21.i19.5985

Zocco, M. A., dal Verme, L. Z., Cremonini, F., Piscaglia, A. C., Nista, E. C., Candelli, M., et al. (2006). Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol. Ther.* 23, 1567–1574. doi: 10.1111/j.1365-2036.2006.02927.x

Zuo, T., and Ng, S. C. (2018). The gut microbiota in the pathogenesis and therapeutics of inflammatory bowel disease. *Front. Microbiol.* 9:2247. doi: 10.3389/fmicb.2018.02247

Zyrek, A. A., Cichon, C., Helms, S., Enders, C., Sonnenborn, U., and Schmidt, M. A. (2007). Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol.* 9, 804–816. doi: 10.1111/j.1462-5822.2006.00836.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 01 February 2019
doi: 10.3389/fphar.2019.00039

Cellular Mechanisms of Etrolizumab Treatment in Inflammatory Bowel Disease

*Charlotte Lichnog[1], Sha Klabunde[2], Emily Becker[1], Franklin Fuh[2], Philipp Tripal[3], Raja Atreya[1], Entcho Klenske[1], Rich Erickson[4], Henry Chiu[5], Chae Reed[4], Shan Chung[4], Clemens Neufert[1], Imke Atreya[1], Jacqueline McBride[2], Markus F. Neurath[1] and Sebastian Zundler[1]**

[1] Department of Medicine 1, Friedrich-Alexander-Universität Erlangen-Nürnberg, Kussmaul Campus for Medical Research and Translational Research Center, Erlangen, Germany, [2] OMNI Biomarker Development, Development Sciences, Genentech, Inc., South San Francisco, CA, United States, [3] Optical Imaging Centre, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, [4] BioAnalytical Sciences, Development Sciences, Genentech, Inc., South San Francisco, CA, United States, [5] Biochemical and Cellular Pharmacology, Genentech, Inc., South San Francisco, CA, United States

Background: Anti-integrin therapy is a new frontline strategy in the treatment of inflammatory bowel diseases (IBD). The anti-β7 integrin antibody etrolizumab is currently being investigated for safety and efficacy in Crohn's disease (CD) and ulcerative colitis (UC) in several phase III trials. Mechanistically, etrolizumab is known to block β7 integrin ligand binding and reduces intestinal trafficking of β7-expressing cells. Etrolizumab blocks β7 integrin ligand binding and reduces β7-positive lymphocyte migration and retention in the inflamed gut mucosa, but the exact mechanisms by which this inhibition occurs are not fully understood.

Methods: Cellular effects of etrolizumab or etrolizumab surrogate antibody (etrolizumab-s) were investigated in cell culture models and analyzed by flow cytometry, fluorescence microscopy, ImageStream®, stimulated emission depletion (STED) microscopy and functional dynamic *in vitro* adhesion assays. Moreover, effects on α4β7 integrin were compared with the pharmacodynamically similar antibody vedolizumab.

Results: As demonstrated by several different approaches, etrolizumab and etrolizumab-s treatment led to internalization of β7 integrin. This resulted in impaired dynamic adhesion to MAdCAM-1. Internalized β7 integrin localized in endosomes and re-expression of β7 was dependent on *de novo* protein synthesis. *In vitro* etrolizumab treatment did not lead to cellular activation or cytokine secretion and did not induce cytotoxicity. Internalization of α4β7 integrin was increased with etrolizumab compared with vedolizumab.

Discussion: Our data suggest that etrolizumab does not elicit secondary effector functions on the single cell level. Integrin internalization may be an important mechanism of action of etrolizumab, which might explain some but not all immunological effects observed with etrolizumab.

Keywords: etrolizumab, inflammatory bowel diseases, internalization, STED microscopy, adhesion

OPEN ACCESS

Edited by:
Luca Antonioli,
University of Pisa, Italy

Reviewed by:
Cristina Giaroni,
University of Insubria, Italy
Maria Cecilia Giron,
University of Padova, Italy

****Correspondence:***
Sebastian Zundler
sebastian.zundler@uk-erlangen.de

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *25 October 2018*
Accepted: *14 January 2019*
Published: *01 February 2019*

Citation:
Lichnog C, Klabunde S, Becker E, Fuh F, Tripal P, Atreya R, Klenske E, Erickson R, Chiu H, Reed C, Chung S, Neufert C, Atreya I, McBride J, Neurath MF and Zundler S (2019) Cellular Mechanisms of Etrolizumab Treatment in Inflammatory Bowel Disease. Front. Pharmacol. 10:39. doi: 10.3389/fphar.2019.00039

INTRODUCTION

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC) are marked by intestinal immune cell infiltration leading to pro-inflammatory signaling and tissue destruction (Strober et al., 2007; Kaser et al., 2010; Neurath, 2014). Such cell accumulation in the gut is controlled by cell trafficking processes including gut homing and intestinal retention (Habtezion et al., 2016; Zundler and Neurath, 2017). Adhesion of lymphocytes dependent on activated α4β7 integrin to mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 expressed on high-endothelial venules in the gut has been identified as an important mechanism of gut homing (Berlin et al., 1993; Zundler et al., 2017a). The translational potential of this mechanism has been impressively demonstrated by the successful clinical implementation of inhibiting the α4β7 integrin by the monoclonal antibody vedolizumab as a therapeutic strategy in IBD (Feagan et al., 2013; Sandborn et al., 2013). In addition to α4, the β7 integrin monomer also pairs with αE to form the αEβ7 heterodimer, which has been shown to control epithelial retention of homed lymphocytes in intestinal inflammation (Cepek et al., 1994). The anti-β7 antibody etrolizumab is currently being investigated in several phase III trials in IBD patients and blocks both α4β7-mediated gut homing as well as αEβ7-controlled retention (Vermeire et al., 2014; Zundler et al., 2017c).

While these mechanisms have been proposed by cell trafficking studies (Zundler et al., 2017c), the molecular mechanisms responsible for the effect of etrolizumab have so far not been described. Here, we addressed the hypothesis derived from previous studies with vedolizumab (Wyant et al., 2013) that one mechanism of action of etrolizumab might be internalization of β7 integrin leading to unavailability of the integrin on the cell surface. We show with complementary techniques including molecular microscopy with stimulated emission depletion (STED) imaging and ImageStream® analyses that etrolizumab leads to internalization of α4β7 integrin, that this functionally impairs β7-dependent adhesion to MAdCAM-1, and that internalization of α4β7 integrin is higher with etrolizumab compared with vedolizumab.

MATERIALS AND METHODS

Patients With IBD

Peripheral blood was collected from patients with CD (n = 53) and UC (n = 44) following prior informed written consent at the Outpatient Department of the Medical Clinic 1 of the University Hospital Erlangen. Control blood was obtained from healthy donors (n = 27). Clinical data of blood donors are summarized in **Table 1**. Blood collection was approved by the Ethics committee of the Friedrich-Alexander University Erlangen-Nuremberg. For some experiments, peripheral blood samples were collected from an anonymous internal Genentech blood donor program of healthy volunteers.

Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation with either Lymphocyte Separation Buffer (Anprotec) or Ficoll Paque (GE). Where indicated, $CD4^+$ T cells were purified from PBMCs with CD4 microbeads (Miltenyi).

Etrolizumab Surrogate Antibody (Etrolizumab-s) Internalization

For assessment of etrolizumab-s internalization, PBMCs were treated with 10 μg/mL of the etrolizumab surrogate rat antibody FIB504 (Genentech) in phosphate buffered saline (PBS) or in RPMI 1640 medium (Thermo Fisher) with 1% penicillin/streptomycin (Biochrom) and 10% FCS (Pan Biotech) at 4°C and/or 37°C for 24 h. Etrolizumab-s is the parent antibody of etrolizumab (Stefanich et al., 2011). For some experiments, etrolizumab-s was labeled with AlexaFluor (AF) 647 with an AF647 labeling kit (Thermo Fisher) according to the manufacturer's instructions.

Acid wash was performed with a solution of 0,5M NaCl + 0,2M acetic acid as previously described (Wyant et al., 2013).

Assessment of Etrolizumab Internalization With ImageStream®

Peripheral blood mononuclear cells were treated with AF488-labeled etrolizumab (10 μg/mL) for 24 h at 37 or 4°C. Cells were washed and stained with CD4 and CD45RA, and subsequently with or without a quenching anti-AF488 antibody (addition of 25 μg to cell pellet on ice for 1 h, Thermo Fisher Scientific). Surface and intracellular fluorescence signals were recorded using the ImageStream®X Mark II Imaging Flow Cytometer (MilliporeSigma).

Flow Cytometry

The following antibodies were used for cell staining according to standard flow cytometry protocols: CD4 (VIT4, VioBlue/FITC, Miltenyi; RPA-T4, PE/V450, BD Biosciences), CD8 (RPA-T8, PE-Cy7, BD Biosciences), CD45RA (HI100, V500/APC/BV510, BD Biosciences/Biolegend), CD19 (SJ25C1, APC-Cy7, BD Biosciences), IgD (IA6-2, V450, BD Biosciences), CD25 (BC96, BV510, Biolegend), CD69 (FN50, APC/Cy7, Biolegend), CD103 (αE integrin) (Ber-ACT8, FITC/APC, BD Biosciences), IFN-γ (B27, PE/Cy7, Biolegend), IL-4 (8D4-8, AF488, Biolegend), IL-17A (BL168, PE, Biolegend), IL-9 (MH9A4, AF647, Biolegend), CD49d (α4) (9F10, FITC/APC, BD Biosciences), 7AAD (BD Biosciences). Additionally, etrolizumab, the anti-β7 integrin antibody FIB504 and the anti-α4β7 integrin antibody Act-1 (all Genentech) labeled with AF647 (AF647 labeling kit, Thermo Fisher) and etrolizumab and the anti-β7 integrin antibody 9D8 (Genentech) labeled with AF488 (AF488 labeling kit, Thermo Fisher) were used.

Where applicable, cells were fixed and permeabilized with the Foxp3 fixation and permeabilization kit (Thermo Fisher).

For analysis of cellular activation (Wyant et al., 2013) upon etrolizumab-s treatment, cells were treated with 1 μg/mL etrolizumab-s for 6 h. Untreated PBMCs cultured for 6 h were

TABLE 1 | Patient characteristics.

		CON	CD	UC
Number		27	53	44
Age [years] (Mean +/− SEM)		26 +/− 1	37 +/− 2	41 +/− 2
Female [%]		63	41	41
HBI (Mean +/− SEM)			4.0 +/− 0.4	
PMS (Mean +/− SEM)				1.7 +/− 0.3
Concomitant therapy [%]	Immunosuppressants		11.3	4.6
	Steroids		1.9	0
	Mesalazin		0	6.8
	Vedolizumab		0	0
	Anti-TNF antibodies		86.8	88.6
Localization [%]			L1: 17.0	E1: 18.6
			L2: 11.3	E2: 32.6
			L3: 41.5	E3: 48.8
			L4: 0	
			L4+: 30.2	

HBI, Harvey-Bradshaw Index; PMS, Partial Mayo Score.

used as negative control, while PBMCs treated with 50 ng/mL PMA (Sigma) and 1 μM ionomycin (Cayman) for 6 h served as positive control. After 2 h of culture, all cells were treated with 10 ng/μL Brefeldin A (Applichem) for the remaining 4 h.

For the analysis of β7 integrin re-expression, baseline expression of β7 was determined in PBMCs. Subsequently, these PBMCs were cultured in the presence of etrolizumab-s at 37°C for 24 h. Next, cells were harvested, washed and then re-seeded in cell culture plates for a further 96 h in the presence or absence of Brefeldin A. The time course of β7 integrin re-expression was determined by flow cytometric analysis of aliquots cells harvested at 24, 48, 72, and 120 h from baseline.

Flow cytometry was performed on MACSQuant X Analyzer (Miltenyi) and BD FACSCanto^{TM} II (BD Biosciences) instruments. Data were analyzed with BD FACSDiva Software v8.0. and FlowJo v7.6.5 and v10.1.

Comparison of Etrolizumab- and Vedolizumab-Induced α4β7 Internalization

Peripheral blood mononuclear cells were pre-incubated with saturating concentrations of unlabeled etrolizumab and vedolizumab at 4°C for 2 h. Cells were then washed and incubated at 4 or 37°C for 24 h prior to a subsequent wash and staining with the non-competing anti-β7 monoclonocal antibody 9D8-AF488. Surface β7 expression was assessed by flow cytometry in subsets of B and T lymphocytes.

Dynamic *in vitro* Adhesion Assay

Peripheral blood mononuclear cells were cultured for 24 h at 37°C in the presence or absence of etrolizumab-s. Next, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies). Suspensions of 1.5 million cells/mL in adhesion buffer (pH 7.4, 150 mM NaCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) were prepared and etrolizumab-s was added or not to aliquots of so far untreated cells. Capillaries for dynamic *in vitro* adhesion assays were prepared as previously described (Binder et al., 2018). In brief, miniature borosilicate capillaries (Vitrocom) were coated with 5 μg/mL rhMAdCAM-1-Fc-chimera (R&D Systems) in 150 mM NaCl with 10 mM HEPES for 1 h at 37°C. Next, unspecific binding sites were blocked with 5% bovine serum albumine (BSA) in phosphate buffered saline (PBS) for 1 h at 37°C.

Perfusion was performed with a peristaltic pump (Shenchen LabV3) at a flow rate of 10 μL/min. Dynamic adhesion was analyzed with time-lapse confocal microscopy (Leica SP8) over 3 min and analyzes with ImageJ (NIH) as previously described (Binder et al., 2018).

Immunofluorescence

Peripheral blood mononuclear cells were treated with AF647-labeled etrolizumab-s for 24 h at 37 or 4°C. In some experiments, cells were permeabilized with 0.1 % Triton X (Roth) after etrolizumab-s incubation and additionally stained with LAMP-1 (H4A3, AF488, Biolegend) or EEA (5632C2, AF488, Novus Bio) to visualize lysosomes and endosomes, respectively. Subsequently, cells were counterstained with Hoechst dye, suspended in Mowiol (Roth) and covered on microscopy slides. Analyses were performed with fluorescence microscopy (Leica DM6000B). Surface and intracellular fluorescence signals were quantified with ImageJ (NIH) by determining the mean fluorescence intensity (MFI) of regions of interest defined around or in projection to the nuclei, respectively.

STED-Microscopy

To increase the number of β7 integrin-expressing cells, PBMCs were stimulated with anti-CD3 (OKT3, eBioscience) and anti-CD28 antibodies (BE0248, inVivoMab) and additionally treated with 20 ng/mL TGF-β for 72 h as previously described (Zundler et al., 2017c).

Subsequently, such cells were treated with a mouse anti-human β7 antibody (473207, R&D systems) or with or without

etrolizumab-s at 37 or 4°C for 24 h. Where indicated, cells treated at 37°C were additionally permeabilized with 0.1% Triton X. Then, secondary staining was performed with goat anti-mouse antibodies and goat anti-rat antibodies labeled with the STED microscopy dye Star 580 (excitation: 594 nm pulsed laser, emission: 605–625 nm) or Star 635P, respectively (both Abberior, excitation: 640 nm pulsed laser, emission: 650–720 nm). Cell suspensions in Mowiol were covered on microscopy slides and analyzed with a STED microscope (Abberior 3D STED 2-Channel Super Resolution Microscope) equipped with a 100× Oil immersion lens (NA: 1.44). Stimulated emission depletion was performed at 775 nm with a pulsed laser. The power of the STED laser was set to 625 mW.

Antibody-Dependent Cytotoxicity (ADCC) Assay With PBMCs

Antibody-dependent cytotoxicity assays were carried out using PBMCs from healthy donors as effector cells and the human lymphoma cell line WIL2-S (ATCC) as target cells. Target cells (4×10^4) in 50 μL assay medium (RPMI-1640 with 1% BSA and 100 U/mL penicillin/streptomycin) were seeded in each well of a 96-well, round-bottom plate. Serial fourfold dilutions (1000 to 0.0038 ng/mL) of etrolizumab and the anti-CD20 antibody rituximab as a positive control (50 μL/well) were added to the plates containing the target cells, followed by incubation at 37°C for 30 min to allow opsonization. Subsequently, 10^6 PBMC effector cells in 100 μL of assay medium were added to each well and the plates were incubated for an additional 4 h. After centrifugation, the supernatants were assayed for lactate dehydrogenase (LDH) activity using a Cytotoxicity Detection Kit (Roche Diagnostics). Cell lysis was quantified through absorbance at 490 nm using a microplate reader. Absorbance of wells containing only the target cells served as Low Control and wells containing target cells lysed with Triton-X100 as High Control. Antibody-independent cellular cytotoxicity (AICC) was measured in wells containing target and effector cells without the addition of antibody. The extent of specific ADCC was calculated as follows:

$$\begin{aligned}\%\ \text{ADCC} = 100 \times (&A_{490\text{nm}}\left(\text{High Control}\right) \\ &- A_{490\text{nm}}\left(\text{Low Control}\right))/(A_{490\text{nm}}\left(\text{Sample}\right) \\ &- A_{490\text{nm}}\left(\text{AICC}\right))\end{aligned}$$

The mean ADCC values from duplicates were plotted against the antibody concentration, and the EC50 values were generated by fitting the data to a four-parameter equation with SoftMax Pro.

Complement-Dependent Cytotoxicity (CDC) Assay

The CDC assays were carried out using WIL2-S cells as target cells and complement derived from human serum (Quidel Corporation). Etrolizumab and the anti-CD20 antibody rituximab were serially diluted in assay medium (RPMI-1640 supplemented with 20 mM HEPES pH 7.2, 0.1% BSA, and 0.1 mg/mL gentamicin), and distributed into a 96-well tissue culture plate (Costar; Corning Inc.). Following the addition of human serum complement (diluted 1:3 in assay medium) and the target cells (10^5 cells/well), the plate was incubated 12 h at 37°C. After the incubation, AlamarBlue was added at 50 μL/well and the plate was incubated for an additional 15–18 h. The CDC of the test antibody was quantified through absorbance at 530 nm excitation with 590 nm emission on a fluorescence plate reader (SpectraMax GeminiXS, Molecular Devices). The EC50 values were generated by fitting the data to a four-parameter equation (SoftMax Pro).

Induction of Pro-inflammatory Cytokine Production

Etrolizumab was evaluated *in vitro* both as a single agent and in the presence of a sub-stimulatory concentration of anti-human CD3 (4 ng/ml) for induction of pro-inflammatory cytokine/chemokine production in purified human PBMCs. PBMCs were assayed in 96-well microtiter plates (either with or without anti-CD3 coating) with trastuzumab, mouse IgG1, or etrolizumab, in solution phase. Positive control wells were coated with both anti-CD3 (4 ng/ml) and anti-CD28 (100 ng/ml), whereas negative control wells remained uncoated. Duplicate wells without anti-CD3 were also assayed, to determine the capability of the solution-phase mAbs to stimulate cytokine/chemokine production in PBMC without a costimulatory signal. Each well contained 4×10^5 PBMC in 100 μL medium and plates were incubated at 37°C. Supernatants were collected following 24 and 48 h of culture and assayed by Luminex assays (Luminex Corp.) for cytokine and chemokine concentrations.

Statistics

Unless otherwise stated, data are displayed as mean with error bars representing the standard error of the mean. Statistical comparisons were performed with Graph Pad Prism software (Graph Pad Software) applying one- or two-way ANOVA as applicable. *Post hoc* testing was done with Newman-Keuls or Bonferroni tests, respectively. Levels of significance are indicated by asterisks ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

β7 Integrin Translocates From the Cell Surface to the Inside of the Cell Upon *in vitro* Treatment of Lymphocytes With Etrolizumab-s

To assess whether β7 integrin is internalized upon etrolizumab treatment, we isolated PBMCs from the peripheral blood of healthy controls and IBD patients and incubated these cells with or without etrolizumab-s *in vitro*. This was performed at 37°C to allow internalization or at 4°C to prevent internalization (Wyant et al., 2013). After 24 h, cells were stained for flow cytometry with an antibody panel including the anti-β7 antibody 9D8 which recognizes β7 in the presence of etrolizumab-s through binding to a different epitope (Stefanich et al., 2011). At 4°C, as expected due to the absence of internalization, no difference in the abundance

of $CD4^+9D8^+$ T cells could be observed between samples treated with or without etrolizumab-s (**Figure 1A**). At 37°C, however, the proportion of $CD4^+$ T cells staining positive for 9D8 was significantly lower after etrolizumab-s treatment compared with no treatment in control donors as well as IBD patients with CD or UC. This suggested that the β7 integrin was reduced on the cell surface following incubation with etrolizumab-s.

To investigate the fate of β7 after such treatment, we performed an additional series of experiments, in which PBMCs were incubated with AF647-labeled etrolizumab-s and stained for flow cytometry after 24 h. We observed that the proportion of etrolizumab-s^+9D8^- CD4 T cells was significantly higher after incubation at 37°C than at 4°C (**Figure 1B**). Therefore, we concluded that the fluorescence signal of etrolizumab-s must originate from the inside of the cells, since the 9D8 antibody was only able to bind surface-expressed β7 integrin. This was confirmed by another approach, in which we incubated PBMCs with AF647-labeled etrolizumab-s at 4 and 37°C for 24 h and applied an acid wash procedure afterwards to remove surface-bound antibody. Unsurprisingly, the substantial proportion of etrolizumab-s^+ cells that could be observed without acid wash treatment at 4°C was almost completely lost, when acid wash was applied (**Figure 1C**). In contrast, a considerable amount of etrolizumab-s^+ cells was observed at 37°C even when acid wash was performed, which similarly argued for an intracellular origin of the fluorescence signal. Thus, taken together, these results supported the conclusion that etrolizumab-s leads to internalization of β7 integrin.

β7 Integrin Is Internalized Following Treatment of Lymphocytes With Etrolizumab

We therefore aimed to complementarily explore the internalization process with fluorescence microscopy. Accordingly, we treated PBMCs from the peripheral blood of controls and IBD patients with AF647-labeled etrolizumab-s at 4 and 37°C for 24 h. After counterstaining with Hoechst, cells were evaluated by fluorescence microscopy. Following incubation at 4°C, we observed a halo of etrolizumab-s fluorescence signal around the cell membrane in a portion of cells consistent with β7 expression on the cell surface (**Figure 2A**). In contrast, the signal observed in cells treated at 37°C was shifted to the inside of the cells. When quantifying the fluorescence signal in the latter compared with the former location by measuring the MFI for AF647, we found a highly significant difference in the ratio of surface and intracellular fluorescence intensity. Consistent with the flow cytometry results, these immunofluorescence images provided further evidence of β7 internalization after etrolizumab-s binding.

Subsequently, we explored whether these observations with etrolizumab-s also applied to the humanized therapeutic antibody etrolizumab. In an approach combining flow cytometry with fluorescence microscopy we analyzed internalization of β7 integrin following treatment with AF488-labeled etrolizumab at 4 and 37°C for 24 h with and without additional anti-AF488 quench (**Figure 2B**). When incubated at 4°C, as expected, superficial AF488 signal could be observed without, but not with quench procedure. At 37°C, however, microscopy demonstrated only minimal superficial AF488 staining that was removed by the quench procedure, but substantial intracellular signal that was not affected by quenching of AF488. Consistently, flow cytometry demonstrated $AF488^+$ cells without and with quench at 37°C.

Additionally, we performed dual staining with AF647-labeled etrolizumab-s and the lysosome marker LAMP-1 or the endosome marker EEA to assess the intracellular location of etrolizumab-s after internalization (**Figure 2C**). At 37°C, but not at 4°C co-localization of both EEA-1 and LAMP-1 with etrolizumab-s could be observed suggesting internalization of the antigen-antibody complex into early endosomes and late endosomes/lysosomes, respectively.

To evaluate the internalization of β7 in even greater detail and on single-molecule level, we made use of STED microscopy, an innovative technology recently introduced to overcome the resolution limitations of conventional microscopic techniques (Blom and Widengren, 2017). Initially, we assessed the expression of β7 integrin induced by TGF-β on PBMCs from IBD patients and controls. Interestingly, β7 expression was not homogenously distributed around the cells, but confined to certain spots (**Figure 3A**). Subsequently, cells were treated with etrolizumab-s at 4 and 37°C for 24 h and secondary staining with Star635P-labeled anti-rat antibodies was performed. After treatment at 4°C, as anticipated, we observed a fraction of cells with a positive signal, which had a "halo" location consistent with cell surface expression of β7 integrin (**Figure 3B**). However, no such signal could be observed on cells treated at 37°C, presumably due to internalization of β7 and, thus, unavailability of etrolizumab-s on the cell surface for secondary staining. To directly demonstrate internalization, we additionally permeabilized cells with Triton-X prior to addition of the secondary antibody. Indeed, this treatment led to detection of a positive signal in projection to the nucleus or directly adjacent, therefore indicating internalized β7 integrin.

Etrolizumab-Driven α4β7 Integrin Internalization Functionally Leads to Decreased Dynamic Adhesion to MAdCAM-1

Next, we sought to determine whether internalization of β7 following etrolizumab treatment equally affects internalization of the β7 integrin monomer paired with α4 or αE. As demonstrated by flow cytometry, we observed a loss of α4β7 following treatment with etrolizumab *in vitro* at 37°C compared with 4°C (**Figure 4A**), although the number of $\alpha4^+$ cells was not substantially affected. Similarly, the abundance of αE remained unchanged, although the MFI of αE^+ cells significantly decreased after treatment at 37°C (**Figure 4B**). Together, these findings suggested that etrolizumab predominantly induces monomer internalization leading to absence of surface α4β7, and possibly also partial co-internalization of αE.

To investigate what α4β7 internalization functionally means for interaction with MAdCAM-1, we used a dynamic adhesion assay to study integrin-addressin interactions under shear stress

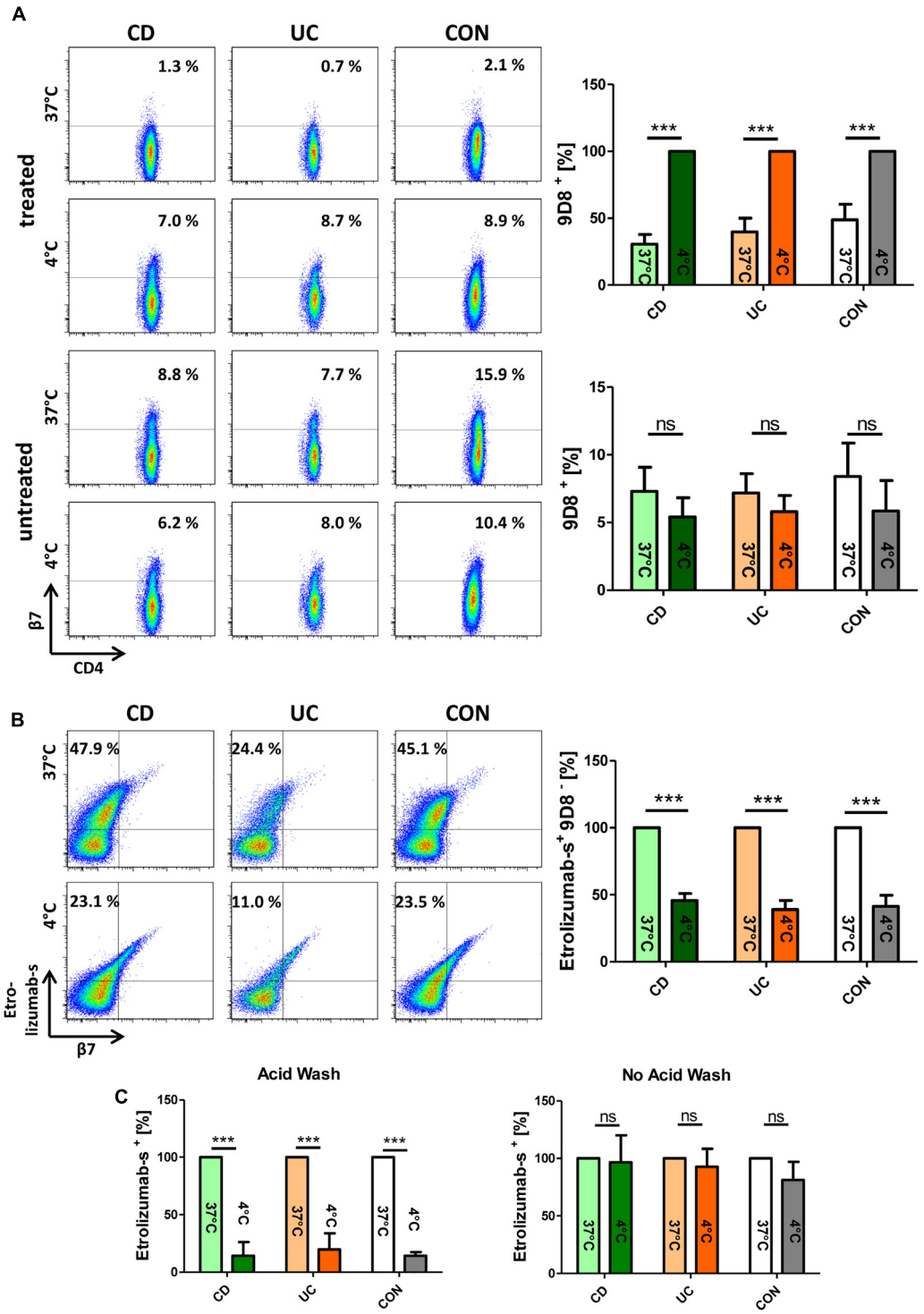

FIGURE 1 | β7 integrin translocates from the cell surface to the inside of the cell upon *in vitro* treatment of lymphocytes with etrolizumab-s. **(A)** Upper panels: Flow cytometry of peripheral blood mononuclear cells (PBMCs) from patients with Crohn's disease (CD), ulcerative colitis (UC), and control donors (CON) treated with etrolizumab-s for 24 h at 4 and 37°C. Left: Representative flow cytometry. The frequency of $9D8^+$ staining on $CD4^+$ T cells is indicated. Right: Quantitative flow cytometry showing surface expression of 9D8 at 37°C relative to expression at 4°C (n = 5 per group). Lower panels: Representative (left) and quantitative flow cytometry (right) of cells cultured at 4 and 37°C without etrolizumab-s treatment. **(B)** Flow cytometry of PBMCs from CD, UC, and CON donors treated with AF-647-labeled etrolizumab-s. Left: Representative flow cytometry. The frequency of etrolizumab-s^+9D8^- cells among $CD4^+$ T cells is indicated. Right: Quantitative flow cytometry showing the proportion of these cells at 4°C in relation to 37°C (n = 5 per group). **(C)** Quantitative flow cytometry of PBMCs treated with AF647-labeled etrolizumab-s at 4 or 37°C and subsequently subjected to acid wash (left) or not (right). n = 5 per group; data are normalized to expression at 37°C. ***$p < 0.001$; ns – not significant.

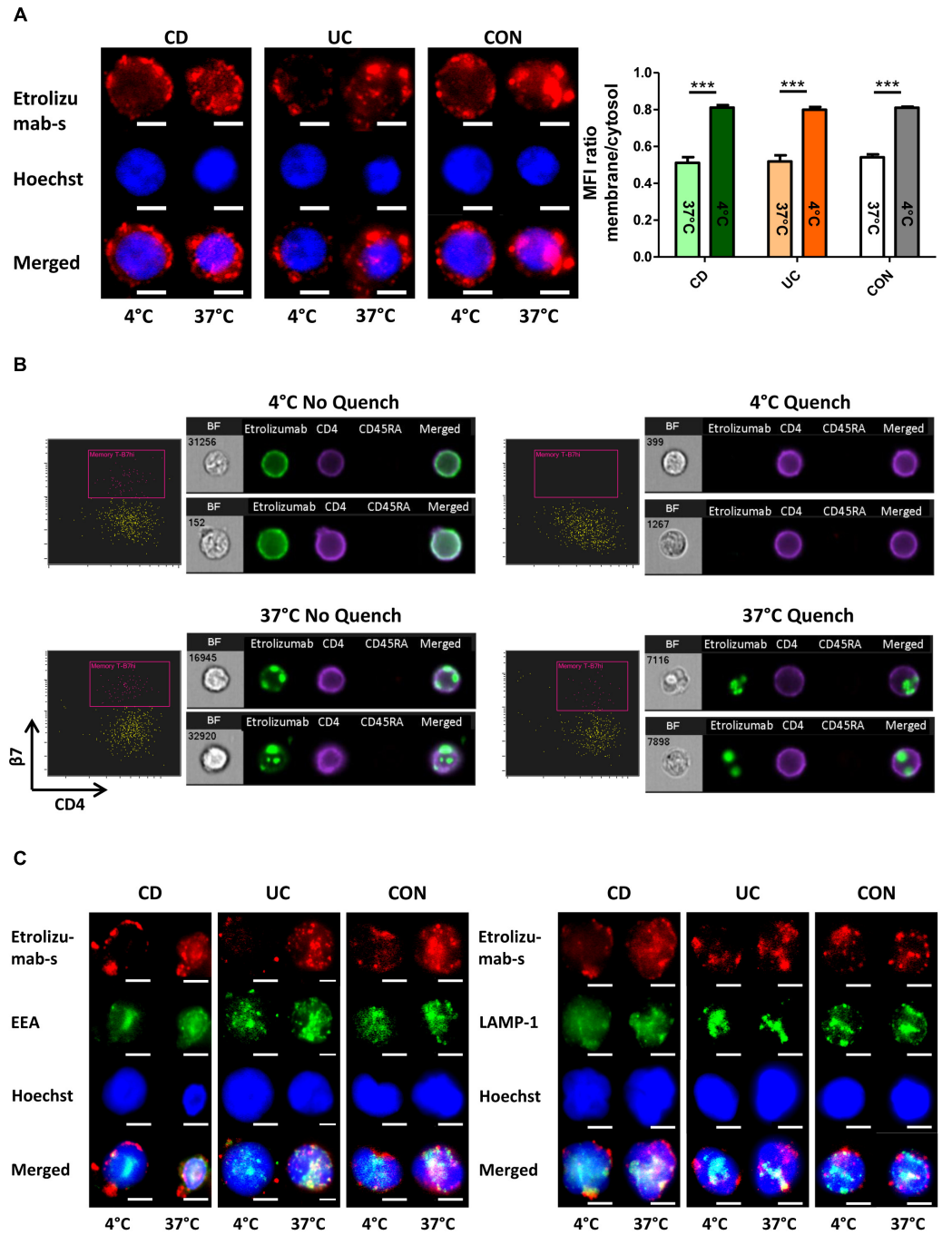

FIGURE 2 | β7 integrin is internalized by treatment of lymphocytes with etrolizumab(-s). **(A)** Left: Representative images showing localization of the fluorescence signal of AF647-labeled etrolizumab-s after treatment of cells from CD, UC, and CON donors at 4 or 37°C for 24 h. Scale bar: 3 μm. Right: Quantification of mean fluorescence intensity (MFI) of AF647 signal in the cytosol relative to the membrane at 4 and 37°C; $n = 5$ per group. ***$p < 0.001$. **(B)** Assessment of etrolizumab internalization with ImageStream®. Representative flow cytometry and microscopy results after cell incubation with AF488-labeled etrolizumab at 4°C (upper row) or 37°C (lower row) and without (left panels) or with (right panels) quench procedure. BF, bright field. Data are representative for two independent experiments with a total of eight samples. **(C)** Representative images showing localization of AF647 fluorescence signal of etrolizumab-s and AF488 fluorescence signal of EEA (left) and LAMP-1 (right) after cell treatment with etrolizumab-s at 4 or 37°C for 24 h. Scale bar = 3 μm. Images are representative for three independent experiments.

(Binder et al., 2018). PBMCs were isolated from the peripheral blood of control donors and IBD patients and incubated with or without etrolizumab-s at 37°C for 24 h to induce internalization of β7 integrin or not, respectively. Subsequently, treated cells were extensively washed to remove any remaining antibody from the cell suspension. Previously untreated cells were either

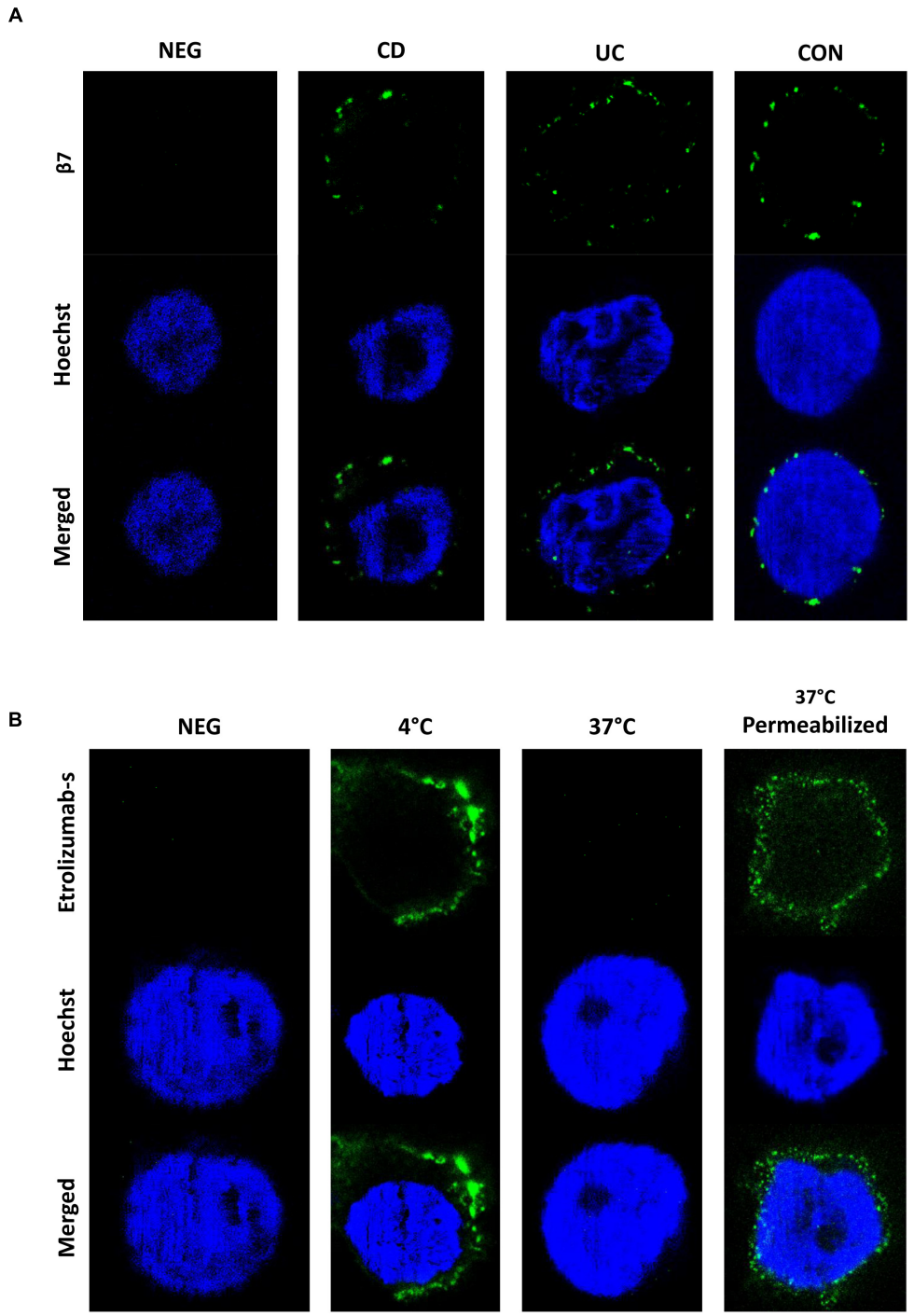

FIGURE 3 | Assessment of etrolizumab-s internalization by STED microscopy. **(A)** Representative STED microscopy images showing localization and distribution of β7 integrin on the surface of cells from CD, UC, and CON donors. Additionally, a negative control (NEG) without primary antibody staining is shown. Images are representative for seven independent experiments. **(B)** Representative images showing etrolizumab-s localization on/in cells incubated with etrolizumab-s at 4°C or cells incubated with etrolizumab-s at 37°C and additionally treated with or without Triton-X. Additionally, a negative control without primary antibody staining is shown. Images are representative for five independent experiments.

left untreated or treated with etrolizumab-s directly prior to perfusion through MAdCAM-1-coated ultrathin glass capillaries in the presence of etrolizumab-s. Substantial adhesion could be observed, when completely untreated cells were perfused. However, when cells treated with etrolizumab-s for 24 h and cells concomitantly treated with etrolizumab-s were used, dynamic

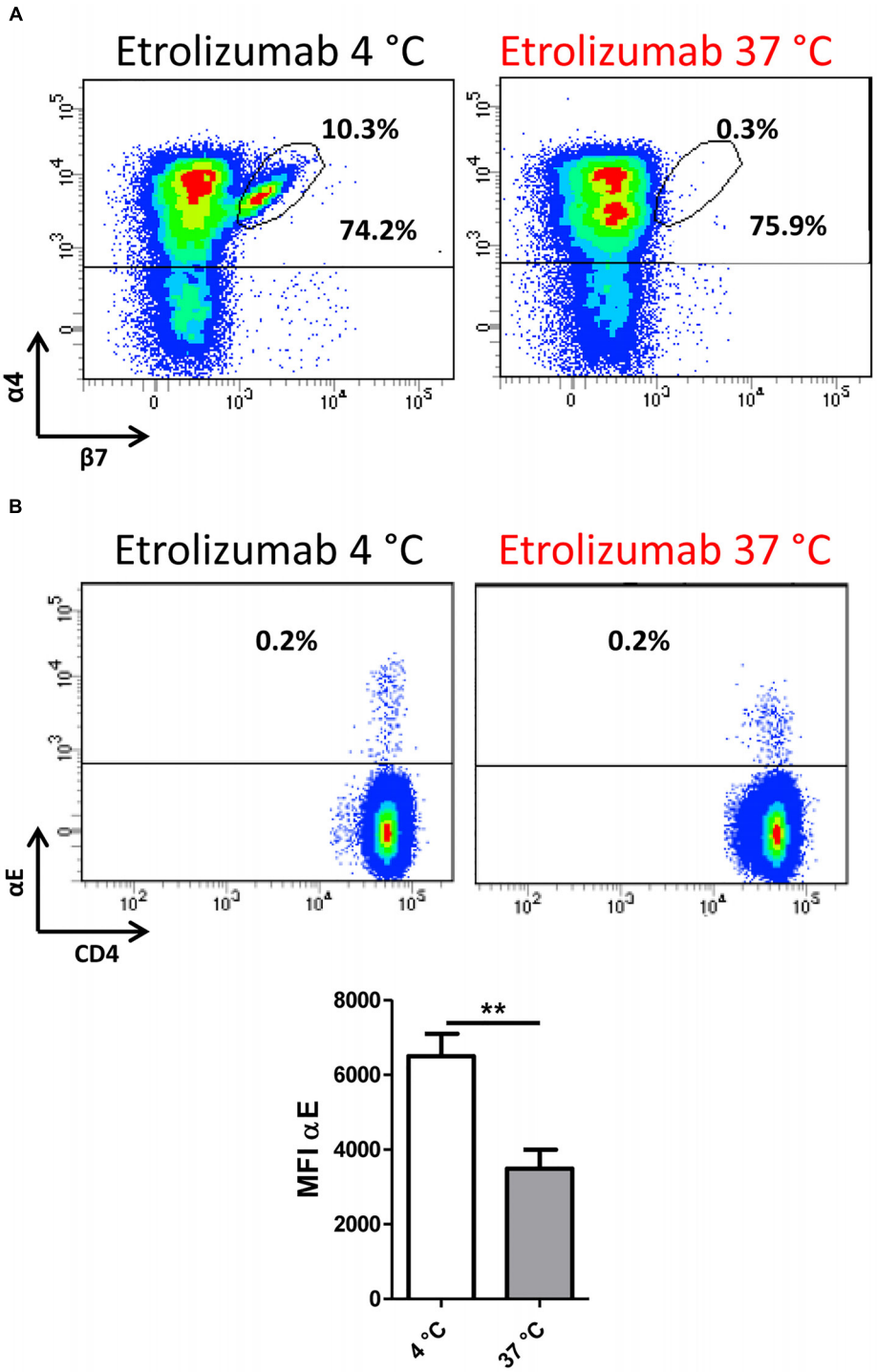

FIGURE 4 | β7 internalization minimizes α4β7 expression on the cell surface. **(A)** Representative flow cytometry plot showing expression of α4β7 integrin after treatment with etrolizumab at 4 and 37°C. Percentages indicate $\alpha4^{+}\beta7^{+}$ and total $\alpha4^{+}$ cells. **(B)** Upper panels: Representative flow cytometry plots showing expression of αE integrin after treatment with etrolizumab at 4 and 37°C. Lower panel: Quantification of β7 mean fluorescence intensity (MFI) of $\beta7^{+}$ cells ($n = 5$). $^{**}p < 0.01$.

adhesion was markedly and similarly reduced (**Figure 5**). Thus, internalization of β7 integrin following incubation with etrolizumab-s led to a decrease in dynamic adhesion to MAdCAM-1 even when no etrolizumab-s was present during perfusion and this effect was comparable to that observed with concomitant treatment. These results indicated that β7 integrin

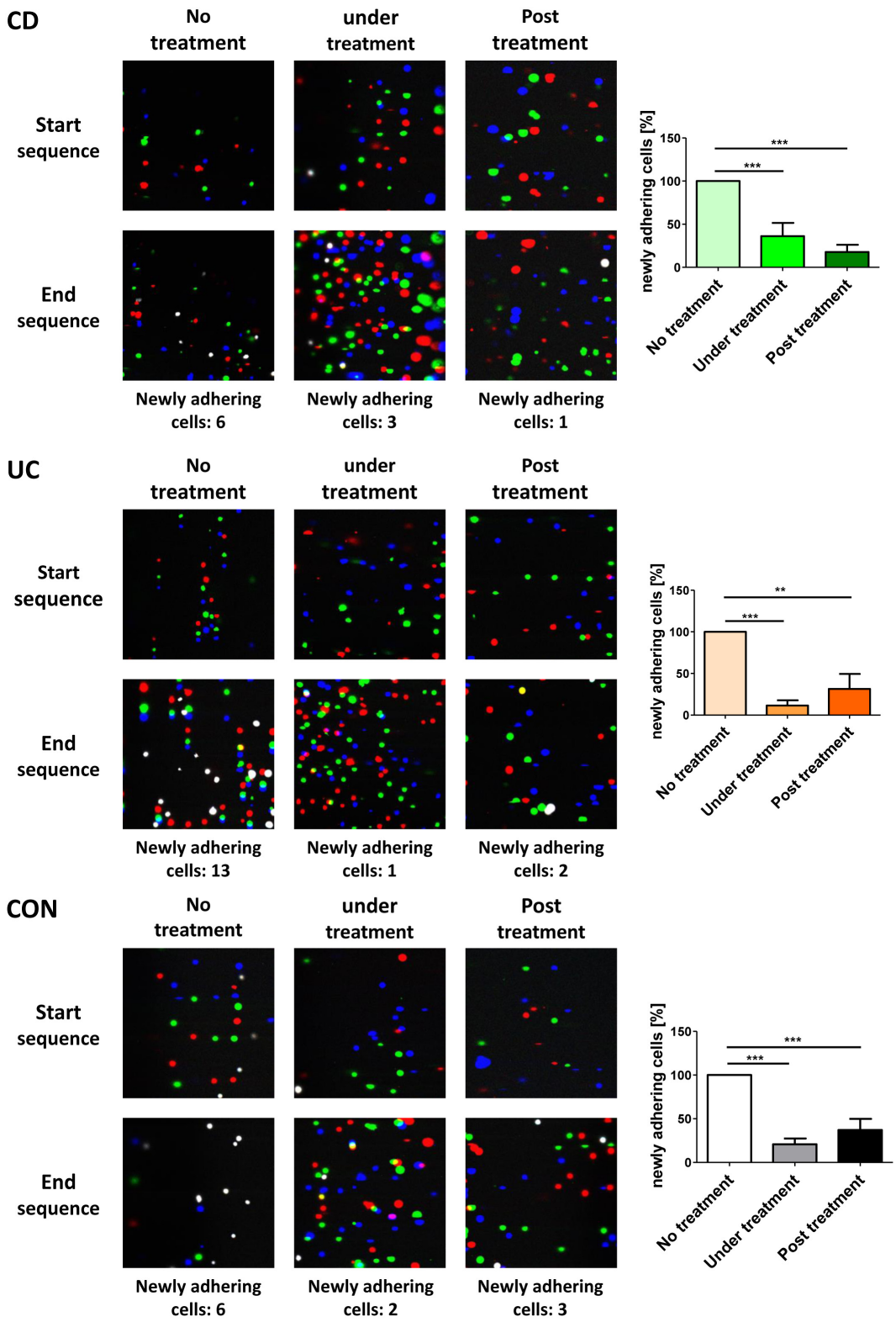

FIGURE 5 | Internalization of β7 integrin functionally leads to decreased dynamic adhesion to MAdCAM-1. Dynamic adhesion assays with untreated PBMCs, PBMCs pre-treated with etrolizumab-s for 24 h or treated with etrolizumab-s during the assay. Left panels: Representative overlays of three differentially colored sequential images collected at the beginning or end of 3 min clips. White cells represent adhering cells. Right panels: Quantitative data normalized to untreated PBMCs (CD $n = 8$, UC $n = 5$, and CON $n = 5$). $^{**}p < 0.01$, $^{***}p < 0.001$.

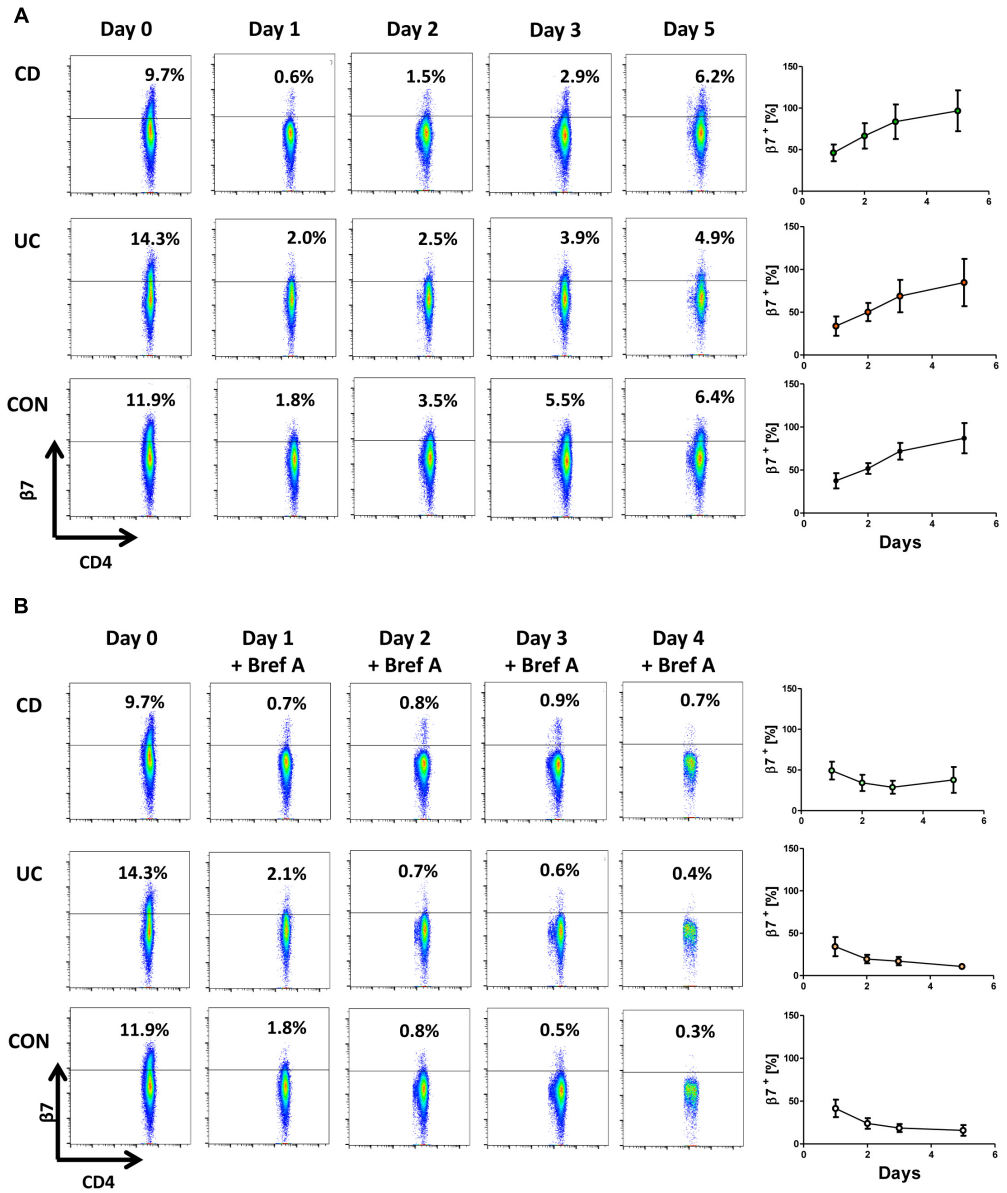

FIGURE 6 | Cell surface expression of β7 integrin is restored after removal of etrolizumab-s. **(A)** Left panels: Representative flow cytometry showing β7 integrin expression at baseline (day 0) and 24 (day 1), 48 (day 2), 72 (day 3) and 120 h (day 5) after treatment with etrolizumab-s for the first 24 h of the experiment. Right panels: Quantitative flow cytometry of β7 surface expression over time relative to day 0 (n = 5–6 per group). **(B)** Left panels: Representative flow cytometry showing β7 integrin expression at baseline (day 0), and 24 (day 1), 48 (day 2), 72 (day 3) and 120 h (day 5) after treatment with etrolizumab-s for the first 24 h of the experiment and treatment with Brefeldin A from day 1 to day 5. Right panels: Quantitative flow cytometry of β7 surface expression over time relative to day 0 (n = 5–6 per group).

internalization induced by etrolizumab is functionally important for the impairment of interactions with MAdCAM-1.

Cell Surface Expression of β7 Integrin Is Restored After Removal of Etrolizumab-s

We then assessed whether β7 integrin is re-expressed on the cell surface after treatment with etrolizumab-s. To analyze this, PBMCs from the peripheral blood were incubated with etrolizumab-s for 24 h and aliquots of the cells were used to determine β7 integrin expression on $CD4^+$ T cells with the 9D8 antibody (labeled with AF488) before and after treatment to confirm downregulation of surface β7 expression (**Figure 6A**). Subsequently, cells were washed to remove excess etrolizumab-s and further cultured for additional 96 h. Cell surface expression of β7 integrin was analyzed after 24, 48, and 96 h and was found to gradually increase until almost reaching pre-treatment levels after 4 days.

To address whether this was due to recycling of internalized β7 integrin or resulting from *de novo* synthesis, we performed

an additional series of experiments, in which etrolizumab-s treatment was followed by application of Brefeldin A to inhibit Golgi transport of freshly translated β7 protein. During such incubation, surface β7 integrin expression persisted on the levels observed directly after treatment with etrolizumab-s (**Figure 6B**). Together, these results suggested that internalized β7-etrolizumab-s aggregates are degraded and β7 expression on the cell surface after removal of etrolizumab-s is restored due to *de novo*-synthesis of β7 integrin.

Etrolizumab-s Does Not Elicit Agonistic Activity

Since monoclonal therapeutic antibodies can potentially have agonistic activity (Winkler et al., 1999), we addressed the expression of activation markers following incubation with etrolizumab-s for six and 24 h. Cells treated with PMA and ionomycin served as positive, untreated cells as negative control. As expected, stimulation with PMA/ionomycin caused a clear upregulation of CD69 on $CD4^+$ T cells after six and 24 h (**Figure 7A**). In contrast, and in accordance with the literature (Wyant et al., 2013), CD25 was only modestly upregulated after six, but markedly increased after 24 h. No induction of CD69 or CD25 could be observed in untreated samples and, similarly, the expression in etrolizumab-s-treated samples was unchanged. These data indicated that binding of etrolizumab-s to β7 integrin does not lead to cell activation.

In a next step, we investigated whether etrolizumab-s has a direct effect on the expression of pro-inflammatory cytokines. Cells were left untreated or treated with etrolizumab-s or PMA/ionomycin for 2 h. Subsequently, Brefeldin A was added for additional 4 h and the expression of cytokines was assessed by flow cytometry. While increased expression of IFN-γ, IL-4, IL-9, and IL-17A could be observed in PMA/ionomycin-treated $CD4^+$ T cells (**Figure 7B**), only very low and comparable production was found in untreated cells and etrolizumab-s-treated cells.

Additionally, we assessed the concentration of several cytokines and chemokines in supernatants of PBMCs following treatment with etrolizumab (**Figure 8A** and **Supplementary Figure S1**). Consistently, cytokine and chemokine levels observed after etrolizumab treatment were similar to negative controls.

As monoclonal antibodies may also induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), we performed ADCC and CDC assays with etrolizumab using the human lymphoma cell line WIL2-S. The anti-CD20 antibody rituximab served as positive control. While substantial ADCC and CDC were observed with rituximab, neither was observed with etrolizumab (**Figure 8B**).

Etrolizumab Is More Effective Than Vedolizumab in Inducing β7 Internalization

The anti-α4β7 antibody vedolizumab and etrolizumab both target the α4β7 integrin and internalization of α4β7 integrin in response to vedolizumab incubation has previously been described (Wyant et al., 2013). Therefore, we aimed to compare the efficacy of both compounds in inducing β7 integrin internalization.

To this end, PBMCs were treated with vedolizumab or etrolizumab at 4°C and 37°C and surface β7 expression was analyzed in T and B cells. Consistent with our previous findings, etrolizumab treatment led to a clear reduction of β7 on all subsets studied (**Figures 9A,B**). Vedolizumab treatment also led to a decrease in expression and fluorescence intensity of surface β7, but this was clearly less marked than the decrease seen after etrolizumab treatment. Importantly, vedolizumab almost completely prevented binding of Act-1 (**Supplementary Figure S2**) indicating full saturation of α4β7 integrin.

DISCUSSION

Anti-integrin therapy has successfully been established as a new concept in the treatment of IBDs (Lobatón et al., 2014; Zundler et al., 2017a). Mechanistically, it is believed that targeted inhibition of the interaction of integrins with respective ligands by monoclonal antibodies leads to impaired cell trafficking and subsequent reduction of pro-inflammatory cell infiltration in the gut (Habtezion et al., 2016; Zundler and Neurath, 2017). However, beyond such mechanisms on the tissue level and regarding cell trafficking, the mode of action of these anti-integrin agents on their integrin target and the cellular consequences of antibody targeting are only partly understood. Here, we investigated the fate of β7 integrin upon binding of both etrolizumab-s and etrolizumab, which is currently being investigated in phase III studies in IBD. Etrolizumab is directly derived from the etrolizumab surrogate rat antibody FIB504 (Andrew et al., 1994) that was used in a part of the studies and has comparable binding properties (Stefanich et al., 2011). Consistently, the effects observed with etrolizumab-s paralleled the effects of etrolizumab, the humanized antibody.

On a single cell level, antibody binding to a target molecule may induce several cellular effects such as induction or blockage of cell activation and consecutive cytokine release. A prominent example for this effect is the anti-CD28 antibody TGN1412, which caused a cytokine storm with severe clinical consequences in participants of a phase I study (Suntharalingam et al., 2006). Increased cytokine secretion has also been observed in patients treated with the anti-CD20 antibody rituximab (Winkler et al., 1999), particularly in individuals with high peripheral cell numbers, and following treatment with the anti-CD3 antibody OKT3 (Gaston et al., 1991). Regarding etrolizumab, our *in vitro* data demonstrate that it does not cause upregulation of cell activation markers like CD25 and CD69, and that cytokine secretion by $CD4^+$ T cells is also unaffected. Thus, agonist activity of etrolizumab on cell activation is highly unlikely. It is important to mention that these observations do not exclude effects of etrolizumab on cytokine expression on the tissue level as suggested by the decline reported in previous studies (Vermeire et al., 2014; Tew et al., 2016). This might be a secondary effect due to altered cell trafficking and reduced numbers of pro-inflammatory cells in the tissue.

Another potential mechanism mediated by therapeutic antibodies is the induction of ADCC or CDC as observed with the above mentioned antibodies rituximab and

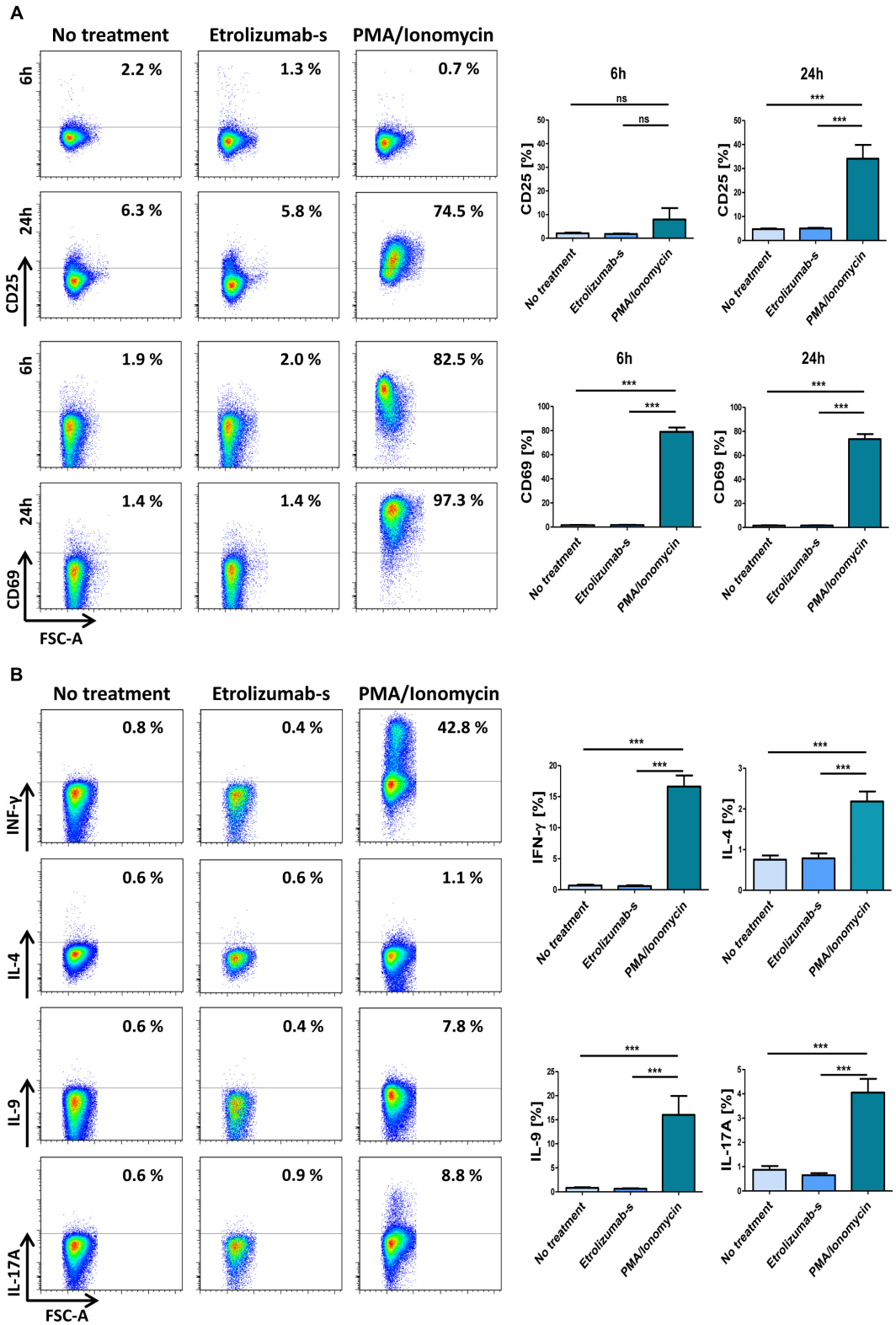

FIGURE 7 | Etrolizumab-s does not elicit agonistic activity and cytokine production. **(A)** Left panels: Representative flow cytometry showing expression of CD25 (upper panels) and CD69 (lower panels) after cell treatment with or without PMA/ionomycin and etrolizumab-s for 6 or 24 h. Right panels: Quantitative flow cytometry (n = 11–26). **(B)** Left panels: Representative flow cytometry showing expression of pro-inflammatory cytokines after cell treatment with or without PMA/ionomycin and etrolizumab-s for 6 h. Right panels: Quantitative flow cytometry (n = 21–23 per group). ***$p < 0.001$; ns – not significant.

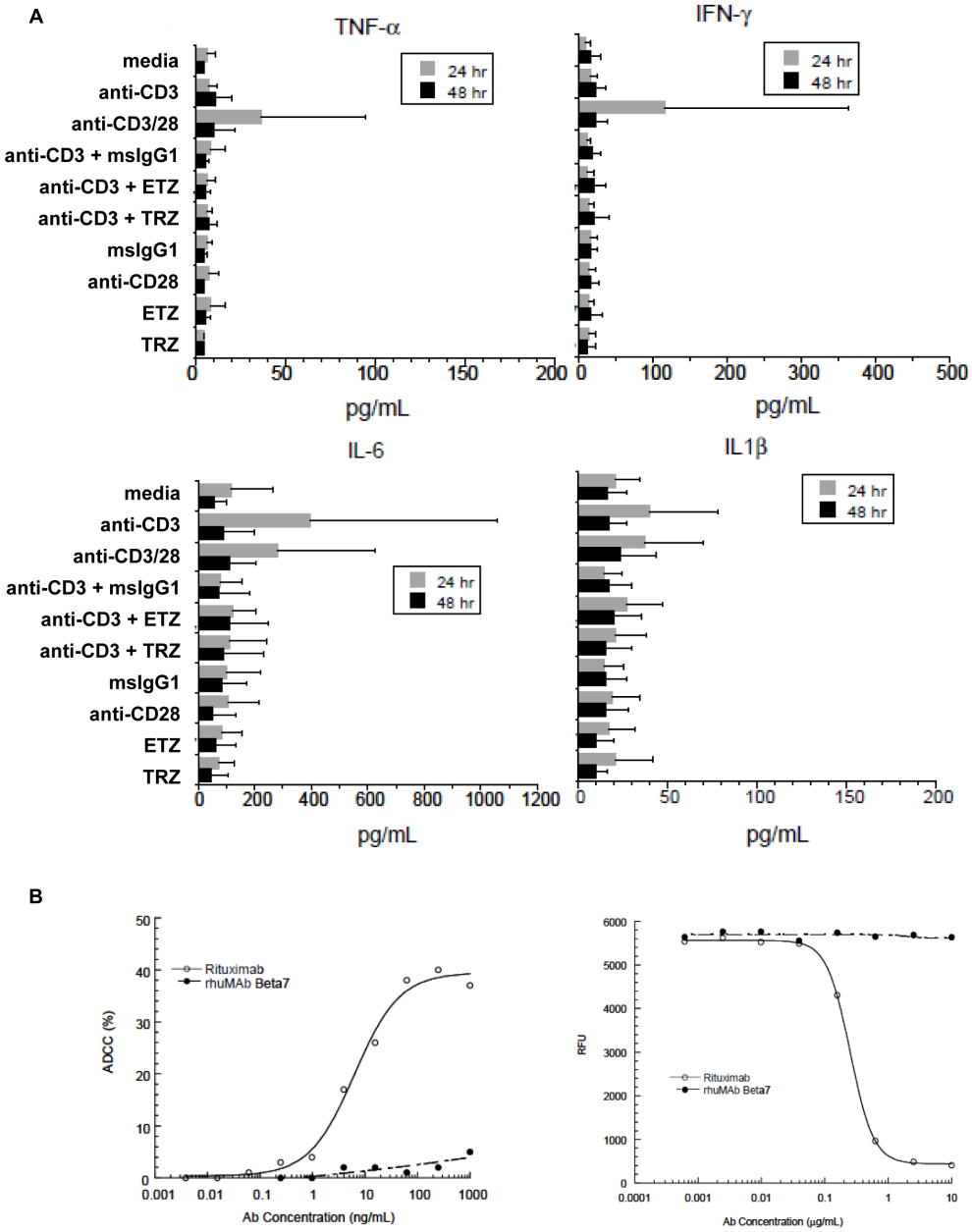

FIGURE 8 | Etrolizumab does not elicit cytokine production and cytotoxicity. **(A)** Concentration of cytokines in supernatants of human PBMCs incubated with different antibodies with or without stimulation with anti-CD3 after 24 and 48 h. *n* = 6, mean +/– SD. **(B)** Left panel: ADCC activity of different concentrations of etrolizumab (rhuMAb Beta7) and rituximab in WIL2-S cells quantified as specified in Methods. Right panel: CDC assay with different concentrations of etrolizumab and rituximab in WIL2-S cells as specified in Methods. Relative fluorescence units (RFU) indicate the number of viable cells. Data are representative for three independent experiments.

OKT3 (Raasveld et al., 1993; Golay et al., 2002). Both effects are mediated by the Fc fragment of the antibodies (Vidarsson et al., 2014). Our data suggest that etrolizumab does not elicit cytotoxicity via either mechanism. The observations reported in preclinical models and clinical studies, where the numbers of peripheral blood lymphocytes were stable or even increasing (Stefanich et al., 2011; Vermeire et al., 2014) under treatment, indicate that this is also not the case *in vivo*.

Internalization of the antibody-antigen complex into the cell is frequently observed in response to antibody treatment (Harper et al., 2013; de Goeij et al., 2014; Xu, 2015). We therefore hypothesized that this might also be true for etrolizumab. Indeed, our microscopic and flow cytometric data strongly support the conclusion that this is case, since, upon treatment with etrolizumab or etrolizumab-s, β7 could be detected intracellularly and reduced presence of β7 integrin on the cell surface was

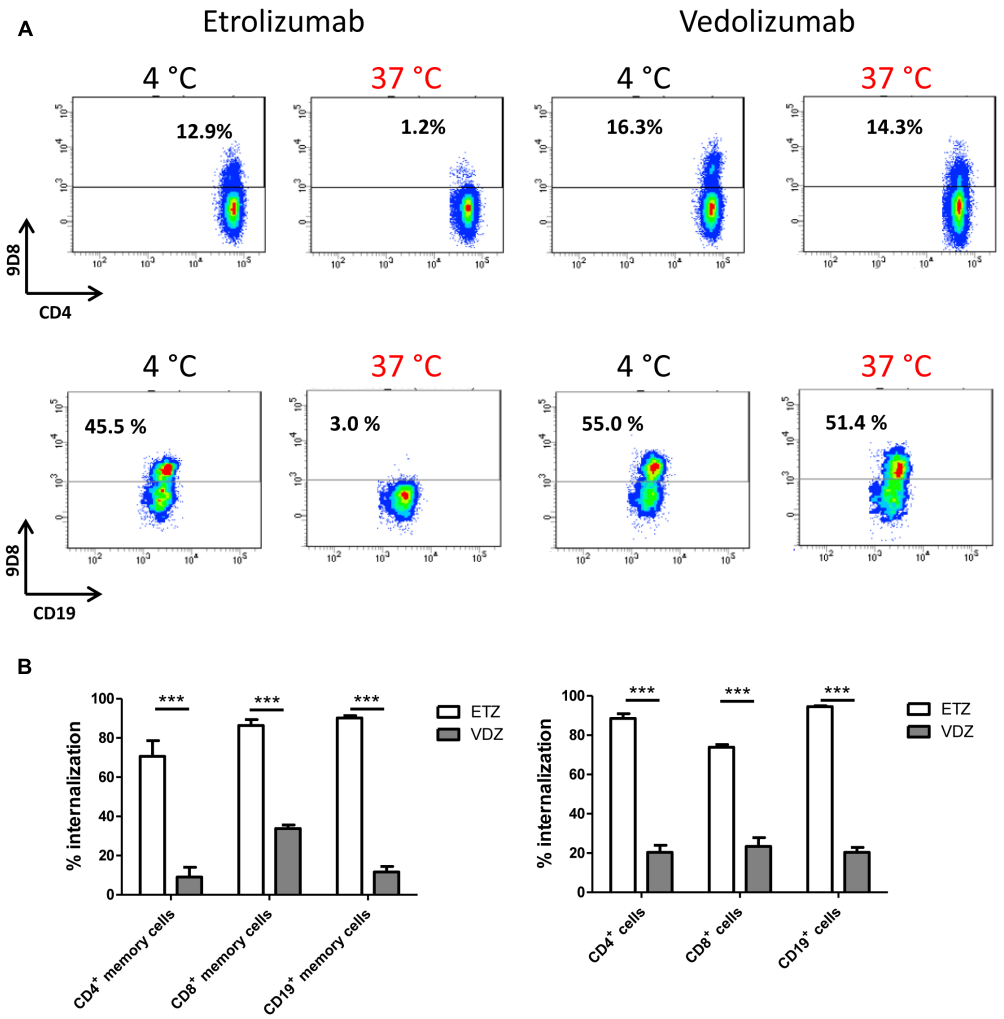

FIGURE 9 | Comparison of β7 internalization after treatment with vedolizumab and etrolizumab *in vitro*. **(A)** Internalization of β7 on $CD4^+$ T and $CD19^+$ B cells from the peripheral blood of human donors. Representative dot plots indicating the percentage of $β7^+$ cells upon treatment with etrolizumab or vedolizumab at 4 or 37°C as indicated. **(B)** Quantification of $β7^+$ internalization (decrease of surface β7 expression related to expression observed after treatment at 4°C) in T and B cell subsets treated with etrolizumab or vedolizumab as indicated. Memory cells were defined as $CD45RA^-$. All analyses were performed on $β7^{high}$ cells (n = 5 donors). ETZ, etrolizumab; VDZ, vedolizumab; n = 10. ***$p < 0.001$.

observed. This was finally confirmed by molecular microscopy using STED imaging, where β7 integrin vanished from the cell surface after etrolizumab-s treatment, but could be detected inside the cells after permeabilization. Thus, internalization seems to be a common feature of integrin ligation by neutralizing antibodies and even natural ligands. E.g., a similar mechanism has been reported for the anti-α4β7 antibody vedolizumab (Wyant et al., 2013) and the anti-rat α4 antibody TA2 (Leone et al., 2003). Moreover, it has been shown that engagement of α4β1 with its endothelial ligand vascular adhesion molecule (VCAM)-1 leads to internalization of the addressin (Ricard et al., 1998).

Using dynamic adhesion assays, we could additionally demonstrate that β7 internalization upon etrolizumab-s treatment has functional consequences and decreases adhesion to MAdCAM-1. Thus, receptor internalization might in fact explain the pre-clinical and clinical effects of etrolizumab (Stefanich et al., 2011; Vermeire et al., 2014).

However, compared to the *in vitro* results, the *in vivo* situation seems to be more complex. As demonstrated by the recent phase II trial with etrolizumab in UC (Vermeire et al., 2014), there was no apparent loss of β7 expression on the cell surface relative to baseline. Our data show that after removal of etrolizumab-s from cell cultures, β7 integrin is newly expressed within few days. Thus, surface-expressed β7 in etrolizumab-treated patients might at least partly result from *de novo* synthesis of the integrin, as it is highly likely that internalization and re-expression occur in parallel *in vivo*. In the light of recent studies suggesting compensatory mechanisms in response to anti-integrin therapies (Fuchs et al., 2017; Zundler et al., 2017b), it is also tempting to speculate that anti-integrin antibody treatment might trigger a

compensatory increase in *de novo* integrin expression. Moreover, it has to be taken into account that anti-β7 treatment is believed to lead to an increased number of target cells in the peripheral blood due to impairment of gut homing (Vermeire et al., 2014; Binder et al., 2018). Thus, in synopsis, in addition to blockade, it is likely that internalization is one of several mechanisms of actions of etrolizumab *in vitro* and *in vivo*.

Our data are also in line with and a potential explanation for the favorable safety profile observed with etrolizumab and gut-specific anti-adhesion therapies in general so far (Lin et al., 2015; Luthra et al., 2015), since they suggest the absence of effector properties affecting key cellular functions other than β7 blockade (Liu et al., 2008).

In a final series of experiments, we compared the efficacy of β7 internalization after treatment with vedolizumab and etrolizumab *in vitro*. Our data indicate that etrolizumab is more effective in this regard and, thus, suggest that these anti-integrin antibodies do not only differ in regard to the overall mechanism of action (Zundler et al., 2017c), but may also act differently on a cellular level. E.g., it seems possible that different mechanisms of endocytosis apply. Although functional short-term effects on α4β7-dependent adhesion to MAdCAM-1 were similar for both antibodies (Binder et al., 2018), differential internalization properties might lead to differences in the efficacy of α4β7 blockade in the longer term.

Taken together, our data suggest that antibody-antigen complex internalization may be an important mechanism of action of etrolizumab and might help us better understand the clinical effects and the safety profile of etrolizumab in IBD.

AUTHOR CONTRIBUTIONS

CL, SK, EB, FF, HC, CR, and SC performed the experiments. RA, EK, CN, IA, MN, and SZ provided clinical samples, protocols, reagents, or designed the experiments. CL, SK, EB, FF, RE, SC, JM, MN, and SZ analyzed and interpreted the data. CL and SZ drafted the manuscript. All authors critically revised the manuscript for important intellectual content.

FUNDING

This research was funded by Hoffmann-La Roche. CL was supported by a Peter Scriba scholarship of the German Society of Internal Medicine (DGIM).

ACKNOWLEDGMENTS

RA, CN, IA, MN, and SZ acknowledge support of their research by the ELAN program and the Interdisciplinary Center for Clinical Research (IZKF) of the University Erlangen-Nuremberg, the Else Kröner-Fresenius-Stiftung, the Fritz-Bender-Stiftung, the Litwin IBD Pioneers Program of the Crohn's and Colitis Foundation of America (CCFA), the German Crohn's and Colitis Association (DCCV), the German Research Foundation (DFG), the DFG topic program on Microbiota, the Emerging Field Initiative and the DFG Collaborative Research Centers 643, 796, 1181, and TRR241. The present work was performed in fulfillment of the requirements for obtaining the degree "Dr. med." for CL. We thank Julia Derdau and Julia Schuster for excellent technical support. Additionally, we also thank the Optical Imaging Center Erlangen (OICE) for excellent scientific and technical support. We also acknowledge support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding program Open Access Publishing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00039/full#supplementary-material

FIGURE S1 | Concentration of cytokines and chemokines in supernatants of human PBMCs incubated with different antibodies with or without stimulation with anti-CD3 after 24 and 48 h.

FIGURE S2 | Incubation with vedolizumab hinders binding of Act-1. Representative dot plots showing binding of AF647-labeled Act-1 to PBMCs treated with (left) or without (right) vedolizumab.

REFERENCES

Andrew, D. P., Berlin, C., Honda, S., Yoshino, T., Hamann, A., Holzmann, B., et al. (1994). Distinct but overlapping epitopes are involved in alpha 4 beta 7-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *J. Immunol.* 153, 3847–3861.

Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D. P., Kilshaw, P. J., Holzmann, B., et al. (1993). Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 74, 185–195.

Binder, M.-T., Becker, E., Wiendl, M., Schleier, L., Fuchs, F., Leppkes, M., et al. (2018). Similar inhibition of dynamic adhesion of lymphocytes from IBD patients to MAdCAM-1 by vedolizumab and etrolizumab-s. *Inflamm. Bowel Dis.* 24, 1237–1250. doi: 10.1093/ibd/izy077

Blom, H., and Widengren, J. (2017). Stimulated emission depletion microscopy. *Chem. Rev.* 117, 7377–7427. doi: 10.1021/acs.chemrev.6b00653

Cepek, K. L., Shaw, S. K., Parker, C. M., Russell, G. J., Morrow, J. S., Rimm, D. L., et al. (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 372, 190–193. doi: 10.1038/372190a0

de Goeij, B. E., Peipp, M., de Haij, S., van den Brink, E. N., Kellner, C., Riedl, T., et al. (2014). HER2 monoclonal antibodies that do not interfere with receptor heterodimerization-mediated signaling induce effective internalization and represent valuable components for rational antibody-drug conjugate design. *mAbs* 6, 392–402. doi: 10.4161/mabs.27705

Feagan, B. G., Rutgeerts, P., Sands, B. E., Hanauer, S., Colombel, J.-F., Sandborn, W. J., et al. (2013). Vedolizumab as induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 369, 699–710. doi: 10.1056/NEJMoa1215734

Fuchs, F., Schillinger, D., Atreya, R., Hirschmann, S., Fischer, S., Neufert, C., et al. (2017). Clinical response to vedolizumab in ulcerative colitis patients is associated with changes in integrin expression profiles. *Front. Immunol.* 8:764. doi: 10.3389/fimmu.2017.00764

Gaston, R. S., Deierhoi, M. H., Patterson, T., Prasthofer, E., Julian, B. A., Barber, W. H., et al. (1991). OKT3 first-dose reaction: association with T cell subsets and cytokine release. *Kidney Int.* 39, 141–148.

Golay, J., Gramigna, R., Facchinetti, V., Capello, D., Gaidano, G., and Introna, M. (2002). Acquired immunodeficiency syndrome-associated lymphomas are efficiently lysed through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity by rituximab. *Br. J. Haematol.* 119, 923–929.

Habtezion, A., Nguyen, L. P., Hadeiba, H., and Butcher, E. C. (2016). Leukocyte trafficking to the small intestine and colon. *Gastroenterology* 150, 340–354. doi: 10.1053/j.gastro.2015.10.046

Harper, J., Mao, S., Strout, P., and Kamal, A. (2013). Selecting an optimal antibody for antibody-drug conjugate therapy: internalization and intracellular localization. *Methods Mol. Biol.* 1045, 41–49. doi: 10.1007/978-1-62703-541-5_3

Kaser, A., Zeissig, S., and Blumberg, R. S. (2010). Inflammatory bowel disease. *Annu. Rev. Immunol.* 28, 573–621. doi: 10.1146/annurev-immunol-030409-101225

Leone, D. R., Giza, K., Gill, A., Dolinski, B. M., Yang, W., Perper, S., et al. (2003). An assessment of the mechanistic differences between two integrin alpha 4 beta 1 inhibitors, the monoclonal antibody TA-2 and the small molecule BIO5192, in rat experimental autoimmune encephalomyelitis. *J. Pharmacol. Exp. Ther.* 305, 1150–1162. doi: 10.1124/jpet.102.047332

Lin, L., Liu, X., Wang, D., and Zheng, C. (2015). Efficacy and safety of antiintegrin antibody for inflammatory bowel disease: a systematic review and meta-analysis. *Medicine* 94:e556. doi: 10.1097/MD.0000000000000556

Liu, X., Pop, L. M., and Vitetta, E. S. (2008). Engineering therapeutic monoclonal antibodies. *Immunol. Rev.* 222, 9–27. doi: 10.1111/j.1600-065X.2008.00601.x

Lobatón, T., Vermeire, S., Van Assche, G., and Rutgeerts, P. (2014). Review article: anti-adhesion therapies for inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 39, 579–594. doi: 10.1111/apt.12639

Luthra, P., Peyrin-Biroulet, L., and Ford, A. C. (2015). Systematic review and meta-analysis: opportunistic infections and malignancies during treatment with anti-integrin antibodies in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 41, 1227–1236. doi: 10.1111/apt.13215

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* 14, 329–342. doi: 10.1038/nri3661

Raasveld, M. H., Bemelman, F. J., Schellekens, P. T., van Diepen, F. N., van Dongen, A., van Royen, E. A., et al. (1993). Complement activation during OKT3 treatment: a possible explanation for respiratory side effects. *Kidney Int.* 43, 1140–1149.

Ricard, I., Payet, M. D., and Dupuis, G. (1998). VCAM-1 is internalized by a clathrin-related pathway in human endothelial cells but its alpha 4 beta 1 integrin counter-receptor remains associated with the plasma membrane in human T lymphocytes. *Eur. J. Immunol.* 28, 1708–1718.

Sandborn, W. J., Feagan, B. G., Rutgeerts, P., Hanauer, S., Colombel, J.-F., Sands, B. E., et al. (2013). Vedolizumab as induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* 369, 711–721. doi: 10.1056/NEJMoa1215739

Stefanich, E., Danilenko, D., Wang, H., O'Byrne, S., Erickson, R., Gelzleichter, T., et al. (2011). A humanized monoclonal antibody targeting the β7 integrin selectively blocks intestinal homing of T lymphocytes. *Br. J. Pharmacol.* 162, 1855–1870. doi: 10.1111/j.1476-5381.2011.01205.x

Strober, W., Fuss, I., and Mannon, P. (2007). The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* 117, 514–521. doi: 10.1172/JCI30587

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., et al. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N. Engl. J. Med.* 355, 1018–1028. doi: 10.1056/NEJMoa063842

Tew, G. W., Hackney, J. A., Gibbons, D., Lamb, C. A., Luca, D., Egen, J. G., et al. (2016). Association between response to etrolizumab and expression of integrin αE and granzyme A in colon biopsies of patients with ulcerative colitis. *Gastroenterology* 150, 477.e9–487.e9. doi: 10.1053/j.gastro.2015.10.041

Vermeire, S., O'Byrne, S., Keir, M., Williams, M., Lu, T. T., Mansfield, J. C., et al. (2014). Etrolizumab as induction therapy for ulcerative colitis: a randomised, controlled, phase 2 trial. *Lancet* 384, 309–318. doi: 10.1016/S0140-6736(14)60661-9

Vidarsson, G., Dekkers, G., and Rispens, T. (2014). IgG subclasses and allotypes: from structure to effector functions. *Front. Immunol.* 5:520. doi: 10.3389/fimmu.2014.00520

Winkler, U., Jensen, M., Manzke, O., Schulz, H., Diehl, V., and Engert, A. (1999). Cytokine-release syndrome in patients with B-cell chronic lymphocytic leukemia and high lymphocyte counts after treatment with an anti-CD20 monoclonal antibody (rituximab, IDEC-C2B8). *Blood* 94, 2217–2224.

Wyant, T., Yang, L., and Fedyk, E. (2013). In vitro assessment of the effects of vedolizumab binding on peripheral blood lymphocytes. *mAbs* 5, 842–850. doi: 10.4161/mabs.26392

Xu, S. (2015). Internalization, trafficking, intracellular processing and actions of antibody-drug conjugates. *Pharm. Res.* 32, 3577–3583. doi: 10.1007/s11095-015-1729-8

Zundler, S., Becker, E., Weidinger, C., and Siegmund, B. (2017a). Anti-Adhesion therapies in inflammatory bowel disease-molecular and clinical aspects. *Front. Immunol.* 8:891. doi: 10.3389/fimmu.2017.00891

Zundler, S., Fischer, A., Schillinger, D., Binder, M.-T., Atreya, R., Rath, T., et al. (2017b). The α4β1 homing pathway is essential for ileal homing of Crohn's disease effector T cells in vivo. *Inflamm. Bowel Dis.* 23, 379–391. doi: 10.1097/MIB.0000000000001029

Zundler, S., and Neurath, M. F. (2017). Novel insights into the mechanisms of gut homing and antiadhesion therapies in inflammatory bowel diseases. *Inflamm. Bowel Dis.* 23, 617–627. doi: 10.1097/MIB.0000000000001067

Zundler, S., Schillinger, D., Fischer, A., Atreya, R., López-Posadas, R., Watson, A., et al. (2017c). Blockade of αEβ7 integrin suppresses accumulation of CD8(+) and Th9 lymphocytes from patients with IBD in the inflamed gut in vivo. *Gut* 66, 1936–1948. doi: 10.1136/gutjnl-2016-312439

Conflict of Interest Statement: SK, FF, RE, CR, HC, SC, and JM are employees of Genentech Inc. MN has served as an advisor for Pentax, Giuliani, MSD, Abbvie, Janssen, Takeda, and Boehringer. SZ and MN received research support from Takeda and Hoffmann-La Roche.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 08 February 2019
doi: 10.3389/fphar.2019.00026

Hsp60 as a Novel Target in IBD Management: A Prospect

Francesco Cappello[1,2,3], Margherita Mazzola[1], Abdo Jurjus[4], Marie-Noel Zeenny[4], Rosalyn Jurjus[5], Francesco Carini[1], Angelo Leone[1], Giuseppe Bonaventura[1], Giovanni Tomasello[1], Fabio Bucchieri[1], Everly Conway de Macario[6] and Alberto J. L. Macario[2,6]*

[1] Department of Experimental Biomedicine and Clinical Neuroscience, University of Palermo (BIONEC-UniPA), Palermo, Italy, [2] Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy, [3] Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA, United States, [4] Department of Anatomy, Cell Biology and Physiology, American University of Beirut, Beirut, Lebanon, [5] Department of Anatomy and Cell Biology, Faculty Development Associate for Education Research, Center for Faculty Excellence, The George Washington University School of Medicine and Health Sciences, Washington, DC, United States, [6] Department of Microbiology and Immunology, School of Medicine, University of Maryland at Baltimore – Institute of Marine and Environmental Technology (IMET), Baltimore, MD, United States

Inflammatory bowel disease (IBD) encompasses various pathological conditions similar but distinct that share a multifactorial etiology, including involvement of the intestinal barrier function, the immune system, and intestinal microorganisms. Hsp60 is a chaperonin component of the chaperoning system, present in all cells and tissues, including the intestine. It plays important roles in cell physiology outside and inside mitochondria, its canonical place of residence. However, Hsp60 can also be pathogenic in many conditions, the Hsp60 chaperonopathies, possibly including IBD. The various clinico-pathological types of IBD have a complicated mix of causative factors, among which Hsp60 can be considered a putatively important driver of events and could play an etiopathogenic role. This possibility is discussed in this review. We also indicate that Hsp60 can be a biomarker useful in disease diagnosing and monitoring and, if found active in pathogenesis, should become a target for developing new therapies. The latter are particularly needed to alleviate patient suffering and to prevent complications, including colon cancer.

Keywords: intestinal wall, microbiota, Hsp60, immune system, chaperoning system, inflammatory bowel disease, chaperonopathy, chaperonotherapy

OPEN ACCESS

Edited by:
Matteo Fornai,
University of Pisa, Italy

Reviewed by:
Maria Cecilia Giron,
University of Padova, Italy
Dapeng Chen,
Dalian Medical University, China

****Correspondence:***
Francesco Cappello
francapp@hotmail.com

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *08 September 2018*
Accepted: *10 January 2019*
Published: *08 February 2019*

Citation:
Cappello F, Mazzola M, Jurjus A, Zeenny M-N, Jurjus R, Carini F, Leone A, Bonaventura G, Tomasello G, Bucchieri F, Conway de Macario E and Macario AJL (2019) Hsp60 as a Novel Target in IBD Management: A Prospect. Front. Pharmacol. 10:26. doi: 10.3389/fphar.2019.00026

THE BOWEL AND ITS INFLAMMATORY DISEASES: A BRIEF OVERVIEW

The intestinal tube is one of the first anatomical structures to form in the embryo, because of the immediate nutritional needs of embryonic cells, and from the intestinal tube other structures originate, such as exocrine and endocrine glands, airways, etc. (Chin et al., 2017). At the end of organogenesis, the intestinal wall is composed of multiple layers, classically described, from the inside, i.e., the lumen, to the outside, as mucosa, submucosa, *muscularis propria* and adventitia (or serosa, when the peritoneum is present). However, this description does not take into account the fact that, in living subjects, the most internal lining of the intestinal lumen consists of the mucus. This is produced by epithelial cells of the mucosa and contains about 100 billion microbial cells encompassing more than 10,000 different species, collectively called the intestinal microbiota.

For this reason, we consider the most internal lining of the intestinal wall in the living organism to be the mucous-microbiota layer. We propose to name this layer "MuMi layer" on account of its two major components, namely the mucous and the microbiota, (**Figure 1A**). This functional layer has a loose (as compared with the other intestinal layers) and changeable structure mostly provided by the biofilms formed by bacteria, archaea, and micro-eukaryotes that constitute the microbiota. However, this layer is not visible under routine histologic examination; it is lost during the processing of the tissue for microscope observation due to the solubility of the mucous in alcoholic solutions. Consequently, this internal lining is systematically missed in histological studies and has, generally, been ignored or forgotten despite its key role in intestinal physiology and pathology.

The intestinal tube establishes multiple relationships with other anatomical districts both through visceral innervation and microvesicular trafficking. Examples of microvesicles are exosomes and outer membrane vesicles produced, respectively, by human and microbial cells. These vesicles can reach through the bloodstream virtually any anatomical districts, including some "protected sanctuaries" such as the brain, testicles, and thymus (Yáñez-Mó et al., 2015). In addition, intestinal mucosa cells produce and release soluble factors with autocrine- and paracrine-like properties with some reaching the general circulation and having systemic effects. This complex homeostasis is subverted in some intestinal pathological disorders, for example in inflammatory bowel disease (IBD).

Inflammatory bowel disease comprises chronic inflammatory pathologies of the intestinal tract, whose etiology is not yet fully understood (Shouval and Rufo, 2017). The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). These two conditions share many clinical and pathological characteristics and are often considered together. However, they present different symptoms and clinical features, reflecting differences in the site of inflammation and in the type of immunological mechanisms involved in the pathogenesis that are characteristic of each of them (Gecse and Vermeire, 2018).

Inflammatory bowel diseases are considered multifactorial diseases triggered by an array of different factors, including immunological and intestinal barrier dysfunction as well as microorganisms (Nagao-Kitamoto et al., 2016; Bernstein and Forbes, 2017; Lanis et al., 2017; Shouval and Rufo, 2017; Gecse and Vermeire, 2018; Yu, 2018). Among these factors, the most prominent are: (i) imbalance of luminal mucosal homeostasis and induction of intestinal inflammation linked to environmental stimuli in genetically susceptible subjects; (ii) altered balance between regulatory mediators of inflammation, such as IFNγ and TNFα, that contribute to an inappropriate and sustained inflammatory response. Noteworthy, the high expression of pro-inflammatory cytokines and the increase of inflammatory cells can, in the long run, contribute to the formation of a microenvironment that supports the growth and proliferation of cancer cells and, for this reason, colorectal cancer (CRC) appears as one of the most serious complications of IBD; and (iii) loss of intestinal mucosal integrity. Consequently, intestinal dysbiosis occurs. This phenomenon is caused by the alteration in the composition of the intestinal microbiota due to exogenous (diet, smoking, antibiotics) and endogenous (psycho-physical stress) factors, **Figure 1**.

It is clear that IBDs have multiple etiopathogenic mechanisms. This complexity in etiology and pathogenic mechanisms, along with the need to design an optimal patient-customized therapy, generates an urgent need for exploring new alternatives. Here, we introduce the chaperonin Hsp60 as a candidate for investigation toward elucidating the pathogenic mechanisms of IBDs.

The reasons to focus on Hsp60 as biomarker useful in patient management and as a potential pathogenic agent in IBD are varied, as follows: (1) Hsp60 can induce production of pro-inflammatory cytokines (Sangiorgi et al., 2017; Cheong et al., 2018; Sun et al., 2018; Swaroop et al., 2018); (2) its quantities in UC mucosa vary in parallel with disease status, high in relapse and low in remission (Rodolico et al., 2010; Tomasello et al., 2011b); (3) its reported role in other conditions with inflammatory component, for instance atherosclerosis (Grundtman et al., 2011; Wick et al., 2014; Wick, 2016; Rahman et al., 2017); (4) there is ample epitope sharing between Hsp60s of various origins and human Hsp60 and other tissues (including intestinal ones). This molecular mimicry (Bachmaier and Penninger, 2005) elicits crossreactive antibodies reacting not-only with the immunizing antigen (e.g., Hsp60 from a bacterium infecting the intestinal or the genitourinary tract), but also with human Hsp60 and intestinal antigens (Füst et al., 2012). Autoimmunity due to molecular mimicry involving Hsp60 has been reported for various pathological conditions such as myasthenia gravis (Cappello et al., 2010; Marino Gammazza et al., 2012), Hashimoto's thyroiditis (Marino Gammazza et al., 2014; Tonello et al., 2015), chlamydial infections (Campanella et al., 2009; Cappello et al., 2009), Guillain Barré syndrome (Loshaj-Shala et al., 2015), and periodontitis (Rizzo et al., 2012; Buhlin et al., 2015); and (5) despite all these revealing clues, research on the possible direct role of Hsp60 in the pathogenesis of IBD is not as abundant as it should be to make progress in disease monitoring and treatment.

In this short review, we aim to gather the few reports available on Hsp60 and IBDs and, thereby, provide a launching platform for innovative research to answer two key questions: (a) Does Hsp60 play a direct pathogenic role? and (b) If yes, What are the molecular mechanisms involved and where do they occur in the intestinal wall?

MOLECULAR CHAPERONES: ROLES IN HEALTH AND DISEASE

Molecular chaperones are essential for cell differentiation, tissue homeostasis, and organ remodeling in virtually all anatomical districts, including the intestinal tract (Rodolico et al., 2010; Tomasello et al., 2011b). They orchestrate the correct folding of many proteins and protect cells from the deleterious consequences of stress by preventing protein misfolding, premature degradation, or aggregation (Macario et al., 2013). The crucial role of molecular chaperones in intestinal tissues homeostasis is illustrated by the fact that their dysregulation in

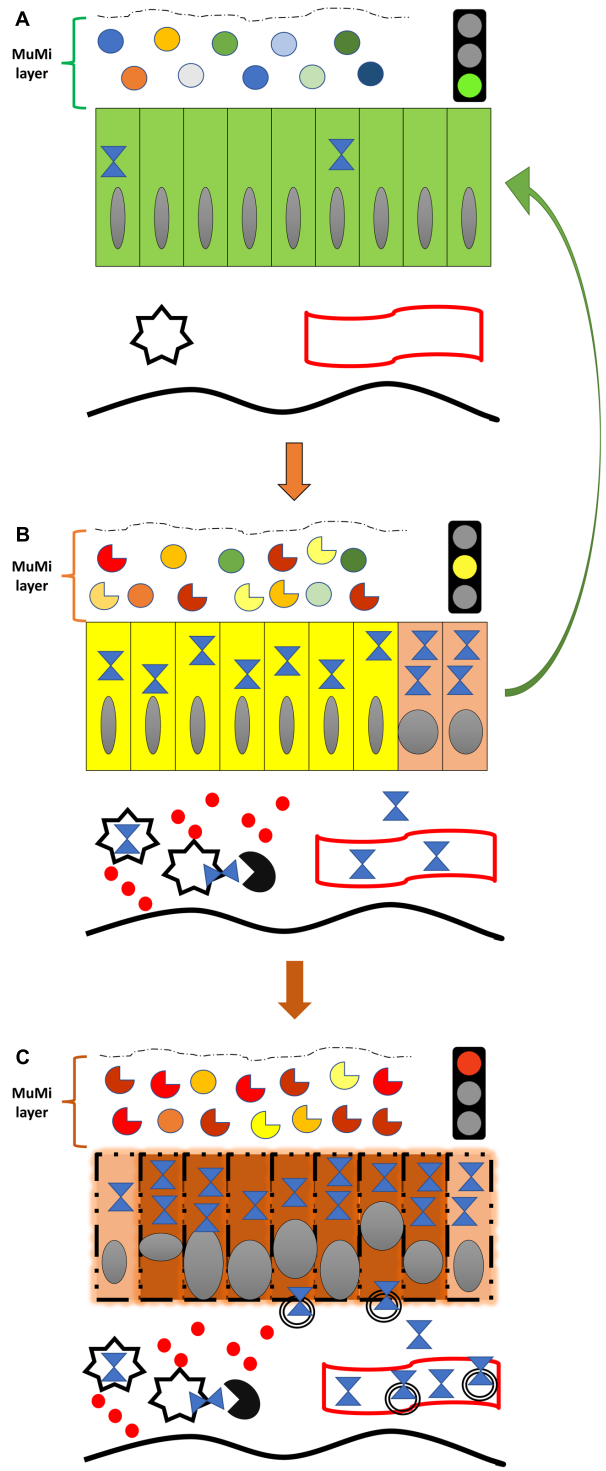

FIGURE 1 | Diagram of the natural history of IBD from the standpoint of Hsp60 and its most feared complication, colorectal cancer. **(A)** Normal colon (or colon of an IBD patient in remission), showing the normal symbiotic flora (top icons; circles of various colors indicate different species of microbes of the normal, healthy microbiota) immersed in the mucous, both forming the functional mucous-microbiota (MuMi) layer, and epithelium with individual cells as rectangles (green) containing few Hsp60 molecules (blue hourglass).

(Continued)

FIGURE 1 | Continued

Also shown are immune system cells (star), e.g., macrophages and dendritic cells, and a vessel (blood or lymphatic; undulating band with red borders) in the lamina propria. The thick, orange vertical arrow pointing downward indicates initiation or relapse of IBD, illustrated in **(B)**. **(B)** Inflamed colon of an IBD patient in relapse. The flora is altered (dysbiosis; while circles indicate normal flora, see **(A)**, the incomplete circles of different colors indicate various microbes that are abnormal, not part of the healthy microbiota, and some may be pathogenic by themselves), and epithelial cells are changed (yellow) affected by the pathologic process with elevated levels of Hsp60, which may be involved in the initiation of carcinogenesis by inhibiting the apoptosis of epithelial cells with malignant DNA transformation, as represented by the two epithelial cells in orange (dysplasia; extreme right). Hsp60 is elevated also in immune system cells and is secreted into the extra-cellular space and/or exposed on the surface and stimulates T lymphocytes (black circle with quadrant missing) and secretion of pro-inflammatory cytokines (solid red circles). Secreted Hsp60 can also reach the general circulation as indicated by the hourglasses inside the lumen of the vessel. The thick vertical reddish arrow pointing downward indicates malignant transformation, illustrated in the **(C)**. The green arrow to the right suggests the expected effect (i.e., the reversal to a normal physiological situation) of IBD treatment aiming at inhibiting/blocking the anti-apoptotic and pro-inflammatory effects of Hsp60 (i.e., negative chaperonotherapy, consisting in blocking the pathogenic action of a chaperone). **(C)** Early (*in situ*) colon carcinoma developed on an IBD patient. The main feature is the profound transformation of many epithelial cells. Malignant cells are represented by reddish rectangles with altered nuclei, while cells still undergoing transformation (dysplasia) are shown in orange, as those shown in **(B)**, extreme right. Transformed cells have also elevated levels of Hsp60 and secrete the chaperonin into the extracellular space, mostly via exosomes (double-bordered circles). The amount of Hsp60 reaching the general circulation in exosomes or free increases considerably, and can be used as a biomarker for patient follow-up and for monitoring response to treatment. Note in the MuMi layer that the predominance of abnormal microbes (incomplete circles) with regard to the normal microbes (circles) is more marked than in **(B)**.

intestinal epithelial cells (including absorptive, Paneth's, goblet, and entero-endocrine cells) drives colitis in experimental animals and patients with IBD (Ma et al., 2017). Specifically Hsp60, the focus of this review, plays a key role in the maintenance of intestinal epithelial stemness and proliferation (Berger et al., 2016), as we will discuss later.

Many chaperones are expressed in inflammation sites, probably participating in tissue regeneration (Hightower et al., 2000; Vitadello et al., 2010; Thanos et al., 2014). Also for this reason, in principle we consider inflammation a physiological phenomenon necessary for tissue repair through the proliferation and differentiation of normal cells but, when inflammation is either defective or excessive, it becomes pathogenic and leads to disease.

A set of proteins belonging to the class of molecular chaperones are heat shock proteins (Hsps), a group of proteins highly conserved during the evolution. The term "Hsp" derives from the observation that the concentration of these proteins increases following exposure to thermal stress (Ritossa, 1996). However, in the last decades it has been shown that Hsp levels can also increase after various stimuli other than thermal stress (Macario and Conway de Macario, 2005). They can be classified into two categories (Macario et al., 2013): (a) constitutive Hsps, are constitutively present inside the cell and act as molecular chaperones to guarantee the correct folding of other proteins,

and the translocation of the mature proteins through the cell membranes, among other functions; and (b) inducible Hsps, that are produced, sometimes in large quantities, under stress conditions, whose primary role is to stabilize other proteins, preventing their denaturation during stress, and other functions unrelated to protein homeostasis.

Hsp chaperones typically reside in the various subcellular compartments such as nucleus, endoplasmic reticulum, microvesicles, and mitochondria (Macario and Conway de Macario, 2005), but can also occur extracellularly. For example, they can be secreted out of the cell via Golgi or in extracellular vesicles (EVs), such as exosomes (Campanella et al., 2012).

Extracellular vesicles are important for cell to cell communication and for this reason Hsps are now considered key proteins in intercellular cross-talk (Caruso Bavisotto et al., 2017). Once released, Hsps can act in two ways: (a) paracrine-like, when their targets are located near the cells that have secreted them, and (b) systemic (i.e., endocrine-like), when they reach their targets through the bloodstream.

Hsps play also an important role in carcinogenesis as many cancer-related proteins have been reported as Hsps clients (Rappa et al., 2012). Therefore, during carcinogenesis Hsps work for the tumor rather than for the host (chaperonopathy by mistake) (Macario et al., 2013). Consequently, cancer treatments targeting Hsps have been developed that can modulate various pathways of cancer progression, including neoplastic growth, angiogenesis, invasiveness, metastasis, and resistance to chemotherapy and radiotherapy (Rappa et al., 2012).

The role of Hsps in the immune response has been the focal point of a variety of studies (Macario et al., 2010). Hsps receptors have been identified on antigen-presenting cells (APCs) and a subset of T cells. It was hypothesized that Hsps are an important element in the defense against damage due to autoimmune mechanisms. Numerous studies have focused on the role of Hsps both as an element of pathophysiology in immune processes and as a potential therapeutic target. Another interesting aspect of the interaction between Hsps and immune system concerns the phenomenon of molecular mimicry (Bachmaier and Penninger, 2005; Cappello et al., 2009). This phenomenon is caused by a structural similarity between Hsp molecules present in pathogenic bacteria and human Hsps and other molecules. An immune response to a foreign Hsp of bacterial origin could result in cross-reaction against the host's ortholog Hsp, generating an autoimmune condition. This mechanism could be the basis of sustained chronic inflammatory processes such as those that occur in IBD.

CHAPERONINS, A UNIQUE CLASS OF CHAPERONES

Hsps are grouped considering their molecular weight into six groups: Super heavy Hsp, Hsp90, Hsp70, Hsp60 (chaperonins), Hsp40, and small Hsp or sHsp (kDa: 100 or higher, 81–99, 65–80, 55–64, 35–54, and 34 or lower, respectively) (Macario et al., 2013), and their nomenclature has been organized (Kampinga et al., 2009). Hsp60 belongs to the group falling within the 55–64 kDa range and has unique characteristics, for instance, it can form very large macromolecular complexes of about ∼ 1 MDa, and has been called "chaperonin" (Hemmingsen et al., 1988).

Classically, there are two groups of chaperonins (Vilasi et al., 2018), although a third group has recently been proposed (Rowland and Robb, 2017). Hsp60 belongs to Group I and it is classically considered a mitochondrial chaperone. Group II is represented by TRiC (TCP-1 Ring Complex), also called CCT (Chaperonin Containing TCP-1), and it works in the cytosol.

Although Hsp60 was initially considered an intramitochondrial protein, nowadays we know that it can also occur beyond mitochondria, for instance in the cytosol, the extracellular space, on the surface of normal and pathological cells (such as cancer cells), and other locales (Merendino et al., 2010). Its translocation to the extracellular medium is possible during stress conditions, such as inflammation and cancer (**Figures 1B,C**).

Hsp60 IN BOWEL PHYSIOLOGY AND PATHOPHYSIOLOGY

Hsp60 is both constitutive and inducible. Under stress conditions its levels increase considerably, accumulates in the cytosol and can be secreted out of the cell via classic (Golgi's) and alternative (exosomal) pathways (Merendino et al., 2010; Campanella et al., 2012).

Although Hsp60 can reside and function in a variety of intra- and extra-cellular locales, its canonical location is the mitochondrial matrix. Inside mitochondria, Hsp60 assists in the folding of all mitochondrial proteins, including those of the electron transfer chain for ATP production, thus playing a crucial role in the maintenance of cellular and tissue (including the intestinal tissue) physiology (Dencher et al., 2007; Takada et al., 2010; Berger et al., 2016). Absence of Hsp60 in intestinal epithelial cells causes the activation of the mitochondrial unfolded protein response (mtUPR) with mitochondrial dysfunction, resulting in loss of stemness and cell-proliferative capacity (Berger et al., 2016). Moreover, mitochondrial dysfunction is associated with paracrine release of WNT-related signals and hyperproliferation of residual stem cells that have escaped Hsp60 deletion (Berger et al., 2016). Hence, Hsp60 is crucial in the control of the epithelial stem cell niche of the bowel.

Upregulation of Hsp60 is a part of the exaggerated inflammatory response in some pathological conditions such as bronchitis (Cappello et al., 2011; Sangiorgi et al., 2017), keratoconjunctivitis (Leonardi et al., 2016), hepatitis (Barone et al., 2016), thyroiditis (Marino Gammazza et al., 2014), periodontitis (Rizzo et al., 2012), and IBD (Rodolico et al., 2010) (for details and illustrative images see Data Availability Statement just prior to the References list). We found Hsp60 in cells typical of inflammation in lamina propria in histological samples of IBDs but not in normal controls (Rodolico et al., 2010). This prompted us to hypothesize that this chaperonin could be implicated in

the activation of the immune system leading to inflammation, particularly when alterations of the MuMi layer are present.

Subsequently, we reported that after treatment of patients with IBD with 5-aminosalicylic acid, alone or in combination with probiotics, amelioration of symptoms was associated with reduction of both inflammation and Hsp60. Interestingly, the levels of Hsp60 positively correlated with those of CD68-positive cells, and double immunofluorescence showed a high index of colocalization of the chaperonin and CD68 in lamina propria (Tomasello et al., 2011a). A correlation between gut microbiota imbalance and chaperoning system malfunction in IBD has also been suggested (Bellavia et al., 2013), as supported by data showing that probiotics supplementation reduce Hsp60 levels as well as its post-translational modifications (Barone et al., 2016); these modifications may be related to its secretion outside cells (Campanella et al., 2016), not only in its free, soluble form but also in the membrane of exosomes.

In agreement with these findings, antibodies against Hsp60 were found in patients with IBD (Elsaghier et al., 1992). These studies are worth expanding because of the potential implications of the findings in the development of therapeutic means for IBD.

Strong positivity staining for Hsp60 in mononuclear cells of the intestinal mucosa of patients with IBDs has been reported (Peetermans et al., 1995). Double staining for B7 and Hsp60 showed that Hsp60 was present in B7-positive cells, thus supporting the hypothesis that Hsp60 may play a role in the initiation and/or maintenance of the inflammatory process. Severe intestinal pathology was induced by the adoptive transfer of an Hsp60-specific CD8+ T-lymphocyte clone pre-activated by bacterial Hsp60, into $TCR^{-/-}$ or SCID mice (Steinhoff et al., 1999). Colitis induction required the presentation of Hsp60 on MHC class I and depended on a functional role of TNF-α. In contrast to the findings obtained in other experimental models, inflammation did not depend on the presence of the resident bacterial flora in the MuMi layer. Thus, the results indicated that autoimmune Hsp60 CD8+ T cells, that were reactive to cellular Hsp60, mediated the pathogenesis of this very severe colitis. Furthermore, anti-inflammatory effects of prozumab, a humanized anti-HSP monoclonal antibody able to bind Hsp60, occurred in murine inflammatory colitis via: (a) induction of IL-10 secretion from naive human peripheral blood mononuclear cells; and (b) suppression of secretion of IFN-γ and IL-6 from anti-CD3-activated cells (Ulmansky et al., 2015).

Hsp60, in contrast to other molecular chaperones, is increased intracellularly in epithelial cells during early stages of colon carcinogenesis, i.e., intestinal adenomatous polyps with dysplasia (Cappello et al., 2003) (**Figure 1B**), a condition in which an alteration of the MuMi layer homeostasis is present (Kang and Martin, 2017). This information has been confirmed in other studies (Cappello et al., 2005; Campanella et al., 2015; Rappa et al., 2016) that correlated also Hsp60 levels to peritumoral inflammation and disease clinical course.

In tumor cells, Hsp60 binds pro-caspase 3, thus blocking apoptosis (Campanella et al., 2008). Hsp60 interacts also with cyclophilin D (Cyp-D), thus preventing Cyp-D dependent tumor-cell death through the formation of a complex with Hsp90 and tumor necrosis factor receptor-associated protein-1 (Ghosh et al., 2010). It has also been shown that Hsp60 plays a role in tumor cell survival through the activation of the NF-$_K$B pathway (Chun et al., 2010). Hsp60 interacts directly with IKKα/β through the activation-dependent serine phosphorylation in a chaperone-independent manner to promote the TNF-α mediated activation of the IKKβ/NF-κB survival pathway (Chun et al., 2010). Finally, in tumor cells, Hsp60 is prone to undergo post-translational modifications that facilitate its secretion in the peritumoral microenvironment with pro-tumoral effects being likely (Gorska et al., 2013; Campanella et al., 2016; Marino Gammazza et al., 2017).

The data discussed in the preceding paragraphs implicate Hsp60 in the mechanisms causing various types of diseases, including IBD. Therefore, the need of more research becomes acutely apparent; research that should aim, for instance, at elucidating the molecular aspects of the Hsp60's pathogenicity directly in the intestinal tissue. The results should help in the discovering of novel and efficacious therapeutic agents. In this regard, it is of interest to report that Hsp60 can be negatively modulated by specific inhibitors, both natural products and synthetic compounds (Pace et al., 2013; Cappello et al., 2014; Meng et al., 2018). These potential therapeutic agents, e.g., mizoribine, epolactaene, myrtucommulone, stephacidin B, avrainvillamide, o-carboranylphenoxyacetanilides, and gold (III) porphyrins were identified by chemoproteomics, and constitute the subject matter of another line of research that also deserves consideration in the efforts to cure IBD.

CONCLUSION AND PERSPECTIVES

Inflammatory bowel disease has a multifactorial etiopathogenesis. The molecular chaperone Hsp60 is emerging as a prominent player not only in the mechanisms of disease but also because of its potential as biomarker useful for diagnosis and patient monitoring and as therapeutic target. Normally, Hsp60 is one of the most important proteins for cell survival, proliferation, and differentiation. This chaperonin plays a key role in the maintenance of protein homeostasis inside mitochondria, including in the intestine. It also functions in many other processes unrelated to protein homeostasis beyond mitochondria. However, Hsp60 can be pathogenic. Of interest for this article are the potential roles of Hsp60 in IBD pathogenesis and complications, including carcinogenesis. Hsp60 has been implicated in IBD pathogenesis, in the initiation and/or maintenance of the inflammatory process. Furthermore, it is most likely involved in the process of bowel carcinogenesis in patients with IBD. Colon rectal cancer is one of the most serious complications of IBD, and Hsp60 seems to be involved in carcinogenesis. With this concept in mind, efforts should be made to elucidate whether or not Hsp60 plays a direct pathogenic role in IBD. If, indeed, Hsp60 does play a pathogenic role in

IBD, research should be done to dissect the pertinent molecular mechanisms and to determine where exactly in the intestinal wall they occur. The results should help in the development of novel therapies targeting Hsp60.

In summary, the data discussed in this review support the notion that Hsp60 is worth investigating as a potential etiopathogenic factor in IBD. It is hoped that the results will provide the basis for IBD treatment focusing on the chaperonin to alleviate inflammation and prevent one of its most feared complications, colon cancer.

DATA AVAILABILITY STATEMENT

Supplementary bibliographic information and images concerning Hsp60 and chronic inflammatory diseases may be found online at: http://www.iemest.eu/en/the-chaperonopathies/28-the-chaperonopathies/224-picture-gallery.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Part of this work was funded by the Italian National Operational Programme (PON) "*Imprese e Competitività*" 2014–2020 FESR, grant awarded by the Italian Ministry of Economic Development to the project titled "*Gestione di un servizio integrato multicentrico di diagnostica e terapia personalizzata in oncologia*" (Project code: F/090012/01-02/X36). AJLM and ECdeM were partially supported by IMET. FC was partially supported by IEMEST and UniPA. This work was done under the agreement between IEMEST (Italy) and IMET (United States) (this is IMET contribution number IMET 19-001).

REFERENCES

Bachmaier, K., and Penninger, J. M. (2005). Chlamydia and antigenic mimicry. *Curr. Top. Microbiol. Immunol.* 296, 153–163.

Barone, R., Rappa, F., Macaluso, F., Caruso Bavisotto, C., Sangiorgi, C., Di Paola, G., et al. (2016). Alcoholic liver disease: a mouse model reveals protection by *Lactobacillus fermentum*. *Clin. Transl. Gastroenterol.* 7:e138. doi: 10.1038/ctg.2015.66

Bellavia, M., Tomasello, G., Romeo, M., Damiani, P., Lo Monte, A. I., Lozio, L., et al. (2013). Gut microbiota imbalance and chaperoning system malfunction are central to ulcerative colitis pathogenesis and can be counteracted with specifically designed probiotics: a working hypothesis. *Med. Microbiol. Immunol.* 202, 393–406. doi: 10.1007/s00430-013-0305-2

Berger, E., Rath, E., Yuan, D., Waldschmitt, N., Khaloian, S., Allgäuer, M., et al. (2016). Mitochondrial function controls intestinal epithelial stemness and proliferation. *Nat. Commun.* 7:13171. doi: 10.1038/ncomms13171

Bernstein, C. N., and Forbes, J. D. (2017). Gut microbiome in inflammatory bowel disease and other chronic immune-mediated inflammatory diseases. *Inflamm. Intest. Dis.* 2, 116–123. doi: 10.1159/000481401

Buhlin, K., Holmer, J., Gustafsson, A., Hörkkö, S., Pockley, A. G., Johansson, A., et al. (2015). Association of periodontitis with persistent, pro-atherogenic antibody responses. *J. Clin. Periodontol.* 42, 1006–1014. doi: 10.1111/jcpe.12456

Campanella, C., Bucchieri, F., Ardizzone, N. M., Marino Gammazza, A., Montalbano, A., Ribbene, A., et al. (2008). Upon oxidative stress, the antiapoptotic Hsp60/procaspase-3 complex persists in mucoepidermoid carcinoma cells. *Eur. J. Histochem.* 52, 221–228.

Campanella, C., Bucchieri, F., Merendino, A. M., Fucarino, A., Burgio, G., Corona, D. F., et al. (2012). The odyssey of Hsp60 from tumor cells to other destinations includes plasma membrane-associated stages and Golgi and exosomal protein-trafficking modalities. *PLoS One* 7:e42008. doi: 10.1371/journal.pone.0042008

Campanella, C., D'Anneo, A., Marino Gammazza, A., Caruso Bavisotto, C., Barone, R., Emanuele, S., et al. (2016). The histone deacetylase inhibitor SAHA induces HSP60 nitration and its extracellular release by exosomal vesicles in human lung-derived carcinoma cells. *Oncotarget* 7, 28849–28867. doi: 10.18632/oncotarget.6680

Campanella, C., Marino Gammazza, A., Mularoni, L., Cappello, F., Zummo, G., and Di Felice, V. (2009). A comparative analysis of the products of GROEL-1 gene from *Chlamydia trachomatis* serovar D and the HSP60 var1 transcript from *Homo sapiens* suggests a possible autoimmune response. *Int. J. Immunogenet.* 36, 73–78. doi: 10.1111/j.1744-313X.2008.00819.x

Campanella, C., Rappa, F., Sciumè, C., Marino Gammazza, A., Barone, R., Bucchieri, F., et al. (2015). Heat shock protein 60 levels in tissue and circulating exosomes in human large bowel cancer before and after ablative surgery. *Cancer* 121, 3230–3239. doi: 10.1002/cncr.29499

Cappello, F., Bellafiore, M., Palma, A., David, S., Marcianò, V., Bartolotta, T., et al. (2003). 60KDa chaperonin (HSP60) is over-expressed during colorectal carcinogenesis. *Eur. J. Histochem.* 47, 105–110.

Cappello, F., Caramori, G., Campanella, C., Vicari, C., Gnemmi, I., Zanini, A., et al. (2011). Convergent sets of data from in vivo and in vitro methods point to an active role of Hsp60 in chronic obstructive pulmonary disease pathogenesis. *PLoS One* 6:e28200. doi: 10.1371/journal.pone.0028200

Cappello, F., Conway de Macario, E., Di Felice, V., Zummo, G., and Macario, A. J. L. (2009). *Chlamydia trachomatis* infection and anti-Hsp60 immunity: the two sides of the coin. *PLoS Pathog.* 5:e1000552. doi: 10.1371/journal.ppat.1000552

Cappello, F., David, S., Rappa, F., Bucchieri, F., Marasà, L., Bartolotta, T. E., et al. (2005). The expression of HSP60 and HSP10 in large bowel carcinomas with lymph node metastase. *BMC Cancer* 5:139. doi: 10.1186/1471-2407-5-139

Cappello, F., Marino Gammazza, A., Palumbo Piccionello, A., Campanella, C., Pace, A., Conway de Macario, E., et al. (2014). Hsp60 chaperonopathies and chaperonotherapy: targets and agents. *Expert Opin. Ther. Targets* 18, 185–208. doi: 10.1517/14728222.2014.856417

Cappello, F., Marino Gammazza, A., Zummo, L., Conway de Macario, E., and Macario, A. J. L. (2010). Hsp60 and AChR cross-reactivity in myasthenia gravis: an update. *J. Neurol. Sci.* 292, 117–118. doi: 10.1016/j.jns.2010.02.021

Caruso Bavisotto, C., Cappello, F., Macario, A. J. L., Conway de Macario, E., Logozzi, M., Fais, S., et al. (2017). Exosomal Hsp60: a potentially useful biomarker for diagnosis, assessing prognosis, and monitoring response to treatment. *Expert Rev. Mol. Diagn.* 17, 815–822. doi: 10.1080/14737159.2017.1356230

Cheong, H. C., Lee, C. Y. Q., Cheok, Y. Y., Shankar, E. M., Sabet, N. S., Tan, G. M. Y., et al. (2018). CPAF, HSP60 and MOMP antigens elicit pro-inflammatory cytokines production in the peripheral blood mononuclear cells from genital *Chlamydia trachomatis*-infected patients. *Immunobiology* doi: 10.1016/j.imbio.2018.10.010 [Epub ahead of print].

Chin, A. M., Hill, D. R., Aurora, M., and Spence, J. R. (2017). Morphogenesis and maturation of the embryonic and postnatal intestine. *Semin. Cell Dev. Biol.* 66, 81–93. doi: 10.1016/j.semcdb.2017.01.011

Chun, J. N., Choi, B., Lee, K. W., Lee, D. J., Kang, D. H., Lee, J. Y., et al. (2010). Cytosolic Hsp60 is involved in the NF-kappaB-dependent survival of cancer cells via IKK regulation. *PLoS One* 5:e9422. doi: 10.1371/journal.pone.0009422

Dencher, N. A., Frenzel, M., Reifschneider, N. H., Sugawa, M., and Krause, F. (2007). Proteome alterations in rat mitochondria caused by aging. *Ann. N. Y. Acad. Sci.* 1100, 291–298.

Elsaghier, A., Prantera, C., Bothamley, G., Wilkins, E., Jindal, S., and Ivanyi, J. (1992). Disease association of antibodies to human and mycobacterial Hsp70 and Hsp60 stress proteins. *Clin. Exp. Immunol.* 89, 305–309.

Füst, G., Uray, K., Bene, L., Hudecz, F., Karádi, I., and Prohászka, Z. (2012). Comparison of epitope specificity of anti-heat shock protein 60/65 IgG type antibodies in the sera of healthy subjects, patients with coronary heart disease and inflammatory bowel disease. *Cell Stress Chaperones* 17, 215–227. doi: 10.1007/s12192-011-0301-7

Gecse, K. B., and Vermeire, S. (2018). Differential diagnosis of inflammatory bowel disease: imitations and complications. *Lancet Gastroenterol. Hepatol.* 3, 644–653. doi: 10.1016/S2468-1253(18)30159-6

Ghosh, J. C., Siegelin, M. D., Dohi, T., and Altieri, D. C. (2010). Heat shock protein 60 regulation of the mitochondrial permeability transition pore in tumor cells. *Cancer Res.* 70, 8988–8993. doi: 10.1158/0008-5472.CAN-10-2225

Gorska, M., Marino Gammazza, A., Zmijewski, M. A., Campanella, C., Cappello, F., Wasiewicz, T., et al. (2013). Geldanamycin-induced osteosarcoma cell death is associated with hyperacetylation and loss of mitochondrial pool of heat shock protein 60 (Hsp60). *PLoS One* 8:e71135. doi: 10.1371/journal.pone.0071135

Grundtman, C., Kreutmayer, S. B., Almanzar, G., Wick, M. C., and Wick, G. (2011). Heat shock protein 60 and immune inflammatory responses in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 31, 960–968. doi: 10.1161/ATVBAHA.110.217877

Hemmingsen, S. M., Woolford, C., Van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., et al. (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 333, 330–334. doi: 10.1038/333330a0

Hightower, L. E., Brown, Renfro, J. L., Perdrizet, G. A., Rewinski, M., Guidon, P. T. Jr., et al. (2000). Tissue-level cytoprotection. *Cell Stress Chaperones* 5, 412–414.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., et al. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 141, 105–111. doi: 10.1007/s12192-008-0068-7

Kang, M., and Martin, A. (2017). Microbiome and colorectal cancer: unraveling host-microbiota interactions in colitis-associated colorectal cancer development. *Semin. Immunol.* 32, 3–13. doi: 10.1016/j.smim.2017.04.003

Lanis, J. M., Kao, D. J., Alexeev, E. E., and Colgan, S. P. (2017). Tissue metabolism and the inflammatory bowel diseases. *J. Mol. Med.* 95, 905–913. doi: 10.1007/s00109-017-1544-2

Leonardi, A., Tarricone, E., Corrao, S., Alaibac, M., Corso, A. J., Zavan, B., et al. (2016). Chaperone patterns in vernal keratoconjunctivitis are distinctive of cell and Hsp type and are modified by inflammatory stimuli. *Allergy* 71, 403–411. doi: 10.1111/all.12814

Loshaj-Shala, A., Regazzoni, L., Daci, A., Orioli, M., Brezovska, K., Panovska, A. P., et al. (2015). Guillain Barré syndrome (GBS): new insights in the molecular mimicry between *C. jejuni* and human peripheral nerve (HPN) proteins. *J. Neuroimmunol.* 289, 168–176. doi: 10.1016/j.jneuroim.2015.11.005

Ma, X., Dai, Z., Sun, K., Zhang, Y., Chen, J., Yang, Y., et al. (2017). Intestinal epithelial cell endoplasmic reticulum stress and inflammatory bowel disease pathogenesis: an update review. *Front. Immunol.* 8:1271. doi: 10.3389/fimmu.2017.01271

Macario, A. J. L., Cappello, F., Zummo, G., and Conway de Macario, E. (2010). Chaperonopathies of senescence and the scrambling of interactions between the chaperoning and the immune systems. *Ann. N. Y. Acad. Sci.* 1197, 85–93. doi: 10.1111/j.1749-6632.2010.05187

Macario, A. J. L., and Conway de Macario, E. (2005). Sick chaperones, cellular stress, and disease. *N. Engl. J. Med.* 353, 1489–1501. doi: 10.1056/NEJMra050111

Macario, A. J. L., Conway de Macario, E., and Cappello, F. (2013). *The Chaperonopathies. Diseases with Defective Molecular Chaperones. Series: Springer Briefs in Biochemistry and Molecular Biology*. Dordrecht: Springer.

Marino Gammazza, A., Bucchieri, F., Grimaldi, L. M., Benigno, A., Conway de Macario, E., Macario, A. J. L., et al. (2012). The molecular anatomy of human Hsp60 and its similarity with that of bacterial orthologs and acetylcholine receptor reveal a potential pathogenetic role of anti-chaperonin immunity in myasthenia gravis. *Cell. Mol. Neurobiol.* 32, 943–947. doi: 10.1007/s10571-011-9789-8

Marino Gammazza, A., Campanella, C., Barone, R., Caruso Bavisotto, C., Gorska, M., Wozniak, M., et al. (2017). Doxorubicin anti-tumor mechanisms include Hsp60 post-translational modifications leading to the Hsp60/p53 complex dissociation and instauration of replicative senescence. *Cancer Lett.* 385, 75–86. doi: 10.1016/j.canlet.2016.10.045

Marino Gammazza, A., Rizzo, M., Citarrella, R., Rappa, F., Campanella, C., Bucchieri, F., et al. (2014). Elevated blood Hsp60, its structural similarities and cross-reactivity with thyroid molecules, and its presence on the plasma membrane of oncocytes point to the chaperonin as an immunopathogenic factor in Hashimoto's thyroiditis. *Cell Stress Chaperones* 19, 343–353. doi: 10.1007/s12192-013-0460-9

Meng, Q., Li, B. X., and Xiao, X. (2018). Toward developing chemical modulators of Hsp60 as potential therapeutics. *Front. Mol. Biosci.* 20:35. doi: 10.3389/fmolb.2018.00035

Merendino, A. M., Bucchieri, F., Campanella, C., Marcianò, V., Ribbene, A., David, S., et al. (2010). Hsp60 is actively secreted by human tumor cells. *PLoS One* 5:e9247. doi: 10.1371/journal.pone.0009247

Nagao-Kitamoto, H., Kitamoto, S., Kuffa, P., and Kamada, N. (2016). Pathogenic role of the gut microbiota in gastrointestinal diseases. *Intest. Res.* 14, 127–138. doi: 10.5217/ir.2016.14.2.127

Pace, A., Barone, G., Lauria, A., Martorana, A., Piccionello, A. P., Pierro, P., et al. (2013). Hsp60, a novel target for antitumor therapy: structure-function features and prospective drugs design. *Curr. Pharm. Des.* 19, 2757–2764.

Peetermans, W. E., D'Haens, G. R., Ceuppens, J. L., Rutgeerts, P., and Geboes, K. (1995). Mucosal expression by B7-positive cells of the 60-kilodalton heat-shock protein in inflammatory bowel disease. *Gastroenterology* 108, 75–82.

Rahman, M., Steuer, J., Gillgren, P., Hayderi, A., Liu, A., and Frostegård, J. (2017). induction of dendritic cell-mediated activation of T cells from atherosclerotic plaques by human Heat Shock Protein 60. *J. Am. Heart. Assoc.* 6:e006778. doi: 10.1161/JAHA.117.006778

Rappa, F., Farina, F., Zummo, G., David, S., Campanella, C., Carini, F., et al. (2012). Hsp-molecular chaperones in cancer biogenesis and tumor therapy: an overview. *Anticancer Res.* 32, 5139–5150.

Rappa, F., Pitruzzella, A., Marino Gammazza, A., Barone, R., Mocciaro, E., Tomasello, G., et al. (2016). Quantitative patterns of Hsps in tubular adenoma compared with normal and tumor tissues reveal the value of Hsp10 and Hsp60 in early diagnosis of large bowel cancer. *Cell Stress Chaperones* 21, 927–933. doi: 10.1007/s12192-016-0721-5

Ritossa, F. (1996). Discovery of the heat shock response. *Cell Stress Chaperones* 1, 97–98.

Rizzo, M., Cappello, F., Marfil, R., Nibali, L., Marino Gammazza, A., Rappa, F., et al. (2012). Heat-shock protein 60 kDa and atherogenic dyslipidemia in patients with untreated mild periodontitis: a pilot study. *Cell Stress Chaperones* 17, 399–407. doi: 10.1007/s12192-011-0315-1

Rodolico, V., Tomasello, G., Zerilli, M., Martorana, A., Pitruzzella, A., Gammazza, A. M., et al. (2010). Hsp60 and Hsp10 increase in colon mucosa of Crohn's disease and ulcerative colitis. *Cell Stress Chaperones* 15, 877–884. doi: 10.1007/s12192-010-0196-8

Rowland, S. E., and Robb, F. T. (2017). "Structure, function and evolution of the Hsp60 chaperonins," in *Prokaryotic Chaperonins. Multiple Copies and Multitude of Functions*, Vol. 11, eds C. M. Santosh Kumar and S. C. Mande (Singapore: Springer Nature Singapore Pte Ltd), 3–20.

Sangiorgi, C., Vallese, D., Gnemmi, I., Bucchieri, F., Balbi, B., Brun, P., et al. (2017). HSP60 activity on human bronchial epithelial cells. *Int. J. Immunopathol. Pharmacol.* 30, 333–340. doi: 10.1177/0394632017734479

Shouval, D. S., and Rufo, P. A. (2017). The role of environmental factors in the pathogenesis of Inflammatory Bowel Diseases: a review. *JAMA Pediatr.* 171, 999–1005. doi: 10.1001/jamapediatrics.2017.2571

Steinhoff, U., Brinkmann, V., Klemm, U., Aichele, P., Seiler, P., Brandt, U., et al. (1999). Autoimmune intestinal pathology induced by Hsp60-specific CD8 T cells. *Immunity* 11, 349–358.

Sun, Y., Zheng, J., Xu, Y., and Zhang, X. (2018). Paraquat-induced inflammatory response of microglia through HSP60/TLR4 signaling. *Hum. Exp. Toxicol.* 37, 1161–1168. doi: 10.1177/0960327118758152

Swaroop, S., Mahadevan, A., Shankar, S. K., Adlakha, Y. K., and Basu, A. (2018). HSP60 critically regulates endogenous IL-1β production in activated microglia by stimulating NLRP3 inflammasome pathway. *J. Neuroinflammation* 15:177.

Takada, M., Otaka, M., Takahashi, T., Izumi, Y., Tamaki, K., Shibuya, T., et al. (2010). Overexpression of a 60-kDa heat shock protein enhances cytoprotective function of small intestinal epithelial cells. *Life Sci.* 86, 499–504. doi: 10.1016/j.lfs.2010.02.010

Thanos, S., Böhm, M. R., Meyer zu Hörste, M., Prokosch-Willing, V., Hennig, M., Bauer, D., et al. (2014). Role of crystallins in ocular neuroprotection and axonal

regeneration. *Prog. Retin. Eye Res.* 42, 145–161. doi: 10.1016/j.preteyeres.2014.06.004

Tomasello, G., Rodolico, V., Zerilli, M., Martorana, A., Bucchieri, F., Pitruzzella, A., et al. (2011a). Changes in immunohistochemical levels and subcellular localization after therapy and correlation and colocalization with CD68 suggest a pathogenetic role of Hsp60 in ulcerative colitis. *Appl. Immunohistochem. Mol. Morphol.* 19, 552–561. doi: 10.1097/PAI.0b013e3182118e5f

Tomasello, G., Sciumé, C., Rappa, F., Rodolico, V., Zerilli, M., Martorana, A., et al. (2011b). Hsp10, Hsp70, and Hsp90 immunohistochemical levels change in ulcerative colitis after therapy. *Eur. J. Histochem.* 55:e38. doi: 10.4081/ejh.2011.e38

Tonello, L., Conway de Macario, E., Marino Gammazza, A., Cocchi, M., Gabrielli, F., Zummo, G., et al. (2015). Data mining-based statistical analysis of biological data uncovers hidden significance: clustering Hashimoto's thyroiditis patients based on the response of their PBMC with IL-2 and IFN-γ secretion to stimulation with Hsp60. *Cell Stress Chaperones* 20, 391–395. doi: 10.1007/s12192-014-0555-y

Ulmansky, R., Landstein, D., Moallem, E., Loeb, V., Levin, A., Meyuhas, R., et al. (2015). A humanized monoclonal antibody against heat shock protein 60 suppresses murine arthritis and colitis and skews the cytokine balance toward an anti-inflammatory response. *J. Immunol.* 194, 5103–5109. doi: 10.4049/jimmunol.1500023

Vilasi, S., Bulone, D., Caruso Bavisotto, C., Campanella, C., Marino Gammazza, A., San Biagio, P. L., et al. (2018). Chaperonin of Group I: oligomeric spectrum and biochemical and biological implications. *Front. Mol. Biosci.* 25:99. doi: 10.3389/fmolb.2017.00099

Vitadello, M., Doria, A., Tarricone, E., Ghirardello, A., and Gorza, L. (2010). Myofiber stress-response in myositis: parallel investigations on patients and experimental animal models of muscle regeneration and systemic inflammation. *Arthritis Res. Ther.* 12:R52. doi: 10.1186/ar2963

Wick, C. (2016). Tolerization against atherosclerosis using heat shock protein 60. *Cell Stress Chaperones* 21, 201–211. doi: 10.107/s12192-015-0659-z

Wick, G., Jakic, B., Buszko, M., Wick, M. C., and Grundtman, C. (2014). The role of heat shock proteins in atherosclerosis. *Nat. Rev. Cardiol.* 11, 516–529. doi: 10.1038/nrcardio.2014.91

Yáñez-Mó, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* 4:27066. doi: 10.3402/jev.v4.27066

Yu, L. C. (2018). Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. *J. Biomed. Sci.* 25:79. doi: 10.1186/s12929-018-0483-8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 13 March 2019
doi: 10.3389/fphar.2019.00212

Targeting Cytokine Signaling and Lymphocyte Traffic via Small Molecules in Inflammatory Bowel Disease: JAK Inhibitors and S1PR Agonists

*Tamara Pérez-Jeldres[1,2,3], Christopher J. Tyler[1,4], Joshua D. Boyer[1,4], Thangaraj Karuppuchamy[1,4], Andrés Yarur[5], Daniel A. Giles[6], Shaila Yeasmin[1], Luke Lundborg[1], William J. Sandborn[1], Derek R. Patel[1] and Jesús Rivera-Nieves[1,4]**

[1] Inflammatory Bowel Disease Center, Division of Gastroenterology, University of California, San Diego, La Jolla, CA, United States, [2] Department of Medicine, Pontifical Universidad Católica de Chile, Santiago, Chile, [3] San Borja Arriarán Clinic Hospital, Santiago, Chile, [4] VA San Diego Healthcare System, San Diego, CA, United States, [5] Division of Gastroenterology and Hepatology, Medical College of Wisconsin, Milwaukee, WI, United States, [6] La Jolla Institute for Allergy and Immunology, San Diego, CA, United States

OPEN ACCESS

Edited by:
Luca Antonioli, University of Pisa, Italy

Reviewed by:
Dapeng Chen, Dalian Medical University, China
Giorgos Bamias, National and Kapodistrian University of Athens, Greece

***Correspondence:**
Jesús Rivera-Nieves
jriveranieves@ucsd.edu

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *20 November 2018*
Accepted: *19 February 2019*
Published: *13 March 2019*

Citation:
Pérez-Jeldres T, Tyler CJ, Boyer JD, Karuppuchamy T, Yarur A, Giles DA, Yeasmin S, Lundborg L, Sandborn WJ, Patel DR and Rivera-Nieves J (2019) Targeting Cytokine Signaling and Lymphocyte Traffic via Small Molecules in Inflammatory Bowel Disease: JAK Inhibitors and S1PR Agonists. Front. Pharmacol. 10:212. doi: 10.3389/fphar.2019.00212

The inflammatory Bowel diseases (IBDs) are a chronic, relapsing inflammatory diseases of the gastrointestinal tract with heterogeneous behavior and prognosis. The introduction of biological therapies including anti-TNF, anti-IL-12/23, and anti-integrins, has revolutionized the treatment of IBD, but these drugs are not universally effective. Due to the complex molecular structures of biologics, they are uniformly immunogenic. New discoveries concerning the underlying mechanisms involved in the pathogenesis of IBD have allowed for progress in the development of new treatment options. The advantage of small molecules (SMs) over biological therapies includes their lack of immunogenicity, short half-life, oral administration, and low manufacturing cost. Among these, the Janus Kinases (JAKs) inhibition has emerged as a novel strategy to modulate downstream cytokine signaling during immune-mediated diseases. These drugs target various cytokine signaling pathways that participate in the pathogenesis of IBD. Tofacitinib, a JAK inhibitor targeting predominantly JAK1 and JAK3, has been approved for the treatment of ulcerative colitis (UC), and there are other specific JAK inhibitors under development that may be effective in Crohn's. Similarly, the traffic of lymphocytes can now be targeted by another SM. Sphingosine-1-phosphate receptor (S1PR) agonism is a novel strategy that acts, in part, by interfering with lymphocyte recirculation, through blockade of lymphocyte egress from lymph nodes. S1PR agonists are being studied in IBD and other immune-mediated disorders. This review will focus on SM drugs approved and under development, including JAK inhibitors (tofacitinib, filgotinib, upadacitinib, peficitinib) and S1PR agonists (KRP-203, fingolimod, ozanimod, etrasimod, amiselimod), and their mechanism of action.

Keywords: IBD, small molecules, JAK inhibitors, S1P agonists, MOA

INTRODUCTION

Inflammatory Bowel diseases (IBDs) is a chronic immune-mediated condition of the gastrointestinal tract (Abraham and Cho, 2009). It is potentially caused by a dysregulated mucosal immune response to intestinal microflora in genetically predisposed hosts (Abraham and Cho, 2009). There are currently no curative therapies, and in most cases, lifelong treatment is required (Abraham and Cho, 2009). Non-specific immunomodulatory drugs such as glucocorticoids, sulfasalazine/5-aminosalicylates, methotrexate, and thiopurines were among the first drugs used to treat IBD (Soendergaard et al., 2018). The introduction of biologics during the last 20 years has revolutionized the treatment of IBD, and several anti-TNF monoclonal antibodies (mAbs) (including infliximab, adalimumab, certolizumab pegol, and golimumab) are commonly used. More recently, antibodies with a different mechanism of action (MOA), such as anti-integrin α4β7 (vedolizumab) and anti-IL12/IL23 (ustekinumab), became available for clinical use (Olivera et al., 2016). However, mAbs have limitations in terms of safety, cost, and sustained efficacy (Hemperly et al., 2018). In fact, around 10–30% of patients treated with anti-TNF are primary non-responders to therapy, and 23–46% are secondary non-responders (Hemperly et al., 2018). For these reasons, novel orally available drugs are still in great need and are being developed to treat IBD. The present review will focus on new families of chemically synthesized SM drugs already available or under development: Janus Kinases (JAK) inhibitors and sphingosine-1-phosphate receptor (S1PR) agonists, with emphasis on their MOA.

Differences Between Small Molecules and Monoclonal Antibodies

Monoclonal antibodies are large molecules with high molecular weights (∼150 kDa) (Samanen, 2013). The mAb structure consists of four polypeptide chains, two identical heavy chains, and two identical light chains. Each mAb molecule has an antigen-binding region (Fab) or variable region, and a constant region or Fc (Ordás et al., 2012). The size and structure of the mAb determines the drug pharmacokinetic, target location, the drug–drug interaction, the antigenicity, and the route of administration. The mAbs are eliminated from the circulation by catabolism, which depends on the rates of proteolysis (extracellular degradation), recycling rates [by interaction with Brambell or the neonatal Fc receptor (FcRn)], and receptor-mediated antibody endocytosis rates (Ordás et al., 2012). Due to the large size of mAb the renal clearance is insignificant (Hemperly et al., 2018). Because of the protein composition of mAbs, the immune system can recognize them as immunogenic foreign antigens, which may lead to the development of specific anti-drug antibodies that nullify their therapeutic effect (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018). This results in increased drug clearance and ultimately may contribute to treatment failure and/or hypersensitivity reactions (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018). The addition of immunomodulators can decrease anti-drug antibody formation but increases the risks associated with inmunosupression (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018).

The term SM typically refers to organic compounds with low molecular weights, usually <1 kDa, which enables them to diffuse easily through cell membranes to reach intracellular targets (Samanen, 2013; Murphy and Zheng, 2015). Many SM inhibitors can function as immunomodulators due to their ability to specifically block intracellular signaling pathways thought to be pivotal to the pathogenesis of IBD (Samanen, 2013; Murphy and Zheng, 2015; Olivera et al., 2016). SMs have several advantages over conventional immunotherapeutic agents, including ease of administration (oral, without infusion costs), stable structures, non-immunogenic, potentially short half-lives, and usually lower manufacturing costs (Samanen, 2013; Murphy and Zheng, 2015; Olivera et al., 2016). **Table 1** compares the main differences between SM and mAb (Samanen, 2013).

JAK-STAT Pathway and JAK Inhibitors

Cytokines are released by the immune system in response to a stimulus (Abbas et al., 2014b). They bind to specific receptors, triggering activation and initiation of intracellular signaling pathways (Abbas et al., 2014b). Cytokines encompass many structurally unrelated proteins that are grouped based on their binding to distinct receptor super families, which

TABLE 1 | Comparison of properties of SM drugs and mAbs (Samanen, 2013).

	Small molecules	Monoclonal antibodies
Molecular weight	Low (<1000 Da)	High (>1000 Da)
Preparation	Chemical synthesis	Biologically produced
Structure	Small organic compounds	Proteins
Route of administration	Oral	Parenteral
Location of target	Intracellular	Extracellular
Distribution	Variable in organs/tissues/cells	Limited to plasma and/or extracellular fluids
Metabolism	Metabolized typically by liver and gut CYPs into no active and active metabolites	Catabolism by proteolytic degradation to peptides and amino acids
Clearance	The clearance can be by renal excretion, biliary excretion, hepatic metabolism, and intestinal transporters	Mainly involves the reticuloendothelial system (RES) through proteolytic catabolism
Toxicity	Can produce specific toxicity due to parent or metabolites (often "off the target")	Receptor-mediated toxicity
Antigenicity–hypersensitivity	No antigenic, but can show unpredictable hypersensitivity	Potential
Drug–drug interaction	Pharmacokinetic interactions by competitive clearance mechanism as: –Decreasing clearance by enzyme inhibition –Increasing clearance by enzyme induction	Infrequent
Mechanism of action	Receptor or enzyme inhibition	Depletion

TABLE 2 | Cytokines, receptors, and transduction pathway.

Ligands	Cytokine receptor	Transduction pathway	Function
	Type I		
Epo, Tpo, G-CSF, GH, and PRL	Homodimer receptor	JAK-STAT (JAK2)	Erythropoiesis Myelopoiesis Megakaryocyte/platelet production Growth Mammary development
IL-3, IL-5, and GM-CSF	Common β chain	JAK-STAT (JAK2)	
IL-6, IL-11, IL-23, and OSM	gp-130	JAK-STAT (mainly JAK1 but also JAK2, TYK2)	Naive T cells differentiation T-cell homeostasis Inflammation Granulopoiesis
IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, and IL-21	Common γ chain	JAK-STAT (JAK1, JAK3)	Growth/maturation lymphoid cells Differentiation/homeostasis T cells, NK cells B cells class switching Inflammation
IFNα,IFNβ, IFNγ, IL-10, and IL-22	Type II	JAK-STAT (JAK1, JAK2, TYK2)	Antiviral Inflammation Antitumor
TNFα, TNFβ, LT, CD40, FasL, BAFF, April, Ox40, GITR, nerve growth factor	TNF receptor family	TRAF	Inflammation
IL-1, IL-18, IL-33	IL-1 receptor family	IRAK	Inflammation
Chemokines	Seven transmembrane G-protein-coupled receptors	G proteins	Chemotaxis and lymphocyte migration

Epo, erythropoietin; Tpo, thrombopoietin; G-CSF, granulocyte-colony stimulating factor; GH, growth hormone; PRL, prolactin; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; OSM, oncostatin M; TNF, tumor necrosis factor; LT, lymphotoxin; FasL, Fas ligand, B-cell activating factor; GITR, glucocorticoid-induced TNF receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; TRAF, TNF receptor-associated factors; IRAK, interleukin-1 receptor-associated kinases (Abbas et al., 2014d).

include Type I cytokine receptors, Type II cytokine receptors, the TNF receptor family, the IL-1 receptor family, and G-protein-coupled receptors. Each family of receptors utilizes different mechanisms of signal transduction (**Table 2**; Abbas et al., 2014b). The cytokines bind to the extracellular domain of the receptor, and trigger intracellular changes, resulting in signal transduction that drives changes in gene expression (Clark et al., 2013; Abbas et al., 2014b). Protein kinases have an essential role in the signal transduction pathway of these receptors, and are an attractive target to regulate the inflammatory response (Clark et al., 2013; Abbas et al., 2014b). However, due to the complexity and redundancy inherent to signal transduction networks, some of these kinases may be better therapeutic targets than others (Clark et al., 2013).

The JAK family is a small family of receptor-associated tyrosine kinases that are essential for the cytokine signaling cascade, downstream of Type I and Type II cytokine receptors (Schwartz et al., 2017). The JAK-signal transducers and activators of transcription (STAT) pathway plays an important role in innate immunity, adaptive immunity, and hematopoiesis, participating in cellular processes such as cell growth, survival, differentiation, and migration (**Table 2**; Banerjee et al., 2017; Olivera et al., 2017). There are four members of the JAK family (JAK1, JAK2, JAK3, and TYK2) and seven signal transducers and transcription activators called signal transducer and activator of transcription, or STAT (STAT 1–4, 5a, 5b, and 6) (Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; **Table 3**).

The unique structure of each JAK clearly distinguishes them from other members of the protein tyrosine kinase family (Banerjee et al., 2017). The JAKs contain four functional domains: the SH2 domain (a scaffold for STAT), the FERM domain (regulates catalytic activity and mediates association with receptors and other proteins), the pseudo-tyrosine kinase domain, and a catalytically active tyrosine kinase domain (Banerjee et al., 2017). These last two domains are the basis for the name of the protein family named Janus (the two-faced Roman god of beginnings, endings, and duality), thus JAK exhibits a domain with kinase activity, while the other negatively regulates the activity of the first (Banerjee et al., 2017).

TABLE 3 | STAT and cellular function.

STAT	Cellular function
1	Cell growth and apoptosis TH1 cell-specific cytokine production Antimicrobial defense
2	Mediation of IFNα/IFNβ signaling
3	Cell proliferation and survival Inflammation Immune response Embryonic development Cell motility
4	TH1 cell differentiation Inflammatory responses Cell proliferation
5A	Cell proliferation and survival IL-2Ra expression in T lymphocytes Mammary gland development Lactogenic signaling
5B	Cell proliferation and survival IL-2Ra expression in T lymphocytes Sexual dimorphism of body growth rate NK cell cytolytic activity
6	Inflammatory and allergic immune response B-cell and T-cell proliferation TH2 cell differentiation

TH, T helper; IFN, interferon; IL, interleukin. Table adapted from Miklossy et al. (2013). Therapeutic modulators of STAT signaling for human diseases (Miklossy et al. 2014).

Canonical JAK-STAT signaling starts with the binding between cytokines and their corresponding transmembrane receptors, allowing receptor dimerization and triggering the transactivation of JAK, followed by phosphorylation of the cytoplasmic tails of the receptors that produce coupling sites for STAT, resulting in the tyrosine-phosphorylation (p-Tyr) of the STAT by JAK (Jatiani et al., 2010; Villarino et al., 2017). After these events, STAT (like homo/heterodimers) translocate

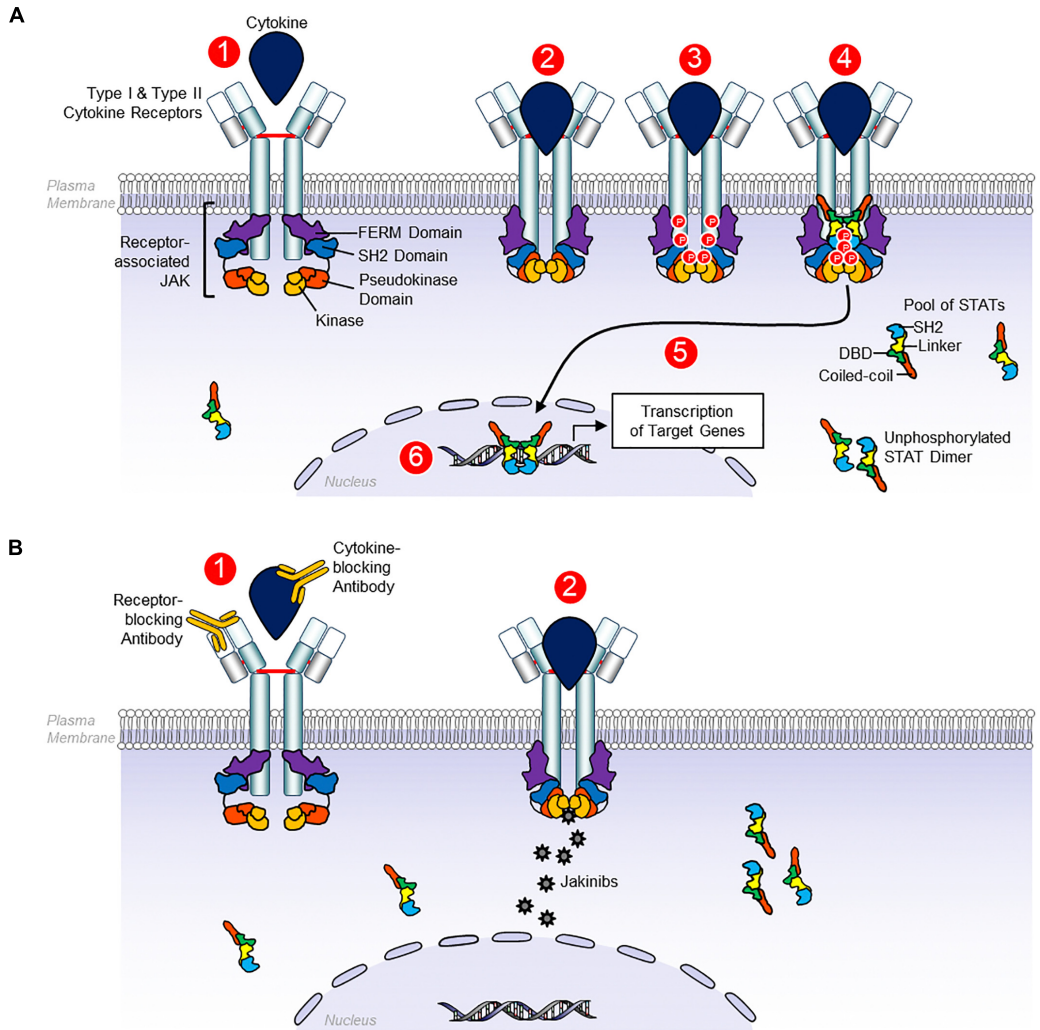

FIGURE 1 | Signaling by receptors Type I and Type II cytokines. **(A)** Type I and type II cytokine receptors comprise subunits that physically associate with Janus kinases (JAKs). Type I and type II cytokine depend on JAKs to transduce intracellular signals. JAK proteins share four components: the kinase domain, the pseudokinase domain, the FERM domain, and the SH2 domain. 1, the canonical JAK-STAT signaling begins with the extracellular association between cytokines and their corresponding transmembrane receptors. 2, the receptor dimerization triggers the transactivation of JAK. 3. Phosphorylation of the cytoplasmic tails of the receptors that create docking sites for STATs. 4–6, STAT binds to JAK, allowing the tyrosine phosphorylation of STAT which results in STAT dimerization, nuclear translocation, DNA binding, and ultimately, modulation of gene transcription. Unphosphorylated STAT dimers also have regulatory functions, although these functions are less well defined. **(B)** 1, monoclonal antibodies can block Type I and Type II cytokines and their receptors. 2, by contrast, JAK inhibitors block cytokine signaling, binding to the kinase domain of JAK in the ATP-binding site, avoiding their phosphorylation and JAK activation, preventing STAT phosphorylation and other substrates, so intracellular signals cannot be transduced (Schwartz et al., 2017).

to the nucleus, bind to DNA, and modulate gene transcription (Jatiani et al., 2010; Villarino et al., 2017). In addition to STAT phosphorylation, other kinases such as Src, phosphoinositide 3-kinases (PI3K), and RAF can be phosphorylated, activating additional signaling pathways involving proteins including Akt, and extracellular signal-regulated kinases (ERK) (**Figure 1**; Jatiani et al., 2010).

Signal transducers and activators of transcription is under the control of physiological negative regulators such as (i) suppressors of cytokine signaling (SOCS), that inhibit the kinase activity, binding phospho-tyrosine residues and competing with STAT at cytoplasmic level, (ii) protein tyrosine phosphatases (PTPs) that inactivate JAK and STAT in both the nucleus and the cytoplasm, (iii) protein inhibitor of activated STAT family (PIAS) that interferes at the nuclear level with STAT-mediated transcription and triggers proteasome degradation, and (iv) the modulators SH2B adaptor protein that increase or decrease JAK activation (Villarino et al., 2017).

Many cytokines implicated in the pathogenesis of immune-mediated diseases use the JAK-STAT pathway, representing a potential therapeutic target for these disorders (Jatiani et al., 2010; Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; Villarino et al., 2017). The mAbs can block Type I and Type II cytokines and their receptors. By contrast, JAK

inhibitors block cytokine signaling, binding to kinase domain of JAK at the ATP-binding site, avoiding their phosphorylation and JAK activation, preventing STAT phosphorylation and other substrates, so intracellular signals cannot be transduced (Jatiani et al., 2010; Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; Villarino et al., 2017). Other potential therapeutic candidates include STAT-binding inhibitory peptides, STAT inhibitors, STAT-targeting small interfering RNA (siRNA), and STAT-binding decoy oligonucleotides (Schwartz et al., 2017; Villarino et al., 2017).

The JAK inhibitors have been used in the treatment of hematologic disorders (Jatiani et al., 2010). In recent years, these inhibitors have received attention for the treatment of autoimmune/immune-mediated disorders such as rheumatoid arthritis (RA) (Vanhoutte et al., 2017), systemic lupus erythematosus (SLE) (ClinicalTrials.gov, ClinicalTrials, 2018e), dermatomyositis (Hornung et al., 2014), Sjogren syndrome (ClinicalTrials.gov, ClinicalTrials, 2018n), vasculitis (Zhang et al., 2018), psoriasis (Hsu and Armstrong, 2014), alopecia areata (Divito and Kupper, 2014), atopic dermatitis (Levy et al., 2015), vitiligo (Liu et al., 2017), and IBD (Panés et al., 2017; Sandborn et al., 2017a; Vermeire et al., 2017a).

JAK Inhibitors for the Treatment of IBD

Typically, IBD is associated with chronic inflammation, defined by a dysregulated response of the innate and adaptive immune systems (Abraham and Cho, 2009; Boland and Vermeire, 2017). Chronic inflammation in Crohn's disease (CD) is characterized by a response of helper T cells type 1 (Th1) and helper T cells type 17 (Th17), with inadequate activity of regulatory T cells (Treg), whereas UC has generally been considered a type 2 T helper cell cytokine profile (Th2) (Boland and Vermeire, 2017). In both diseases, many of the cytokines produced by these T cells signal through JAK receptors; therefore, JAK proteins have an important place in the signaling of inflammation in IBD (Boland and Vermeire, 2017).

Key cytokines in the pathogenesis of IBD belong to Type I and Type II cytokines receptors [i.e., IL-6, IL-5, IL-9, IL-10, IL-13, IL-12/23, IL-22, granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ] (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017). These cytokines all signal through the JAK/STAT pathway. In contrast, the cytokines TNF, IL-1, and IL-17, which are the major drivers of IBD, do not use the JAK-STAT pathway in their signaling pathways (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017). However, these cytokines induce the expression of a wide range of downstream pro-inflammatory cytokines, that in turn depend on JAK/STAT signaling (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017).

Interleukin-6 (IL-6) along with oncostatin M (OSM) and IL-11 signal through the gp130-associated receptor family. IL-6 activates JAK1, JAK2, and TYK2 leading to STAT3 transduction, which promotes T cell proliferation, favoring the polarization of Th17 cells (Zhou et al., 2007; Banerjee et al., 2017). Notably, IL-6 can also promote Th2 differentiation (Dienz and Rincon, 2009). In addition, IL-6 has other functions relevant to IBD, such as regulating intestinal permeability, by its effects on tight junctions, regulating the proliferation of epithelium, and healing of wounds (Flamant et al., 2017).

Interleukin-12 and IL-23 also play an important role in IBD, and JAK2 and tyrosine kinases type 2 (TYK2) are involved in the signaling of these cytokines by activating STAT3 and STAT4, promoting inflammatory reactions through their ability to induce Th1 and Th17 polarization, respectively, and production of IFN-γ, IL-21, and IL-22 (Flamant et al., 2017).

Interleukin-10 is an anti-inflammatory cytokine produced by many immune cell populations, including activated macrophages, dendritic cells, regulatory T cells, Th1 and Th2 cells. IL-10 activates JAK1 and TYK2 proteins, leading to STAT3 phosphorylation (Abbas et al., 2014a). The anti-inflammatory effects of IL-10 results, in part, from its ability to inhibit the production of IL-12 by activated macrophages and dendritic cells as well as inhibiting the expression of costimulatory and class II MHC molecules in these cells (Abbas et al., 2014a).

Interleukin-22 is produced in epithelial tissues, especially in the skin and gastrointestinal tract. IL-22 activates JAK1 and TYK2, transducing signals via STAT3, STAT1, and STAT5. IL-22 has a role in maintaining epithelial integrity, mainly by promoting the barrier function of epithelial cells and by inducing production of anti-microbial peptides (Abbas et al., 2014c). However, IL-22 contributes to inflammation, in part by stimulating epithelial production of chemokines, and may therefore be involved in tissue injury in inflammatory diseases (Abbas et al., 2014c).

Interleukin-9 binding to its receptor leads to activation of JAK1 and JAK3, which in turn phosphorylates STAT1/STAT3 and STAT1/STAT5, respectively (Flamant et al., 2017). IL-9 has been associated with deleterious impact on intestinal epithelial wound healing (Flamant et al., 2017).

Interferon-γ activates JAK1 and JAK2, inducing STAT1 activation, resulting in macrophage activation, Th1 polarization, and increased expression of several proinflammatory cytokines. However, IFN-γ also has a protective function in epithelial healing (Flamant et al., 2017). Moreover, IFN-γ protects from tissue destruction by inhibiting the expression of genes that code for tissue destructive factors such as matrix metalloproteinases (MMPs), serine proteases, coagulation factors, complement components, and enzymes involved in the metabolism of prostaglandin. In addition, IFN-γ decreases neutrophil and monocytes infiltration (Hu and Ivashkiv, 2009). GM-CSF activates JAK2 which phosphorylates STAT5, and STAT3 promoting monocyte/macrophage/granulocyte survival and activation (Kimura et al., 2009; Flamant et al., 2017).

Drugs that block JAK/STAT signaling have the potential to alter multiple inflammatory pathways, being less specific in their action than drugs that target specific cytokines or their receptors (Soendergaard et al., 2018). This complexity is clear for IL-6 (pro-inflammatory) and IL-10 (anti-inflammatory) signaling, where both ligands, despite activating JAK1 and STAT3, have opposing functions (Soendergaard et al., 2018). Consequently, blocking JAK1 affects both IL-6 and IL-10, and may alter the inflammatory balance in both directions (Soendergaard et al., 2018). Additionally, JAK inhibitors can

result in undesirable adverse effects like cytopenia and infectious complications, through its blockade of GM-CSF and IFN-γ signaling, respectively (Clark et al., 2013). On the other hand, a major strength is their effectiveness. Through adequate plasma levels, these drugs induce partial and reversible inhibition of cytokine signaling, resulting in a better balance between the inflammatory and immunomodulatory response (Clark et al., 2013). More selective inhibition of the JAK-STAT pathway is being developed and may overcome the challenges of less selective inhibitors.

The US Food and Drug Administration (FDA), in May 2018, approved tofacitinib as the first JAK inhibitor to treat moderate severely active UC (Soendergaard et al., 2018). Similar to the FDA, the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMA) had a favorable opinion, and recommended their use in adult patients with moderately to severely active UC with inadequate or loss of response or intolerance to either conventional therapy or biological agents. Currently, no JAK inhibitors are approved for CD; however, other selective JAK inhibitors are in the pipeline for CD (Soendergaard et al., 2018).

Tofacitinib

Tofacitinib (Xeljanz, Pfizer) is a pan-JAK inhibitor, that preferentially inhibits JAK1 and JAK3, in a dose-dependent fashion (Sandborn et al., 2017a). Tofacitinib has a predicted gut availability of 93%, and the clearance is 70% hepatic, whereas the remaining 30% is cleared by renal metabolism (Hemperly et al., 2018). Tofacitinib's half-life is 3 h and neither age, gender, body weight, or disease severity at baseline have an effect on its clearance or plasma levels (Dowty et al., 2014).

A double-blind, placebo-controlled phase 2 study evaluated the efficacy of tofacitinib in patients with UC (n = 194) with moderate to severe activity (Sandborn et al., 2012). The patients were randomly assigned during 8 weeks to different tofacitinib doses (0.5, 3, 10, and 15 mg each 12 h) or placebo. The primary outcome at 8 weeks (clinical response established as the decrease of at least three points and at least 30% from the baseline total Mayo score, and decrease of at least one point or an absolute rectal bleeding sub-score of 0 or 1) reported a statistically significant response between the higher doses versus placebo (78% versus 42%, respectively) (Sandborn et al., 2012). These data were supported by phase 3, double-blind placebo-controlled studies; OCTAVE induction 1, 2, and OCTAVE sustain. In the induction trials; OCTAVE 1 (n = 598) and 2 (n = 591) trials, the patients were randomly assigned to receive 10 mg of tofacitinib twice daily or placebo during 8 weeks (Sandborn et al., 2017a). The primary endpoint was remission at week 8 (a total Mayo score of $\leq$2, with no subscore $>$ 1 and a rectal bleeding sub-score of 0). This endpoint was achieved in 18.5% in the tofacitinib-treated group versus 8.2% in the placebo group (P = 0.007); in the OCTAVE Induction 2 trial, remission was achieved in 16.6% versus 3.6% (P < 0.001). A total of 593 patients achieved clinical response after the induction therapy and were recruited in the OCTAVE Sustain trial to randomly receive tofacitinib as maintenance therapy (5 or 10 mg twice daily) or placebo during 52 weeks. The aim endpoint (remission at 52 week) was achieved in 34.3 and 40.6% (5 and 10 mg twice daily, respectively) versus 11.1% placebo (P < 0.001) (Sandborn et al., 2017a). Furthermore, mucosal healing was more frequent in the tofacitinib group, and tofacitinib was effective in both treated and naïve to anti-TNF patients. The safety and efficacy data were evaluated in a phase 3, multicenter, open-label, long-term extension study in patients with severe to moderate UC (n = 946). Preliminary data showed that no new safety concerns emerged, compared with those observed in RA. Efficacy results from OLE study (NCT01470612) support sustained efficacy with tofacitinib at both 5 and 10 mg doses twice daily (Lichtenstein et al., 2017).

Similar studies were conducted in patients with moderate to severe CD; In a phase II (n = 139) study, patients were randomly assigned to receive tofacitinib (1, 5, or 15 mg twice daily) or placebo during 4 weeks. This study did not show a significant clinical response or remission response (Sandborn et al., 2014). Subsequently, another phase IIb study was performed. In this study, patients were randomized, during 8 induction weeks, to tofacitinib 5 mg twice per day (n = 86) or placebo (n = 91). The responders were included in the maintenance phase, during 26 weeks, to receive tofacitinib 5 or 10 mg daily or placebo. The majority of enrolled patients were previously treated with anti-TNF (76–79%). In this study, the results were also disappointing, despite the long duration of treatment, the remission rates did not reach significant differences (Panés et al., 2017).

These discouraging results in CD may be due to high placebo response rates or differences in the fundamental immunopathogenesis of CD and UC. Several factors may have contributed to the high placebo response observed, including lack of centralized reading endoscopy and absence of baseline objective markers of disease activity (Panés et al., 2017).

Filgotinib (GLPG0634, Galapagos/Gilead Sciences) is an oral JAK1 inhibitor, with enhanced selectivity for JAK1 over JAK2 and JAK3 (30 and 50 times, respectively) in blood (Vermeire et al., 2017a,b; Hemperly et al., 2018). Filgotinib dosing leads to the formation of active metabolite which exhibits a similar JAK1 selectivity profile as the parent compound, but has less potency (Vermeire et al., 2017a,b; Hemperly et al., 2018). Still, both contribute to the clinical activity of filgotinib. The half-life of filgotinib is 6 h, while the metabolite has a terminal elimination half-life of 21–27 h. Filgotinib and its metabolites are predominantly cleared renally (>80%) (Vermeire et al., 2017a,b; Hemperly et al., 2018).

FITZROY, a double-blind, placebo-controlled study, examined the efficacy and safety of filgotinib for the treatment of active moderate to severe CD (Vermeire et al., 2017a). A total of 174 patients with active CD were enrolled. Disease activity was confirmed by centrally read endoscopy. A proportion of patients achieved clinical remission with filgotinib 200 mg once a day, compared with placebo (47 versus 23%; p = 0.077) at week 10. Data also suggested that filgotinib is effective in anti-TNF exposed and naïve patients, being twofold higher in TNF-naïve group (Vermeire et al., 2017a). In addition, a recent *post hoc* analysis showed that clinical remission is still seen in CD, regardless of the disease location or duration (Vermeire et al., 2017b). Currently, there are phase III trials underway in both a CD and UC (ClinicalTrials.gov, ClinicalTrials, 2018h,i,j,m).

Peficitinib (GLPG1205, Janssen) is JAK1 and JAK3 inhibitor (Sands et al., 2018; Soendergaard et al., 2018). The efficacy and safety of the drug has been evaluated for the treatment of moderate to severe UC (n = 219) in a multicenter, randomized, double blind, placebo-controlled, phase IIb trial. Nevertheless, the development of this drug was discontinued in 2017 due to disappointing efficacy results (Sands et al., 2018; Soendergaard et al., 2018).

Upadacitinib (UPA) (ABT-494, Abbvie) is a JAK1-selective inhibitor. It is a non-sensitive substrate for cytochrome P450, approximately 20% is eliminated, unchanged, in urine (Hemperly et al., 2018). Its efficacy and safety were assessed in patients with moderate-to-severe CD who had inadequate response, or intolerance, to anti-TNF (Sandborn et al., 2017b). In this study, patients receiving 6 mg twice daily (27%) achieved clinical remission at a higher rate than placebo (11%). A significant dose–response relationship for endoscopic remission was observed in the UPA arm (Sandborn et al., 2017b). In addition, patients with moderate-to-severely active UC (n = 250), and history of inadequate response, loss of response or intolerance to corticosteroids, immunosuppressant, or biologic therapies, were included in a phase IIb double-blind placebo-controlled dose-ranging induction study, to assess the safety and efficacy of UPA. At week 8, both the primary objective: clinical remission per Adapted Mayo Score (stool frequency subscore $\leq$ 1, rectal bleeding score = 0, endoscopic score $\leq$ 1) and the secondary objectives: clinical remission per full Mayo score, clinical response per adapted Mayo score and endoscopic improvement were evaluated (Sandborn et al., 2018b). All of these objectives were achieved with different doses ranging from 15 to 45 mg QD. The tolerance to UPA was good and safety was similar to that of other UPA studies (Sandborn et al., 2018b). Phase III studies in CD and UC are ongoing (ClinicalTrials.gov, ClinicalTrials, 2018g,l,o,p).

TD-1473 (Theravance, Biopharma) is a new oral pan-JAK inhibitor being investigated (ClinicalTrials.gov, ClinicalTrials, 2018f; Soendergaard et al., 2018). Unlike other JAK inhibitors, its distribution is limited to the gastrointestinal tract, minimizing systemic toxicity and side effects (ClinicalTrials.gov, ClinicalTrials, 2018f; Soendergaard et al., 2018). Data from phase I study in healthy volunteers have shown that treatment with TD-1473 is safe and well-tolerated (Beattie et al., 2018). The safety, tolerability, and pharmacokinetics of TD-1473 were assessing in a double-blind placebo-controlled multicenter phase Ib study in subjects with moderately to severely active UC (n = 40) (ClinicalTrials.gov, ClinicalTrials, 2018f; Sandborn et al., 2018a). In this study, TD-1473 was generally well tolerated over 4 weeks with evidence of intestinal restriction, low systemic exposure, and signals for clinical and biomarker activity in subjects with moderately to severely active UC (ClinicalTrials.gov, ClinicalTrials, 2018f; Sandborn et al., 2018a).

Pf-06651600/Pfizer (JAK3 inhibitor) and **Pf-06700841/Pfizer** (TYK2/JAK1 inhibitor) are being tested in clinical trials to be completed by early 2020 (ClinicalTrials.gov, ClinicalTrials, 2018k,q).

Adverse Effects: Experience From IBD and Rheumatoid Arthritis

Most of the safety information currently for JAK inhibitors belongs to RA and psoriasis literature. For S1PR agonists most of the safety data originated from Multiple Sclerosis and IBD trials, as tofacitinib and fingolimod were approved for those applications years earlier. Otherwise, post-marketing real-world data from clinical practice after the approval of tofacitinib in immune-mediated disease as RA and IBD are available (Hsu and Armstrong, 2014; Charles-Schoeman et al., 2015; Liu et al., 2017; Schwartz et al., 2017; Winthrop, 2017; Cohen et al., 2018; Kang et al., 2018; Verden et al., 2018).

Tofacitinib is the JAK inhibitor whose side effects are best known compared to other more specific JAK inhibitors. Still is unknown if the higher selectivity of the new JAK will result in fewer adverse effects (Winthrop, 2017).

Infections

The risk of serious infections during JAK inhibitor treatments is similar to that of biologics and most infections do not require treatment discontinuation. Nasopharyngitis and influenza are the most frequently reported infection-related adverse events. Tuberculosis and osteomyelitis are infrequent infections that also have been identified, and in this circumstance, the therapy must be interrupted (Winthrop, 2017). In addition, JAK inhibitors increase the risk of herpes zoster infection. However, Shingrix (recombinant zoster vaccine, GlaxoSmithKline) can reduce risk of infection and associated complications in patients treated with JAK inhibitors (Winthrop, 2017).

Other serious viral infections like nephropathy by BK virus (a polyoma virus) have been identified with the use of tofacitinib during renal trasplantation (Schwartz et al., 2017). A few cases of cytomegalovirus (CMV) infections, including CMV retinitis, have occurred in patients under treatment with tofacitinib in the long-term extension studies (Sandborn et al., 2014; Schwartz et al., 2017; Winthrop, 2017). Also cases of abscesses, cellulitis, *Clostridium difficile* infection, pneumonia by *Pneumocystis jiroveci*, candida infections, urinary tract infections, and histoplasmosis have been reported (Sandborn et al., 2014).

Malignancy

All immunosuppressants have the potential to increase cancer risk. Accordingly, JAK inhibitors could interfere with T and NK immune vigilance against cancer and the antineoplastic role of IFN-γ (Schwartz et al., 2017).

Recently, the post marketing surveillance (PMS) data of worldwide tofacitinib use in RA, obtained from Pfizer safety database during a 3-year reporting period, was published. The estimated exposure to tofacitinib was 34,223 patient years. The overall relative risk was 0.45 per 100 patients-year, being highest during the first year and stabilizing later. The most notified neoplasms were lymphoma, skin, lung, breast, brain, prostate, uterine and colon cancer, malignant melanoma, squamous, and basal cell carcinoma. During the PMS, the most reported cancer in RA patients receiving tofacitinib therapy was the non-melanoma skin cancer (NMSC) (Cohen et al., 2018).

Dyslipidemia and Cardiovascular Events

A dose-dependent increase in HDL, LDL, and total cholesterol has been observed. Levels normalized after cessation of treatment. This change in lipid profiles has not been found to be associated with an increase of adverse cardiovascular events (Charles-Schoeman et al., 2015).

Thromboembolic events were reported during a placebo-controlled trial of baricitinib, a JAK inhibitor tested in RA. A post-marketing adverse event report from the FDA's Adverse Event Reporting System did not show increased risk of thromboembolic events for tofacitinib, tofacitinib extended-release, or ruxolitinib. However, the data indicated that pulmonary thrombosis and portal vein thrombosis may be a class-wide risk for JAK inhibitors (Kang et al., 2018; Verden et al., 2018).

Anemia and Leukopenia

Because hematopoietic growth factors signal through JAK2, cytopenia is frequent with the use of the first-generation pan-JAK inhibitors. These alterations are usually well tolerated and do not require treatment discontinuation (Schwartz et al., 2017).

Pregnancy

There is a lack of information about the effect of JAK inhibitors during pregnancy since most studies exclude pregnant women, and there is little data available from patients who became pregnant while receiving the medication (Winthrop, 2017). The pregnancy results from patients with UC under tofacitinib exposure were reported. Mahadevan et al. (2018) notified that from 1157 UC patients recruited in interventional trials, 25 cases were reported (11 maternal, 14 paternal) with exposure to tofacitinib. These results include 15 healthy neonates, 2 spontaneous abortions, and 2 medical interruptions. Cases of fetal death, neonate death, and congenital malformations were not described.

The data available to date does not allow to definitive position regarding the tofacitinib effect on pregnancy, and its use is not recommended (Mahadevan et al., 2018).

Others Adverse Events

Intestinal perforation and elevated serum liver enzymes have been reported with the use of tofacitinib (Olivera et al., 2016).

Future Perspectives

The pathogenesis of UC and CD involve different signaling pathways, which may explain the differential response to diverse drugs. The use of a drug with different MOA could be an effective alternative (i.e., tofacitinib for anti-TNF non-responders in UC). Further understanding the main pathways involved in the pathogenesis of IBD may predict the efficacy of specific drugs based on their MOA in the near future (Jabeen et al., 2015).

Janus kinases inhibitors target a broad spectrum of cytokines, and are a safe and effective treatment for immune-mediated disorders, such as IBD. As stated previously the JAK-STAT pathway plays an important role in innate and adaptive immunity, cell growth, survival, differentiation, and migration; hence, there are concerns of potential off-target effects. However, the safety profile to date is similar to other biological agents (Winthrop, 2017). Selectivity of the new JAK inhibitors may improve safety, while maintaining efficacy. The development of drugs such as TD-1473, with action limited to the gastrointestinal tract and less systemic exposure, may also improve safety.

In cases of refractory illness, an emergent idea is the combined use of drugs that target distinct pathways, such as inhibitors of kinase PI3K or receptor tyrosine kinases (RTKs) (Montor et al., 2018).

Signal transducers and activators of transcriptions do not have intrinsic catalytic activity unlike JAK and RTKs, whose kinase domains are an obvious therapeutic target. A potential and attractive approach is the inhibition of STAT using oligonucleotides, which would sequester STAT away from the nucleus. Small molecules (SMs), inhibitory peptides, and siRNAs that target STATs are also undergoing clinical trials for other diseases (Villarino et al., 2015; ClinicalTrials.gov, ClinicalTrials, 2018b).

The relative risk and benefits of these drugs as monotherapy, combination, or sequential therapy with other drugs remain incompletely characterized (Barroso et al., 2017).

S1P/S1PR Targeting

Sphingosine-1-phosphate (S1P) is a sphingosine-derived phospholipid that binds to 5 G-protein-coupled receptors (S1PR1-5) (Park and Im, 2017). The S1P receptors are involved in several physiological events and cellular processes, such as adhesion, migration, lymphocyte/hematopoietic cell trafficking, endocytosis, vascular tone and permeability, embryogenesis, angiogenesis, and cardiac function (Sanchez and Hla, 2004; Gonzalez-Cabrera et al., 2014).

Sphingolipids are important elements in the structure of cell membrane, and S1P is a sphingolipid metabolite derived from sphingosine. S1P is phosphorylated by sphingosine kinases 1 and 2, and reversibly dephosphorylated by sphingosine phosphatases 1,2 and by the action of S1P lyase, S1P is irreversibly degraded (Le Stunff et al., 2004; **Figure 2**).

Sphingosine-1-phosphate/S1PR1 interactions are relevant for lymphocyte trafficking through the thymus, secondary lymphoid organs, circulation, and tissues. S1P mediates the traffic of dendritic cells, B cells, and T cells (naive and central memory-CCR7-positive), but does not have a significant role in the chemotaxis of effector memory CCR7-negative T cells, which maintain tissue immune-surveillance (Abbas et al., 2014a; Perez-Jeldres et al., 2018). The S1P lyase distribution, higher in tissues but absent in the vasculature, favors an S1P concentration gradient between the blood (higher levels), lymph, secondary lymphoid organs, and tissues (lower levels), determining the movement from the areas with low concentration to high S1P concentration (Abbas et al., 2014a; Perez-Jeldres et al., 2018). Elevated S1P levels in blood induce S1PR1 internalization, whereas in the lymph node and tissues S1PR1 is re-expressed after some hours, and during this time the T cell is able to interact with antigen-presenting cells (Abbas et al., 2014a; Perez-Jeldres et al., 2018). Once S1PR1 re-appears on the surface of lymphocyte, these cells can leave the lymph node or tissue by

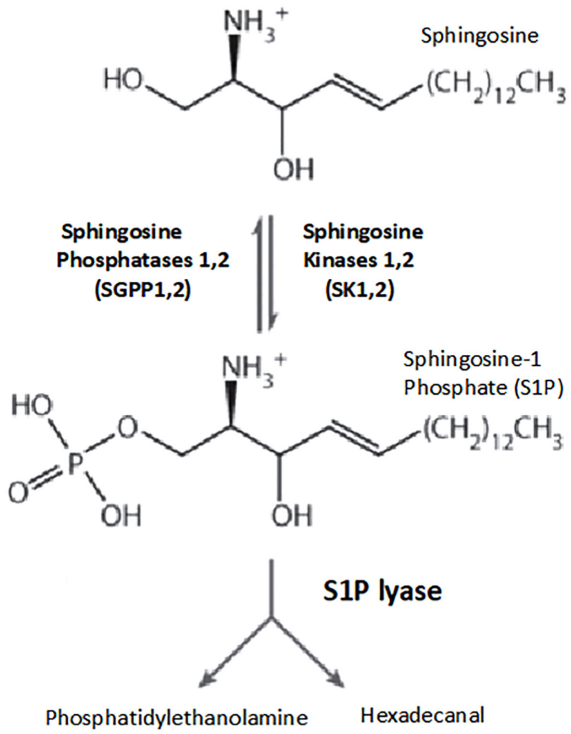

FIGURE 2 | Pathways of sphingosine-1-phosphate metabolism. Key enzymes for the formation and degradation of S1P. S1P is produced by the phosphorylation of sphingosine by sphingosine kinase. S1P can then be metabolized by S1P lyase to phosphoethanolamine and hexadecanal, which are then further metabolized into glycerophospholipids and phosphatidylethanolamine, respectively. Conversely, S1P phosphohydrolase regenerates sphingosine by dephosphorylating S1P. SphK, sphingosine kinase; SPP, sphingosine-1-phosphate phosphohydrolase; SPL, sphingosine-1-phosphate lyase (Le Stunff et al., 2004).

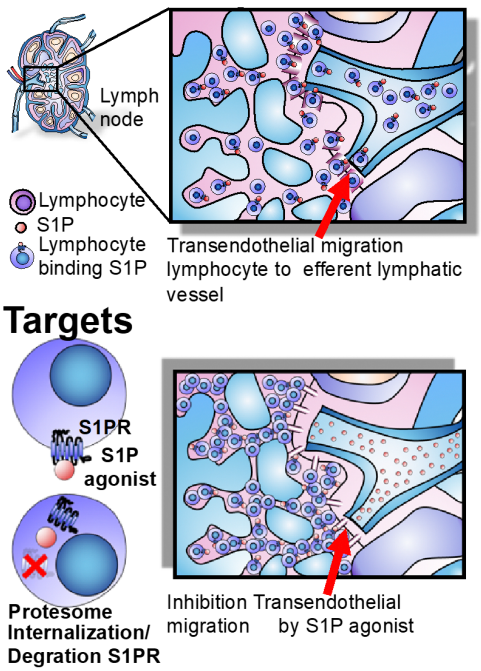

FIGURE 3 | Lymph node egress and targets. The egress of lymphocytes from lymph nodes is dependent on the S1P gradient, whose concentration is higher in blood than lymph nodes and effector tissues. S1PR agonists induce long-lasting receptor downregulation and lymphocyte sequestration in lymphoid tissues and inhibit transendothelial migration of T cells across the lymphatic endothelial barrier in the lymph node, where they remain sequestered (Perez-Jeldres et al., 2018).

sensing the higher S1P concentration in the blood, determining immune cell egress into the circulation (Horga and Montalban, 2008; Olivera et al., 2016).

Mechanisms of Action of S1PR Modulators

The native ligand S1P indices internalization of S1PR, which are recycled back to the cell surface within several hours, achieving a transitory lymphopenia (Park and Im, 2017). By contrast, S1PR1 agonists lead to the internalization of the receptor and subsequent ubiquitination and proteasome degradation of the receptor, producing sustained lymphopenia that renders lymphocytes incapable of following the S1P gradient and exiting the lymph node. This sequestration potentially prevents their access to sites of inflammation (Abbas et al., 2014a; Park and Im, 2017; Perez-Jeldres et al., 2018). In addition, S1PR1 is strongly expressed by lymphatic endothelium, where it tightens the lymphatic endothelial barrier. S1PR1 agonists can therefore interfere with lymphocyte trafficking by inhibiting transendothelial migration and blocking lymphocyte egress from the lymph node. These effects are reversed upon withdrawal of the agent (Horga and Montalban, 2008; Perez-Jeldres et al., 2018; **Figure 3**).

Sphingosine-1-phosphate signaling is involved in multiple immune functions. Therapies targeting the S1P axis may be applicable to treat autoimmune/immune-mediated diseases and have been tested in MS, RA, SLE, psoriasis, and IBD (Perez-Jeldres et al., 2018).

S1PR Modulators in IBD

Fingolimod/FTY720 (Gilenya^{TM}) is an S1P-analog, acting as non-selective potent agonist of S1PR1,3,4,5. The first S1PR modulator approved for the treatment of relapsing MS was fingolimod (Currò et al., 2017; Peyrin-Biroulet et al., 2017a).

Various preclinical studies have demonstrated its efficacy at ameliorating colitis in animal models of IBD. Treatment of IL-10 knockout mice for 4 weeks efficiently reduced the number of CD4+ T cells in the colonic lamina propria and decreased the production of IFN-γ in the colon (Mizushima et al., 2004; Huwiler and Zangemeister-Wittke, 2018). Similar data were reported with other colitis models, such as dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid, and T cell transfer into immunocompromised mice (Deguchi et al., 2006; Daniel et al., 2007; Radi et al., 2011; Montrose et al., 2013; Huwiler and Zangemeister-Wittke, 2018). The clinical use of fingolimod in IBD has not been tested, and other, more selective,

S1PR modulators are being developed for clinical use in IBD (Currò et al., 2017).

KRP-203 (Novartis™) is a S1PR1,4,5 agonist and partial agonist of S1PR3. The safety, tolerance, and efficacy of KRP203 were tested in 27 patients with active moderate UC, in a multicenter, double-blind, placebo-controlled study (Radeke et al., 2016; Perez-Jeldres et al., 2018). KRP203 demonstrated adequate tolerance and safety. While KRP203 was shown to be minimally effective with regards to the clinically relevant threshold (novel Bayesian trial design), a 14% in the KRP203 group achieved clinical remission in comparison a 0% in the placebo group (Radeke et al., 2016; Perez-Jeldres et al., 2018). Based on the results of this small study, further development of KRP-203 for ulcerative colitis (UC) was terminated (ClinicalTrials.gov, ClinicalTrials, 2018a).

Ozanimod/RPC1063 (Celgene™) is a S1PR agonist, with enhanced selectivity for S1PR1 and S1PR5 (Perez-Jeldres et al., 2018). Ozanimod is metabolized in humans to form one major active metabolite (CC-112273) and other minor active metabolites (RP101988 and RP101075). CC-112273 is responsible for much of the total activity of ozanimod in human with similar potency and selectivity to ozanimod to S1P1 and S1P5 (Scott et al., 2016). Ozanimod is eliminated primarily via biotransformation, followed by biliary excretion. Renal excretion is limited (Hemperly et al., 2018). Its half-life is 19 h, thus upon drug discontinuation, lymphocyte counts return to normal within 72 h (Scott et al., 2016; Hemperly et al., 2018; seekingalpha.com, 2018). However, new data show that the effect could be prolonged by the metabolite CC112273, which has a long 10–13 day half-life, reducing the competitive advantage of ozanimod on the key safety feature of the lymphocyte recovery profile (Scott et al., 2016; Hemperly et al., 2018; seekingalpha.com, 2018). Currently, there is an ongoing phase 1, randomized, parallel-group, open-label study to evaluate the effect of the modulators of the cytochrome P450 (CYP) 2C8 and/or 3A on the single-dose pharmacokinetics of ozanimod and CC112273 in healthy adult subjects (Scott et al., 2016; ClinicalTrials.gov, ClinicalTrials, 2018r; seekingalpha.com, 2018). The TOUCHSTONE phase 2 trial randomized 197 adults with moderate-to-severe UC to either ozanimod 0.5 or 1 mg or placebo daily for up to 32 weeks (Sandborn et al., 2016; White et al., 2018). After 8 weeks induction, 13.8 and 16.4% of patients (0.5 and 1 mg, respectively) reported clinical remission, versus 6.2% in the placebo group. At the same time, clinical response rates were achieved in 57, 54, and 37% for 1, 0.5, and placebo groups, respectively. Mucosal improvement/healing was observed in approximately 30% of the patients treated in each dose group in comparison with 12% in the placebo group. Moreover, at 32 weeks there was an observed improvement in the rates of clinical remission (21, 26, and 6% for 1, 0.5, and placebo, respectively), and 51% of the patients treated with 1 mg had a clinical response, versus 35 and 20% in the groups treated with 0.5 mg and placebo, respectively. Mucosal improvement/healing did not show major differences in comparison with 8 weeks. Ozanimod treatment (1 mg/kg dose) was associated with a greater proportion of histological remission (defined as a Geboes score ≤ 2) at both 8 and 32 weeks (Sandborn et al., 2016). The long-term follow-up of patients involved in TOUCHSTONE study demonstrated that ozanimod was safe, effective, and well tolerated (Sandborn et al., 2016; White et al., 2018).

Initial results of a phase 2, open-label study in 69 patients treated with ozanimod for moderate-to-severe CD demonstrated a meaningful clinical improvement at week 4 and endoscopic improvement at week 12 (Feagan et al., 2017). Phase 3 studies investigating the role of ozanimod in IBD are in progress (White et al., 2018).

Etrasimod/APD334 (Arena™) is a S1PR1,4,5 selective agonist. Preliminary data from phase 2 OASIS trial in moderate-to-severe UC were reported (prnewswire.com, 2018). The primary objective, defined as an improvement in 3-component Mayo Clinic Score (stool frequency, rectal bleeding, and endoscopy), was met at 12 weeks. In addition, the 2 mg group achieved significant endoscopic improvement compared with placebo (41.8 versus 17.8%), and also the clinical remission was significant in the 2 mg group compared with placebo (24.5 versus 6.0%). Etrasimod was well tolerated and few patients had serious adverse events (SAEs). Arena plans to start a Phase 3 trial for UC (prnewswire.com, 2018).

Amiselimod/MT-1303 (Mitsubishi Tanabe Pharma Corporation™) is an oral selective S1PR1,5 receptor developed for the therapy of autoimmune/immune-mediated disorders. The efficacy and safety of MT-1303 were studied in a phase 2 trial in CD, but the results have not yet been published (ClinicalTrials.gov, ClinicalTrials, 2018c,d). Amiselimod was also investigated for UC, MS, and other immune-mediated diseases; however, its development was discontinued (Peyrin-Biroulet et al., 2017a).

Safety and Adverse Events

Infections

In general, S1PR modulators maintain immune surveillance against pathogens because their effects on effector memory T cell traffic are limited (Perez-Jeldres et al., 2018). However, serious infections, such as disseminated varicella zoster and herpes are rare, but have been reported with fingolimod (Pelletier and Hafler, 2012). In post-marketing surveillance studies, there have been cases of progressive multifocal leukoencephalopathy (PML) and cryptococcal meningitis with the treatment of fingolimod, and without previous use of natalizumab (Olivera et al., 2016; fda.gov, 2018). However, it is necessary to emphasize that the risk to develop PML is low with fingolimod in the absence of prior natalizumab treatment. It is estimated that the risk with fingolimod use is less 1:10,000 patients. The Novartis safety database has identified 15 cases of PML with the use of fingolimod in monotherapy, as of August 2017 (Berger et al., 2018).

Cardiovascular Events

Reported cardiovascular events include transient bradycardia, atrioventricular block, and hypertension with the fingolimod use (Olivera et al., 2016; Sandborn et al., 2016). These side effects are attributed to S1PR2 and S1PR3 modulation. The development of selective S1PR1 modulators could theoretically bypass these side effects. However, S1PR1

is found in atrial cardiomyocytes, leading to a dose-dependent reduction in heart rate. In the TOUCHSTONE trial, a patient with preexisting bradycardia developed an asymptomatic, transient bradycardia, and first-degree AV block. The episode was self-limited without the need for treatment. These side effects could be minimized with a gradual dose titration regimen (Olivera et al., 2016; Sandborn et al., 2016).

Malignancy

Isolated cases of breast and skin cancer have been identified (Pelletier and Hafler, 2012). Squamous-cell carcinoma of the skin was reported in the TOUCHSTONE trial in one patient on 1 mg of ozanimod. This patient had also received mercaptopurine for more than 2 years (Sandborn et al., 2016).

Leukopenia

A dose-dependent and sustained decrease in lymphocyte count has been reported, consistent with the drug's MOA. However, it is reversible with drug discontinuation (Peyrin-Biroulet et al., 2017b).

Pregnancy

The teratogenicity risk of this group of drugs is unknown, so it is not recommended for use during pregnancy (Scott et al., 2016).

Others Adverse Events

Pulmonary disorders, elevated liver enzymes, and macular edema have been reported (Olivera et al., 2016).

Future Directions for the S1P Pathway

Ponesimod, Ceralifimod, Siponimod AUY954, SEW2871, AUY954, W061, CS-0777, and GSK2018682 are currently being investigated for use in other autoimmune/immune-mediated disorders (Park and Im, 2017). The pathways involved in the synthesis, degradation, and the mechanism of transport of these molecules represent an attractive new area of research (Perez-Jeldres et al., 2018).

Sphingosine Kinases

There are two isoforms of sphingosine kinase (SphK), SphK1 and SphK2. TNF induces SphK1 activation, leading to cyclooxygensase-2 (COX-2) expression and production of prostaglandin E2 (PGE2) that may contribute to mucosal inflammation (Pettus et al., 2003). Moreover, SphK1 expression was found to be elevated in both colonic epithelial cells and inflammatory cells in patients with UC patients correlating with COX2 overexpression (Snider et al., 2009). Data from mice indicate that the SphK1/S1P pathway participates in the development and maintenance of intestinal inflammation (Snider et al., 2009; Wollny et al., 2017). Thus, inhibition of this enzyme could represent a potential new target.

Sphingosine Phosphatase

This enzyme, expressed in the gastrointestinal tract, catalyzes dephosphorylation of S1P to sphingosine, resulting in regulation of S1P levels. Elevated sphingosine phosphatase expression has been demonstrated in colitis and contributes to its pathogenesis by disrupting barrier integrity, indicating that its inhibition may have beneficial effects in IBD (Huang et al., 2016).

S1P Lyase

Sphingosine-1-phosphate lyase degrades S1P irreversibly. This enzyme is abundant in tissues (Wollny et al., 2017), maintaining low levels S1P in the colonic mucosa in relation with the blood. This favors lymphocyte recirculation from the intestine back into circulation. Its inhibition may impair intestinal lymphocyte egress, but its effect still remains unclear with evidence that shows amelioration of DSS colitis, while other studies show worsening disease (Degagné et al., 2014; Shirakabe et al., 2018).

Spinster Homolog 2

The expression of this intra- and extracellular S1P transporter is upregulated in patients with IBD. Thus, it may represent another way to regulate S1P levels for therapeutic purposes (Miklossy et al., 2013).

Positioning of Small Molecules in the Therapeutic Algorithm of IBD

The choice of IBD treatment must be personalized according to the activity, severity, phenotype, preferences of the patients, comorbidities, history of the therapies used previously, and surgery (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018).

The current treatment for IBD is based on aminosalicylates, steroids, immunosuppressants, and biologic therapies (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). The 5-ASA compounds are used as first line in mild-to-moderate UC, and in some cases of IBD-associated arthritis (sulfasalazine). These drugs have an excellent safety profile. Immunosuppressants can be added during maintenance therapy in cases of moderate severity, or in combination with biologic therapy in moderate-to-severe cases due to their synergism or to decrease the immunogenicity of the biologic (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). In recent years, measuring drug and antibody levels has allowed optimization of biological therapies and assisted in avoiding misuse of biologics by under dosing or drug failure (absence of response despite adequate drug level) (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). The calcineurin inhibitors have a limited role in the treatment due their narrow therapeutic window and side effects. Thus, they are mostly being used as a bridge to another maintenance drug in cases of acute severe colitis refractory to corticosteroid. However, in this last case infliximab seems to be a better option, due to less toxicity in comparison with cyclosporine (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018).

New SM offers an alternative to the current therapeutic arsenal, especially in cases of steroid-resistance and cases of nonresponse and/or are intolerance to conventional therapies.

Precise positioning the new small drugs in the therapeutic armament for IBD is difficult in the absence of head-to-head randomized controlled trials. The SM have a role in the treatment of moderate-to-severe IBD due to the lack of immunogenicity, and potential intermittent "on-off" dosing without resultant antibody formation and loss of response.

Most information available is for tofacitinib, approved for UC moderate-to-severe active, being a good option in cases refractory to anti-TNFa. Its effectiveness in comparison with anti-TNF as first-line therapy in moderate-to-severe UC, their use as combination therapy for example with other drugs as vedolizumab, its sequential use with other drugs (for example, induction with tofacitinib, followed by vedolizumab), or even it uses in acute severe colitis refractory to steroids, must also be evaluated in clinical trials, before authoritative consensus recommendations. In the absence of head-to-head comparisons, the evidence favors the use of infliximab in hospitalized patients with acute severe colitis in perianal disease.

Furthermore, it is important to consider tofacitinib's safety profile and may be premature recommend its use in combination with immunomodulators, anti-TNFa, and/or cyclosporine, until additional safety information is available.

Improved knowledge of the mechanisms regulating disease, by genome sequencing analysis, improved comprehension of the immunological pathways, and further understanding of the role of the microbiome, may lead to new targets. In fact, it is possible that future therapies will be chosen not only by considering traditional patient characteristics, but also based on the patient's microbiome and immune genotype, as well as predictive modeling of drug responses validated prospectively.

CONCLUSION

Novel, orally available drugs represent a new and exciting option in the IBD therapeutic arsenal, showing efficacy and reasonable safety. However, more studies are required to define their safety related to infection, malignancy, and pregnancy. One of the clear advantages of SMs is their lack of immunogenicity and their short half-life which represents an advantage when adverse events may mandate interruption of therapy. Other advantages include the administration route, maintenance of T cell effector memory response, potentially lower manufacturing cost, and finally, the new agents are more receptor-specific (Perez-Jeldres et al., 2018). The positioning of these new drugs with relation to existing treatment paradigm remains uncertain.

AUTHOR CONTRIBUTIONS

JR-N, TP-J, WS conception of work. JR-N, TP-J design of work. JR-N, TP-J drafting of manuscript. JR-N, WS, DP, AY, DG, JB, TK, CT, SY, and LL critical revision of manuscript. JR-N, TP-J, WS, DP, AY, JB, TK, CT, DG, SY, and LL final approval of work. JR-N, TP-J, WS, DP, AY, JB, TK, CT, DG, LL,and SY agreement to be accountable for all aspects of presented work.

FUNDING

This work was funded by grants from the National Institutes of Health (DK108670) and VA Merit grant (BLRD-I01 BX003436).

REFERENCES

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2014a). *Cellular and Molecular Immunology. Chapter 3*. Amsterdam: Elsevier.

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2014b). *Cellular and Molecular Immunology. Chapter 7*. Amsterdam: Elsevier.

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2014c). *Cellular and Molecular Immunology. Chapter 10*. Amsterdam: Elsevier.

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2014d). *Cellular and Molecular Immunology. Chapter 15*. Amsterdam: Elsevier.

Abraham, C., and Cho, J. H. (2009). Inflammatory bowel disease. *New Engl. J. Med.* 361, 2066–2078. doi: 10.1056/NEJMra0804647

Banerjee, S., Biehl, A., Gadina, M., Hasni, S., and Schwartz, D. M. (2017). JAK–STAT signaling as a target for inflammatory and autoimmune diseases: current and future prospects. *Drugs* 77, 521–546. doi: 10.1007/s40265-017-0701-9

Barroso, N. S., Miller, E. Z., and Furst, D. E. (2017). A case of series on patients on tofacitinib in combinaton with a biologic. *J. Clin. Rheumatol.* 24, 349–351. doi: 10.1097/RHU.0000000000000663

Beattie, D., Tsuruda, P., Shen, F., Brassil, P., Langrish, C., Janc, J., et al. (2018). *P069 TD-1473, a Novel, Potent, and Orally Administered, GI-Targeted, Pan-Janus Kinase (JAK) Inhibitor*. Available at: https://www.ecco-ibd.eu/publications/congress-abstract-s/abstracts-2016/item/p069-td-1473-a-novel-potent-and-orally-administered-gi-targeted-pan-janus-kinase-jak-inhibitor.html [accessed August 26, 2018].

Berger, J. R., Cree, B. A., Greenberg, B., Hemmer, B., Ward, B. J., Dong, V. M., et al. (2018). Progressive multifocal leukoencephalopathy after fingolimod treatment. *Neurology* 90, e1815–e1821. doi: 10.1212/WNL.0000000000005529

Boland, B. S., and Vermeire, S. (2017). Janus kinase antagonists and other novel small molecules for the treatment of crohn's disease. *Gastroenterol. Clin. North Am.* 46, 627–644. doi: 10.1016/j.gtc.2017.05.015

Charles-Schoeman, C., Fleischmann, R., Davignon, J., Schwartz, H., Turner, S. M., Beysen, C., et al. (2015). Potential mechanisms leading to the abnormal lipid profile in patients with rheumatoid arthritis versus healthy volunteers and reversal by tofacitinib. *Arthritis Rheumatol.* 67, 616–625. doi: 10.1002/art.38974

Clark, J. D., Flanagan, M. E., and Telliez, J. (2013). Discovery and development of janus kinase (JAK) inhibitors for inflammatory diseases. *J. Med. Chem.* 57, 5023–5038. doi: 10.1021/jm401490p

ClinicalTrials.gov (2018a). *NCT01375179. A Multi-centre, Double-blind, Placebo Controlled, Parallel Group, Proof of Concept Study to Evaluate the Efficacy, Safety and Tolerability of KRP203 in Subjects With Moderately Active Refractory Ulcerative Colitis*. Available at: https://clinicaltrials.gov/ct2/show/NCT01375179 [accessed August 25, 2018].

ClinicalTrials.gov (2018b). *NCT01563302. Phase 1/2, Open-label, Dose-escalation Study of IONIS-STAT3Rx, Administered to PAtients with Advanced Cancers*. https://clinicaltrials.gov/ct2/show/results/NCT01563302 [accessed August 25, 2018].

ClinicalTrials.gov (2018c). *NCT02378688. Safety and Efficacy of MT-1303 in Subjects With Moderate to Severe Active Crohn's Disease*. Available at: https://clinicaltrials.gov/ct2/show/NCT02378688?term = MT-1303&cond = Inflammatory+Bowel+Diseases&rank = 4 [accessed August 20, 2018].

ClinicalTrials.gov (2018d). *NCT02389790. Extension Study of MT-1303 in Subjects With Crohn's Disease*. Available at: https://clinicaltrials.gov/ct2/show/NCT02389790?term = MT-1303&cond = Inflammatory+Bowel+Diseases&rank = 3 [accessed August 20, 2018].

ClinicalTrials.gov (2018e). *NCT02535689. Safety of Tofacitinib, an Oral Janus Kinase Inhibitor, in Systemic Lupus Erythematosus* Available at: https://clinicaltrials.gov/ct2/show/NCT02535689 [accessed August 12, 2018].

ClinicalTrials.gov (2018f). *NCT02818686. TD-1473 for Active Ulcerative Colitis (UC)*. Available at: https://clinicaltrials.gov/ct2/show/NCT02818686 [accessed August 26, 2018].

ClinicalTrials.gov (2018g). *NCT02819635. A Study to Evaluate the Safety and Efficacy of Upadacitinib (ABT-494) for Induction and Maintenance Therapy in Subjects with Moderately to Severely Active Ulcerative Colitis (UC)*. Available at: https://clinicaltrials.gov/ct2/show/NCT02819635?term = upaDACITINIBandcond = Inflammatory+Bowel+Diseasesandrank = 6 [accessed August 26, 2018].

ClinicalTrials.gov (2018h). *NCT02914522. Filgotinib in the Induction and Maintenance of Remission in Adults With Moderately to Severely Active Ulcerative Colitis (SELECTION1)*. Available at: https://clinicaltrials.gov/ct2/show/NCT02914522?term = filgotinibandcond = Inflammatory+Bowel+Diseasesandrank = 8 [accessed August 26, 2018].

ClinicalTrials.gov (2018i). *NCT02914535. Filgotinib in Long-Term Extension Study of Adults With Ulcerative Colitis (SELECTIONLTE)*. Available at: https://clinicaltrials.gov/ct2/show/NCT02914535?term = filgotinibandcond = Inflammatory+Bowel+Diseasesandrank = 7 [accessed August 26, 2018].

ClinicalTrials.gov (2018j). *NCT02914600. Filgotinib in Long-Term Extension Study of Adults With Crohn's Disease (DIVERSITYLTE)* Available at: https://clinicaltrials.gov/ct2/show/NCT02914600 [accessed August 26, 2018].

ClinicalTrials.gov (2018k). *NCT02958865. Study to Compare Oral PF-06651600, PF-06700841 and Placebo in Subjects with Moderate to Severe Ulcerative Colitis*. https://clinicaltrials.gov/ct2/show/NCT02958865 [accessed August 26, 2018].

ClinicalTrials.gov (2018l). *NCT03006068. A Study to Evaluate the Long-Term Safety and Efficacy of Upadacitinib (ABT-494) in Subjects with Ulcerative Colitis (UC)*. Available at: https://clinicaltrials.gov/ct2/show/NCT03006068?term = upaDACITINIBandcond = Inflammatory+Bowel+Diseasesandrank = 5 [accessed August 26, 2018].

ClinicalTrials.gov (2018m). *NCT03046056. Efficacy and Safety of Filgotinib in the Treatment of Small Bowel Crohn's Disease (SBCD)*. Available at: https://clinicaltrials.gov/ct2/show/NCT03046056?term = filgotinibandcond = Inflammatory+Bowel+Diseasesandrank = 1 [accessed August 26, 2018].

ClinicalTrials.gov (2018n). *NCT03100942.Safety and Efficacy Study of Filgotinib, GS-9876 and Tirabrutinib in Adults With Active Sjogren's Syndrome* Available at: https://clinicaltrials.gov/ct2/show/NCT03100942 [accesed August 25, 2018].

ClinicalTrials.gov (2018o). *NCT03345836. A Study of the Efficacy and Safety of Upadacitinib (ABT-494) in Subjects With Moderately to Severely Active Crohn's Disease Who Have Inadequately Responded to or Are Intolerant to Biologic Therapy*. Available at: https://clinicaltrials.gov/ct2/show/NCT03345836?term = upaDACITINIBandcond = Inflammatory+Bowel+Diseasesandrank = 1 [accessed August 26, 2018].

ClinicalTrials.gov (2018p). *NCT03345849. A Study of the Efficacy and Safety of Upadacitnib (ABT-494) in Subjects With Moderately to Severely Active Crohn's Disease Who Have Inadequately Responded to or Are Intolerant to Conventional Therapies But Have*. Available at: https://clinicaltrials.gov/ct2/show/NCT03345849?term = upaDACITINIBandcond = Inflammatory+Bowel+Diseasesandrank = 4 [accessed August 26, 2018].

ClinicalTrials.gov (2018q). *NCT03395184. Study to Evaluate the Efficacy and Safety of Oral PF-06651600 And PF-06700841 In Subjects with Moderate to Severe Crohn's Disease*. Available at: https://clinicaltrials.gov/ct2/show/NCT03395184?term = Pf-06700841andcond = Inflammatory+Bowel+Diseasesandrank = 1 [accessed August 26, 2018].

ClinicalTrials.gov (2018r). *NCT03624959. Drug-drug Interaction Study of Ozanimod With Inhibitor or Inducer of CYP2C8 and/or CYP3A*. Available at: https://seekingalpha.com/article/4173916-2-cents-ozanimod-sagahttps://clinicaltrials.gov/ct2/show/NCT03624959?term=CC-112273&rank=1 [accessed November 11, 2018].

Cohen, S., Curtis, J. R., DeMasi, R., Chen, Y., Fan, H., Soonasra, A., et al. (2018). Worldwide, 3-year, post-marketing surveillance experience with tofacitinib in rheumatoid arthritis. *Rheumatol. Ther.* 5, 283–291. doi: 10.1007/s40744-018-0097-3

Currò, D., Pugliese, D., and Armuzzi, A. (2017). Frontiers in drug research and development for inflammatory bowel disease. *Front. Pharmacol.* 8:400. doi: 10.3389/fphar.2017.00400

Daniel, C., Sartory, N., Zahn, N., Geisslinger, G., Radeke, H. H., and Stein, J. M. (2007). FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4+CD25+ regulatory T cells. *J. Immunol.* 178, 2458–2468. doi: 10.4049/jimmunol.178.4.2458

Degagné, E., Pandurangan, A., Bandhuvula, P., Kumar, A., Eltanawy, A., Zhang, M., et al. (2014). Sphingosine-1-phosphate lyase downregulation promotes colon carcinogenesis through STAT3-activated microRNAs. *J. Clin. Invest.* 124, 5368–5384. doi: 10.1172/JCI74188

Deguchi, Y., Andoh, A., Yagi, Y., Bamba, S., Inatomi, O., Tsujikawa, T., et al. (2006). The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice. *Oncol. Rep.* 16, 699–703. doi: 10.3892/or.16.4.699

Dienz, O., and Rincon, M. (2009). The effects of IL-6 on CD4 T cell responses. *Clin. Immunol.* 130, 27–33. doi: 10.1016/j.clim.2008.08.018

Divito, S. J., and Kupper, T. S. (2014). Inhibiting Janus kinases to treat alopecia areata. *Nat. Med.* 20, 989–990. doi: 10.1038/nm.3685

Dowty, M. E., Lin, J., Ryder, T. F., Wang, W., Walker, G. S., Vaz, A., et al. (2014). The pharmacokinetics, metabolism and clearance mechanisms of tofacitinib, a janus kinase inhibitor, in humans. *Drug Metab. Dispos.* 42, 759–773. doi: 10.1124/dmd.113.054940

fda.gov (2018). *FDA. FDA Drug Safety Communication: FDA Warns About Cases of Rare Brain Infection with MS Drug Gilenya (fingolimod) in Two Patients With no Prior Exposure to Immunosuppressant Drugs*. Available at: https://www.fda.gov/Drugs/DrugSafety/ucm456919.htm [accessed August 20, 2018].

Feagan, B. G., Sandborn, W. J., Danese, S., D'Haens, G., Levesque, B. G., Wolf, D. C., et al. (2017). *P1272 - Endoscopic and Clinical Efficacy Demonstrated With Oral Ozanimod in Moderately to Severely Active Crohn's Disease. WCG at ACG*. Available at: http://gi.org/wp content/uploads/2017/02/ACG17Exhibitor-Prospectus-lo.pdf [accessed August 26, 2018].

Flamant, M., Rigaill, J., Paul, S., and Roblin, X. (2017). Advances in the development of janus kinase inhibitors in inflammatory bowel disease: future prospects. *Drugs* 77, 1057–1068. doi: 10.1007/s40265-017-0755-8

Gonzalez-Cabrera, P. J., Brown, S., Studer, S. M., and Rosen, H. (2014). S1P signaling: new therapies and opportunities. *F1000 Prime Rep*. 6:109. doi: 10.12703/P6-109

Hemperly, A., Sandborn, W. J., and Vande Casteele, N. (2018). Clinical pharmacology in adult and pediatric inflammatory bowel disease. *Inflamm. Bowel Dis.* doi: 10.1093/ibd/izy189 [Epub ahead of print].

Horga, A., and Montalban, X. (2008). FTY720 (fingolimod) for relapsing multiple sclerosis. *Expert Rev. Neurother.* 8, 699–714. doi: 10.1586/14737175.8.5.699

Hornung, T., Janzen, V., Heidgen, F. J., Wolf, D., Bieber, T., Wenzel, J., et al. (2014). Remission of recalcitrant dermatomyositis treated with ruxolitinib. *N. Engl. J. Med.* 371, 1324–1331. doi: 10.1056/NEJMc1412997

Hsu, L., and Armstrong, A. W. (2014). JAK inhibitors: treatment efficacy and safety profile in patients with psoriasis. *J. Immunol. Res.* 2014:283617. doi: 10.1155/2014/283617

Hu, X., and Ivashkiv, L. B. (2009). Cross-regulation of signaling and immune responses by IFN-γ and STAT1. *Immunity* 31, 539–550. doi: 10.1016/j.immuni.2009.09.002

Huang, W. C., Liang, J., Nagahashi, M., Avni, D., Yamada, A., Maceyka, M., et al. (2016). Sphingosine-1-phosphate phosphatase 2 promotes disruption of mucosal integrity and contributes to ulcerative colitis in mice and humans. *FASEB J.* 30, 2945–2958. doi: 10.1096/fj.201600394R

Huwiler, A., and Zangemeister-Wittke, U. (2018). The sphingosine 1-phosphate receptor modulator fingolimod as a therapeutic agent: recent findings and new perspectives. *Pharmacol. Ther.* 185, 34–49. doi: 10.1016/j.pharmthera.2017.11.001

Jabeen, R., Miller, L., Yao, W., Gupta, S., Steiner, S., and Kaplan, M. H. (2015). Altered STAT4 isoform expression in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* 21, 2383–2392. doi: 10.1097/MIB.0000000000000495

Jatiani, S. S., Baker, S. J., Silverman, L. R., and Premkumar Reddy, E. (2010). JAK/STAT pathways in cytokine signaling and myeloproliferative disorders: approaches for targeted therapies. *Genes Cancer* 1, 979–993. doi: 10.1177/1947601910397187

Kang, E. H., Liao, K. P., and Kim, S. C. (2018). Cardiovascular safety of biologics and JAK inhibitors in patients with rheumatoid arthritis. *Curr. Rheumatol. Rep.* 20:42. doi: 10.1007/s11926-018-0752-2

Kimura, A., Rieger, M. A., Simone, J. M., Chen, W., Wickre, M. C., Zhu, B. M., et al. (2009). The transcription factors STAT5A/B regulate GM-CSF-mediated granulopoiesis. *Blood* 114, 4721–4728. doi: 10.1182/blood-2009-04-216390

Kornbluth, A., and Sachar, D. B. (2010). Practice parameters committee of the american college of gastroenterology. *Am. J. Gastroenterol.* 105, 501–523. doi: 10.1038/ajg.2009.727

Le Stunff, H., Milstien, S., and Spiegel, S. (2004). Generation and metabolism of bioactive sphingosine-1-phosphate. *J. Cell. Biochem.* 92, 882–899. doi: 10.1002/jcb.20097

Levy, L. L., Urban, J., and King, B. A. (2015). Treatment of recalcitrant atopic dermatitis with the oral Janus kinase inhibitor tofacitinib citrate. *J. Am. Acad. Dermatol.* 73, 395–399. doi: 10.1016/j.jaad.2015.06.045

Lichtenstein, G. R., Loftus, E. V., Bloom, S., Lawendy, N., Friedman, G. S., Zhang, H., et al. (2017). Tofacitinib, an oral Janus Kinase inhibitor, in the treatment of ulcerative colitis: open-label, long-term extension study. *United Eur. Gastroenterol. J.* 5, A39–A40. doi: 10.1016/j.cgh.2018.11.035

Lichtenstein, G. R., Loftus, E. V., Isaacs, K. L., Regueiro, M. D., Gerson, L. B., and Sands, B. E. (2018). ACG clinical guideline: management of crohn's disease in adults. *Am J Gastroenterol.* 113, 481–517. doi: 10.1038/ajg.2018.27

Liu, L. Y., Strassner, J. P., Refat, M. A., Harris, J. E., and King, B. A. (2017). Repigmentation in vitiligo using the Janus kinase inhibitor tofacitinib may require concomitant light exposure. *J. Am. Acad. Dermatol.* 77, 675.e1–682.e1. doi: 10.1016/j.jaad.2017.05.043

Mahadevan, U., Dubinsky, M. C., Su, C., Lawendy, N., Jones, T. V., Marren, A., et al. (2018). Outcomes of pregnancies with maternal/paternal exposure in the tofacitinib safety databases for ulcerative colitis. *Inflamm. Bowel Dis.* doi: 10.1093/ibd/izy160 [Epub ahead of print].

Miklossy, G., Hilliard, T. S., and Turkson, J. (2013). Therapeutic modulators of STAT signalling for human diseases. *Nat. Rev. Drug Discov.* 12, 611–629. doi: 10.1038/nrd4088

Mizushima, T., Ito, T., Kishi, D., Kai, Y., Tamagawa, H., Nezu, R., et al. (2004). Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. *Inflamm. Bowel Dis.* 10, 182–192. doi: 10.1097/00054725-200405000-00002

Montor, W. R., Salas, A. R. O. S. E., and Melo, F. H. M. (2018). Receptor tyrosine kinases and downstream pathways as druggable targets for cancer treatment: the current arsenal of inhibitors. *Mol. Cancer* 17, 1–18. doi: 10.1186/s12943-018-0792-2

Montrose, D. C., Scherl, E. J., Bosworth, B. P., Zhou, X. K., Jung, B., Dannenberg, A. J., et al. (2013). S1P1 localizes to the colonic vasculature in ulcerative colitis and maintains blood vessel integrity. *J. Lipid Res.* 54, 843–851. doi: 10.1194/jlr.M034108

Murphy, A. G., and Zheng, L. (2015). Small molecule drugs with immunomodulatory effects in cancer. *Hum. Vaccin. Immunother.* 11, 2463–2468. doi: 10.1080/21645515.2015.1057363

Olivera, P., Danese, S., and Peyrin-biroulet, L. (2016). Next generation of small molecules in inflammatory bowel disease. *Gut* 66, 199–209. doi: 10.1136/gutjnl-2016-312912

Olivera, P., Danese, S., and Peyrin-Biroulet, L. (2017). JAK inhibition in inflammatory bowel disease. *Expert Rev. Clin. Immunol.* 13, 693–703. doi: 10.1080/1744666X.2017.1291342

Ordás, I., Mould, D. R., Feagan, B. G., and Sandborn, W. J. (2012). Anti-TNF monoclonal antibodies in inflammatory bowel disease: pharmacokinetics-based dosing paradigms. *Clin. Pharmacol. Ther.* 91, 635–646. doi: 10.1038/clpt.2011.328

Panes, J., and Alfaro, I. (2017). New treatment strategies for ulcerative colitis. *Expert Rev. Clin. Immunol.* 13, 963–973. doi: 10.1080/1744666X.2017.1343668

Panés, J., Sandborn, W. J., Schreiber, S., Sands, B. E., Vermeire, S., D'Haens, G., et al. (2017). Tofacitinib for induction and maintenance therapy of Crohn's disease: results of two phase IIb randomised placebo-controlled trials. *Gut* 66, 1049–1059. doi: 10.1136/gutjnl-2016-312735

Park, S. J., and Im, D. S. (2017). Sphingosine 1-phosphate receptor modulators and drug discovery. *Biomol. Ther.* 25, 80–90. doi: 10.4062/biomolther.2016.160

Pelletier, D., and Hafler, D. A. (2012). Fingolimod for multiple sclerosis. (2012). *N. Engl. J. Med.* 366, 339–347. doi: 10.1056/NEJMct1101691

Perez-Jeldres, T., Tyler, C. J., Boyer, D. J., Karuppuchamy, T., Bamias, G., Dulai, P. S., et al. (2018). Cell trafficking interference in inflammatory bowel disease: therapeutic interventions based on pathogenesis concepts. *Inflamm. Bowel Dis.* doi: 10.1093/ibd/izy269 [Epub ahead of print].

Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., et al. (2003). The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J.* 17, 1411–1421. doi: 10.1096/fj.02-1038com

Peyrin-Biroulet, L., Christopher, R., Behan, D., and Lassen, C. (2017a). Modulation of sphingosine-1-phosphate in inflammatory bowel disease. *Autoimmun. Rev.* 16, 495–503. doi: 10.1016/j.autrev.2017.03.007

Peyrin-Biroulet, L., Christopher, R., Trokan, L., Lassen, C., Adams, J., and Kühbacher, T. (2017b). *Safety, Tolerability and Lymphocyte-Lowering Properties of Etrasimod (APD334), an Oral, Potent, Next-Generation, Selective S1P Receptor Modulator, After Dose Escalation in Healthy Volunteer*. Available at: http://www.arenapharm.com/wp-content/uploads/2017/05/ECCO-Poster-Safety-and-Lymphocyte-Lowering-Final.pdf. [accessed August 26, 2018].

prnewswire.com (2018). *Arena Pharmaceuticals Reports Positive Phase 2 Results from the OASIS Trial for Etrasimod in Patients with Ulcerative Colitis*. Available at: https://www.prnewswire.com/news-releases/arena-pharmaceuticals-reports-positive-phase-2-results-from-the-oasis-trial-for-etrasimod-in-patients-with-ulcerative-colitis-300616131.html. [accessed August 20, 2018].

Radeke, H. H., Stein, J., and Kruis, W. (2016). P372. A multicentre, double-blind, placebo-controlled, parallel group, proof of concept study to evaluate the efficacy, safety and tolerability of the S1P receptor modulator KRP203 in subjects with moderately active refractory ulcerative colitis. *J. Crohn's Colitis* 10, S285–S286.

Radi, Z. A., Heuvelman, D. M., Masferrer, J. L., and Benson, E. L. (2011). Pharmacologic evaluation of sulfasalazine, FTY720, and anti-IL-12/23p40 in a TNBS-induced Crohn's disease model. *Dig. Dis. Sci.* 56, 2283–2291. doi: 10.1007/s10620-011-1628-8

Samanen, J. (2013). "Similarities and differences in the discovery and use of biopharmaceuticals and small-molecule chemotherapeutics," in *Introduction to Biological and Small Molecule Drug Research and Development: Theory and Case Studies*, eds R. Ganellin, S. Roberts, and R. Jefferis (Waltham, MA: Elsevier).

Sanchez, T., and Hla, T. (2004). Structural and functional characteristics of S1P receptors. *J. Cell. Biochem.* 92, 913–922. doi: 10.1002/jcb.20127

Sandborn, W. J., Bhandari, R., Leighton, J., Ganeshappa, R., Nguyen, D., Ferslew, B., et al. (2018a). *The Intestinally Restricted, Orally Administered, Pan-Jak Inhibitor TD-1473 Demonstrate Favorable Safety, Tolerability, Pharmacokinetics, and Signal for Clinical Activity in Subjects With Moderately-to-Severely Active Ulcerative Colitis*. Available at: https://www.ueg.eu/education/document/the-intestinally-restricted-orally-administered-pan-jak-inhibitor-td-1473-demonstrates-favorable-safety-tolerability-pharmacokinetics-and-signal-for-clinical-activity-in-subjects-with-moderately-to-severely-active-ulcerative-colitis/180035/ [accessed November 8, 2018].

Sandborn, W. J., Feagan, B. G., Panes, J., D'Haens, G. R., Colombel, J. F., Zhou, Q., et al. (2017b). Safety and efficacy of ABT-494 (Upadacitinib), an oral jak1 inhibitor, as induction therapy in patients with crohn's disease: results from Celest. *Gastroenterology* 152, S1308–S1309. doi: 10.1016/S0016-5085(17)34357-3

Sandborn, W. J., Feagan, B. G., Wolf, D. C., D'Haens, G., Vermeire, S., Hanauer, S. B., et al. (2016). Ozanimod induction and maintenance treatment for ulcerative colitis. *N. Engl. J. Med.* 374, 1754–1762. doi: 10.1056/NEJMoa1513248

Sandborn, W. J., Ghosh, S., Panes, J., Schreiber, S., D'Haens, G., et al. (2018b). *Efficacy and Safety of Upadacitinib as an Induction Therapy For Patients With Moderately-to Severely Active Ulcerative Colitis: Data From the Phase 2B*

Study U-ACHIEVE. Available at: https://www.ueg.eu/education/document/efficacy-and-safety-of-upadacitinib-as-an-induction-therapy-for-patients-with-moderately-to-severely-active-ulcerative-colitis-data-from-the-phase-2b-study-u-achieve/180240/ [accessed November 8, 2018].

Sandborn, W. J., Ghosh, S., Panes, J., Vranic, I., Wang, W., and Niezychowski, W. (2014). A phase 2 study of Tofacitinib, an oral janus kinase inhibitor, inpatients with crohn's disease. *Clin. Gastroenterol. Hepatol.* 12, 1485.e2–1493.e2. doi: 10.1016/j.cgh.2014.01.029

Sandborn, W. J., Su, C., Sands, B. E., D'Haens, G. R., Vermeire, S., Schreiber, S., et al. (2017a). Tofacitinib as induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 376, 1723–1736. doi: 10.1056/NEJMoa1606910

Sandborn, W. J., Subatra, G., Panes, J., Vranic, I., Su, C., Rousell, S., et al. (2012). Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *New Engl. J. Med.* 367, 616–624. doi: 10.1056/NEJMoa1112168

Sands, B. E., Sandborn, W. J., Feagan, B. G., Lichtenstein, G. R., Zhang, H., Strauss, R., et al. (2018). Peficitinib, an oral janus kinase inhibitor, in moderate-to-severe ulcerative colitis: results from a randomized, phase 2 study. *J. Crohns Colitis* doi: 10.1093/ecco-jcc/jjy085 [Epub ahead of print].

Schwartz, D. M., Kanno, Y., Villarino, A., Ward, M., Gadina, M., and O'Shea, J. J. (2017). JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nat. Rev. Drug Discov.* 16, 843–862. doi: 10.1038/nrd.2017.201

Scott, F. L., Clemons, B., Brooks, J., Brahmachary, E., Powell, R., Dedman, H., et al. (2016). Ozanimod (RPC1063) is a potent sphingosine-1-phosphate receptor-1 (S1P1) and receptor-5 (S1P5) agonist with autoimmune disease-modifying activity. *Br. J. Pharmacol.* 173, 1778–1792. doi: 10.1111/bph.13476

seekingalpha.com (2018). *My 2 Cents On the Ozanimod Saga*. Available at: https://seekingalpha.com/article/4173916-2-cents-ozanimod-saga [accessed November 11, 2018].

Shirakabe, K., Higashiyama, M., Furuhashi, H., Takajo, T., Maruta, K., Okada, Y., et al. (2018). Amelioration of colitis through blocking lymphocytes entry to Peyer's patches by sphingosine-1-phosphate lyase inhibitor. *J. Gastroenterol. Hepatol.* doi: 10.1111/jgh.14092 [Epub ahead of print].

Snider, A. J., Kawamori, T., Bradshaw, S. G., Orr, K. A., Gilkeson, G. S., Hannun, Y. A., et al. (2009). A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. *FASEB J.* 23, 143–152. doi: 10.1096/fj.08-118109

Soendergaard, C., Bergenheim, F. H., Bjerrum, J. T., and Nielsen, O. H. (2018). Targeting JAK-STAT signal transduction in IBD. *Pharmacol. Ther.* 192, 100–111. doi: 10.1016/j.pharmthera.2018.07.003

Vanhoutte, F., Mazur, M., Voloshyn, O., Stanislavchuk, M., Van der Aa, A., Namour, F., et al. (2017). Efficacy, safety, pharmacokinetics, and pharmacodynamics of filgotinib, a selective Janus kinase 1 inhibitor, after short-term treatment of rheumatoid arthritis: results of two randomized Phase IIA trials. *Arthritis Rheumatol.* 69, 1949–1959. doi: 10.1002/art.40186

Verden, A., Dimbil, M., Kyle, R., Overstreet, B., and Hoffman, K. B. (2018). Analysis of spontaneous postmarket case reports submitted to the FDA regarding thromboembolic adverse events and JAK inhibitors. *Drug Saf.* 41, 357–361. doi: 10.1007/s40264-017-0622-2

Vermeire, S., Schreiber, S., Petryka, R., Kuehbacher, T., Hebuterne, X., Roblin, X., et al. (2017a). Clinical remission in patients with moderate-to-severe Crohn's disease treated with filgotinib (the FITZROY study): results from a phase 2, double-blind, randomised, placebo-controlled trial. *Lancet* 389, 266–275. doi: 10.1016/S0140-6736(16)32537-5

Vermeire, S., Van Der Aa, A., Jamoul, C., Tasset, P., Harrison, G., and D'Haens, R. (2017b). Effect of disease duration and location on clinical remission in Crohn's disease patients treated with Filgotinib, a selective JAK1 inhibitor: post-hoc analysis from the phase 2 FITZROY study. *United Eur. Gastroenterol. J.* 5, A1–A160.

Villarino, A. V., Kanno, Y., Ferdinand, J. R., and O'Shea, J. J. (2015). Mechanisms of Jak/STAT signaling in immunity and disease. *J. Immunol.* 194, 21–27. doi: 10.4049/jimmunol.1401867

Villarino, A. V., Kanno, Y., and O'Shea, J. J. (2017). Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat. Immunol.* 18, 374–384. doi: 10.1038/ni.3691

White, J. R., Phillips, F., Monaghan, T., Fateen, W., Samuel, S., Ghosh, S., et al. (2018). Review article: novel oral-targeted therapies in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 47, 1610–1622. doi: 10.1111/apt.14669

Winthrop, K. L. (2017). The emerging safety profile of JAK inhibitors in rheumatic disease. *Nat. Rev. Rheumatol.* 13, 234–243. doi: 10.1038/nrrheum.2017.23

Wollny, T., Wątek, M., Durnaś, B., Niemirowicz, K., Piktel, E., Żendzian-Piotrowska, M., et al. (2017). Sphingosine-1-phosphate metabolism and its role in the development of inflammatory bowel disease. *Int. J. Mol. Sci.* 18:741. doi: 10.3390/ijms1804074

Yarur, A. J., and Rubin, D. T. (2015). Therapeutic drug monitoring of anti-tumor necrosis factor agents in patients with inflammatory bowel diseases. *Inflamm. Bowel Dis.* 21, 1709–1718. doi: 10.1097/MIB.0000000000000380

Zhang, H., Watanabe, R., Berry, G. J., Tian, L., Goronzy, J. J., and Weyand, C. M. (2018). Inhibition of JAK-STAT signaling suppresses pathogenic immune responses in medium and large vessel vasculitis. *Circulation* 137, 1934–1948. doi: 10.1161/CIRCULATIONAHA.117.030423

Zhou, L., Ivanov, I. I., Spolski, R., Min, R., Shenderov, K., Egawa, T., et al. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8, 967–974. doi: 10.1038/ni1488

Conflict of Interest Statement: WS reports research grants from Atlantic Healthcare Limited, Amgen, Genentech, Gilead Sciences, Abbvie, Janssen, Takeda, Lilly, Celgene/Receptos; consulting fees from Abbvie, Allergan, Amgen, Boehringer Ingelheim, Celgene, Conatus, Cosmo, Escalier Biosciences, Ferring, Genentech, Gilead, Gossamer Bio, Janssen, Lilly, Miraca Life Sciences, Nivalis Therapeutics, Novartis Nutrition Science Partners, Oppilan Pharma, Otsuka, Paul Hastings, Pfizer, Precision IBD, Progenity, Prometheus Laboratories, Ritter Pharmaceuticals, Robarts Clinical Trials (owned by Health Academic Research Trust or HART), Salix, Shire, Seres Therapeutics, Sigmoid Biotechnologies, Takeda, Tigenix, Tillotts Pharma, UCB Pharma, Vivelix; and stock options from Ritter Pharmaceuticals, Oppilan Pharma, Escalier Biosciences, Gossamer Bio, Precision IBD, Progenity. AJ-R is consultant and part of the speaker bureau for Prometheus Laboratories and employee of Prometheus Laboratories. DP has been a speaker bureau ABBVIE.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 19 March 2019
doi: 10.3389/fphar.2019.00247

Is There a Risk of Lymphoma Associated With Anti-tumor Necrosis Factor Drugs in Patients With Inflammatory Bowel Disease? A Systematic Review of Observational Studies

Sara Ferraro[1], *Luca Leonardi*[1], *Irma Convertino*[1], *Corrado Blandizzi*[1,2] *and Marco Tuccori*[1,2*]

[1] *Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy,* [2] *Unit of Adverse Drug Reaction Monitoring, University Hospital of Pisa, Pisa, Italy*

OPEN ACCESS

Edited by:
Luca Antonioli,
University of Pisa, Italy

Reviewed by:
Emanuel Raschi,
University of Bologna, Italy
Janet Sultana,
University of Messina, Italy

****Correspondence:***
Marco Tuccori
m.tuccori@ao-pisa.toscana.it;
marco.tuccori@gmail.com

Specialty section:
This article was submitted to Inflammation Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *08 January 2019*
Accepted: *26 February 2019*
Published: *19 March 2019*

Citation:
Ferraro S, Leonardi L, Convertino I, Blandizzi C and Tuccori M (2019) Is There a Risk of Lymphoma Associated With Anti-tumor Necrosis Factor Drugs in Patients With Inflammatory Bowel Disease? A Systematic Review of Observational Studies. Front. Pharmacol. 10:247. doi: 10.3389/fphar.2019.00247

Background: Inflammatory bowel diseases (IBDs) are generally not considered a risk factor for the development of lymphoma. When considering IBD treatments, there is good evidence supporting thiopurines (azathioprine, 6-mercaptopurine) as a risk factor for lymphoma. Conversely, the association between the use of anti-TNF agents and the development of lymphoma remains undetermined. In this systematic review, we analyzed the evidence coming from observational studies supporting an association between the use of anti-TNF drugs and lymphoma in patients with IBDs.

Methods: This systematic review was performed according with MOOSE and PRISMA statements. We searched observational studies conducted on IBD patients, using MEDLINE, EMBASE, and Google Scholar, published in English language, within the period ranging from January 1st, 1999 to June 30th, 2018. An assessment of the methodologic shortcomings of selected studies was performed as well.

Results: Fourteen studies met the eligibility criteria and were included in the review. Only four studies found a significant association of anti-TNF drug with lymphoma or groups of cancers including lymphoma. However, the methodologic shortcomings of all the included studies made their results unreliable, irrespectively of whether their findings supported an association or not.

Conclusions: Current evidence from observational studies does not allow excluding or confirming an association of the exposure to anti-TNF treatments with lymphoma in IBD patients.

Keywords: anti-TNF, lymphoma, observational study, inflammatory bowel disease, Crohn's disease, ulcerative colitis

INTRODUCTION

The term "inflammatory bowel disease" (IBD) describes a group of immune disorders characterized by chronic inflammation of the digestive tract. The main types of IBD include ulcerative colitis (UC) and Crohn's disease (CD) (Khor et al., 2011). The major complications associated with IBDs are thrombosis, primary sclerosing cholangitis, skin, eye and joint inflammation and even colonic cancer (Rothfuss et al., 2006). When considering other neoplastic complications, there is some evidence that chronic inflammation might be a risk factor for lymphoma (Ekström Smedby et al., 2008). However, at variance with other immune-mediated inflammatory disorders, such as rheumatoid arthritis (RA) (Simon et al., 2015; Mercer et al., 2017), the evidence supporting an association of IBDs with the development of lymphoma is still scarce (Williams et al., 2014). Furthermore, some authors suggested that pharmacological treatments for IBDs and RA (e.g., anti-tumor necrosis factor drugs, TNF), could promote the development of lymphoma (Herrinton et al., 2011; Parakkal et al., 2011). However, owing to the intrinsic risk for background diseases, it is difficult to establish an association between pharmacological treatments and the onset of lymphoma in these categories of patients, as well as to identify clear underlying determinants of biological plausibility (Baecklund et al., 2014).

When IBDs treatments have been considered in details, thiopurines (azathioprine, 6-mercaptopurine) were found to increase the risk of lymphoma (Kotlyar et al., 2015), while the association between the use of anti-TNF agents and the development of lymphoma remains questionable (Herrinton et al., 2011; Lichtenstein et al., 2012; Nyboe Andersen et al., 2014; Williams et al., 2014). A disproportionality analysis conducted on suspected adverse drug reactions recorded in the FDA Adverse Event Reporting System (FAERS) on patients with IBD, suggested a signal of risk for thiopurines, alone or in combination with anti-TNF drugs, but not with anti-TNF drugs alone (Deepak et al., 2013). Moreover, data from randomized clinical trials (RCT) are generally conflicting, likely because of the long term and rarity of the outcome (Chen et al., 2016). Likewise, available observational studies have provided conflicting results. In this regard, a recently published observational study (Lemaitre et al., 2017) showed a significant risk of lymphoma in patients with IBDs receiving anti-TNF monotherapy, thiopurine monotherapy or combination therapies, as compared with unexposed patients, thus fostering further the debate about the safety surrounding these treatments.

In light of the above-mentioned conflicting knowledge, we performed a systematic review of observational studies in patients with IBDs, focused on the association of lymphoma with the use of anti-TNF drugs, whatever the comparator, in order to analyze the solidity of evidence supporting this relationship.

METHODS

Search Strategy and Study Selection

The present systematic review was performed in accordance with PRISMA (Shamseer et al., 2015) and MOOSE (Stroup et al., 2000) statements. We conducted a literature search in MEDLINE, EMBASE, and Google Scholar by a combination of the following keywords: ("infliximab" OR "adalimumab" OR "certolizumab pegol" OR "golimumab") AND ("lymphoma"). We examined databases for all indexed articles, restricted to the English language, with publication dates falling in the period from January 1st, 1999 (year of infliximab approval) to June 30th, 2018. Duplicates were removed primarily by Mendeley auto-deduplication tool and then by manual assessment. Three reviewers (I.C., S.F., L.L.) examined the retrieved papers. The reviewers assessed the relevance of the collected studies by the title and abstract. If the study eligibility remained unclear, the full text was checked. Any disagreement was resolved by discussion with a senior reviewer (M.T.).

Study Inclusion and Exclusion Criteria

We included only observational studies that evaluated the risk of lymphoma associated with the four TNF inhibitors of interest, namely infliximab, adalimumab, certolizumab pegol and golimumab, currently approved for treatment of patients affected by CD and UC. In particular, studies were included only if they reported lymphoma incident rate ratio (95% Confidence Interval [CI]) or hazard ratio (95% CI) in CD or UC patients exposed to anti-TNF drugs. We did not consider any restriction about comparator groups. Notably the accepted studies reported lymphoma as specific (all types of lymphoma) or composite (i.e., lymphoma was included in a larger cluster of malignancy) outcomes. However, we included studies with composite endpoints only when they reported the overall number of lymphoma cases. Articles focused exclusively on special populations (i.e., pediatric patients) were excluded. Review articles, randomized trials, open-label extension studies, case series, articles based on questionnaires, case reports, unpublished studies, and conference abstracts were not included.

Study Classification and Definitions

The following information were extracted from each selected study: authors and publication year, type of source used to collect patient clinical data, design and main methodologic characteristics (information about adjustments and matchings, presence of lag period, inclusion of prevalent patients -considered as patients who were already users of the drug of interest at the cohort entry-), observation period, patient disease (IBD, CD or UC), and drug exposure. The observation period was defined by the time interval in which patients were followed for the outcomes of interest. The lag period was defined as the time window in which a patient should be considered "not exposed" to the potential risk factor (i.e., a drug) for the event of interest, since the temporal relationship would not be supportive of a causative role. Each study was read in full by two experts and the study designs methodology was assessed carefully by expert judgment, particularly for relevant biases, such as immortal time bias (Targownik and Suissa, 2015) and time-window bias (Suissa et al., 2011). Any disagreement was resolved with a third expert. We extracted also outcome measures [number of lymphoma events, number of person-year, incidence rate (95% CI) and

hazard ratio (95% CI) values], when available. This review was not submitted in advance to any public repository.

RESULTS

Figure 1 summarizes the search strategy and the selection process. Among 3,724 screened records, 3,684 were excluded after reviewing title and abstract. Forty-one full-text publications were assessed for eligibility. Overall, fourteen full-text articles met the eligibility criteria and were analyzed in detail. **Table 1** summarizes the characteristics of the included studies. Among the selected articles, five included lymphoma as a specific endpoint. Nine studies (Biancone et al., 2006, 2016; Fidder et al., 2009; Haynes et al., 2013; Beigel et al., 2014; Lichtenstein et al., 2014; Nyboe Andersen et al., 2014; Liu et al., 2015; D'Haens et al., 2017) performed an assessment of cancer risk that included lymphoma cases among the endpoints, but only two (Lichtenstein et al., 2014; Liu et al., 2015) of them provided a specific assessment of lymphoma (**Tables 2A,B**).

Lymphoma as Specific Outcome

Among the selected publications, seven studies (Herrinton et al., 2011; Afif et al., 2013; Lichtenstein et al., 2014; Kopylov et al., 2015; Liu et al., 2015; Lemaitre et al., 2017; D'Haens et al., 2018) assessed lymphoma as a specific outcome (**Table 2A**).

Only two (Herrinton et al., 2011; Lemaitre et al., 2017) out of the seven studies found a significant association of the drugs with the outcomes of interest, and they both examined a general exposure to the class of anti-TNF drugs. Both studies included prevalent patients. Only the study by Lemaitre et al. included a lag period in a sensitivity analysis (Lemaitre et al., 2017). Herrinton et al. estimated the exposure time for both anti-TNF drugs and thiopurines based on treatment coverage while the exposure time after treatment discontinuation was allocated to the unexposed group (Herrinton et al., 2011).

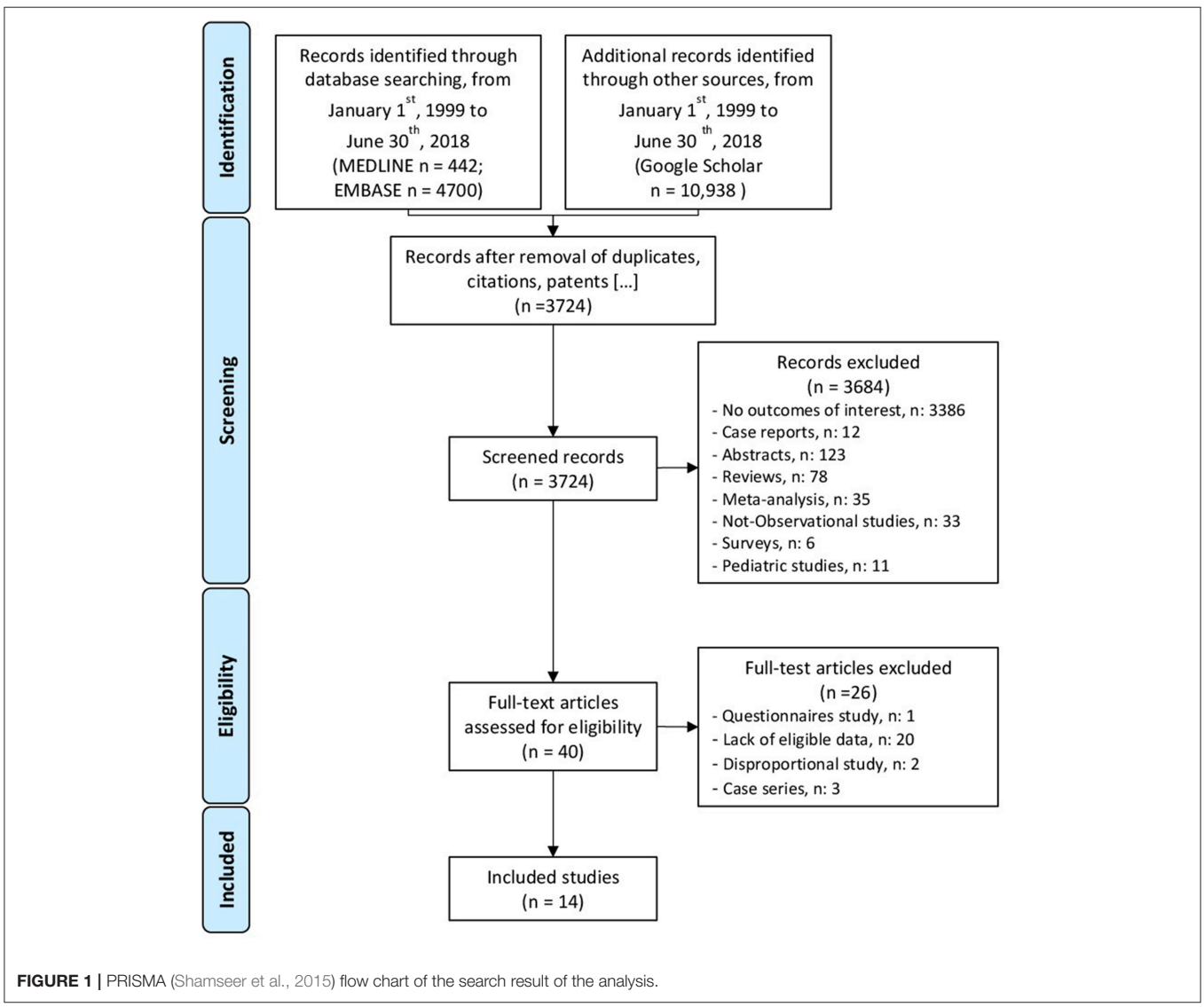

FIGURE 1 | PRISMA (Shamseer et al., 2015) flow chart of the search result of the analysis.

TABLE 1 | Characteristics of the selected studies.

References	Design	Data source	Observation Period	Patients (*n*, disease)
Biancone et al., 2006	Prospective matched pair cohort study	Italian IBD referral centers	1999–2004	◦ 404, CD patients exposed to infliximab ◦ 404, CD patients never exposed to infliximab
Fidder et al., 2009	Retrospective cohort study	Medical records of the Gasthuisberg University Hospital, Belgium	1994–2008	◦ 743, IBD patients exposed to infliximab ◦ 666, IBD patients unexposed to infliximab
Herrinton et al., 2011	Retrospective cohort study	Kaiser Permanente IBD and cancer registry	2000–2006	◦ 4,918, CD patients ◦ 9,499, UC patients ◦ 1,606, IBD not further specified patients
Afif et al., 2013	Nested case-control study	Mayo Clinic Rochester diagnostic index	1980–2009	◦ 80, lymphoma cases (44 CD, 36 UC) ◦ 159, IBD controls
Haynes et al., 2013	Retrospective cohort study	Medicare and Medicaid databases, Kaiser permanent Northern California Registry	1998–2007	◦ 2,657, IBD patients exposed to anti-TNF drugs ◦ 3,700, IBD patients unexposed to anti-TNF drugs
Nyboe Andersen et al., 2014	Prospective cohort study	Danish Nationwide Registry	1999–2012	◦ 4,553, IBD patients exposed to anti-TNF drugs ◦ 51,593, IBD patients unexposed to anti-TNF drugs
Beigel et al., 2014	Retrospective cohort study	Medical records and histopathological reports from a German IBD center	2000–2010	◦ 404, IBD exposed to anti-TNF drugs ◦ 262, IBD patients never exposed to anti-TNF drugs
Lichtenstein et al., 2014	Prospective cohort study	Crohn' s Therapy, Resource, Evaluation, and Assessment Tool (TREAT) Registry	1999–2010	◦ 3,420, CD patients exposed to infliximab ◦ 2,509, CD patients unexposed to infliximab
Kopylov et al., 2015	Nested case-control study	Québec health insurance claims database and registry	1996–2009	◦ 121 IBD, lymphoma cases ◦ 1,201 controls
Liu et al., 2015	Retrospective matched cohort study	Health Core Integrated Research Database, health insurance claims database	2007–2011	◦ 515 CD patients exposed to infliximab ◦ 515 CD patients exposed to adalimumab and certolizumab pegol
Biancone et al., 2016	Nested case-control study	Clinical records of Italian IBD referral centers	2012–2014	◦ 174 IBD malignancy cases (6 lymphoma) ◦ 378 IBD controls
D'Haens et al., 2017	Prospective cohort study	ENCORE Registry (European safety registry)	2003–2013	◦ 1,541 CD patients exposed to infliximab ◦ 1,121 CD patients initially unexposed to infliximab (298 switches to infliximab)
Lemaitre et al., 2017	Retrospective cohort study	SNIIRAM French National Health Insurance claim database	2009–2015	◦ 30,294 IBD patients exposed to anti-TNF drugs ◦ 50,405 IBD patients unexposed to anti-TNF drugs
D'Haens et al., 2018	Prospective cohort	Multicentre CD registry of adult patients treated with adalimumab: PYRAMID	2007–2015	◦ 5,025 CD patients exposed to adalimumab

IBD, inflammatory bowel disease; CD, crohn's disease; UC, ulcerative colitis.

The remaining five studies (Afif et al., 2013; Lichtenstein et al., 2014; Kopylov et al., 2015; Liu et al., 2015; D'Haens et al., 2018) assessing the specific risk of lymphoma in anti-TNF users did not find any association. Three of them evaluated the exposure to the overall anti-TNF class (Afif et al., 2013; Lichtenstein et al., 2014; Kopylov et al., 2015). Two (Afif et al., 2013; Kopylov et al., 2015) out of these three studies included prevalent patients and only one (Kopylov et al., 2015) considered a lag period. The results of all these studies are likely affected by time-related biases. One study (Kopylov et al., 2015) likely had no sufficient power to assess the risk of lymphoma. Only the study by D'Haens et al. (2018) assessed the risk of lymphoma for a specific anti-TNF drug (adalimumab). This study included prevalent patients, did not consider a lag period and assessed the risk of lymphoma in adalimumab patients without a comparison group, but comparing the rate of lymphoma with an estimated background lymphoma rate in the general population, adjusted for thiopurine use. Liu et al. (2015) estimated the frequency of lymphoma in two populations of anti-TNF users, stratified by the route of administration (infliximab and adalimumab/certolizumab pegol, respectively). This study included prevalent patients and was likely not powered enough to detect the risk of lymphoma (**Table 3**).

TABLE 2A | Studies with lymphoma as specific outcome.

References	Exposure	Events (*n*)	Person-year (PY)	Incident rate (95% CI)	Hazard ratio (95%)
Herrinton et al., 2011	Never anti-TNF and total (never, past, current) thiopurines	38	85.09	44.7 PY	SIR 1.0 (0.96–1.1)
	Past anti-TNF and total (never, past, current) thiopurines	3	2,217	135.3 PY	SIR 5.5 (4.5–6.6)
	Current anti-TNF and total (never, past, current) thiopurines	2	1,757	113.8 PY	SIR 4.4 (3.4–5.4)
Afif et al., 2013	Anti-TNF (infliximab, adalimumab)	9	NA	NA	OR: 2.04 (0.32–12.79)
	Unexposed	71	NA	NA	NA
Lichtenstein et al., 2014	Infliximab	8	17,712	0.05/100 PYs	HR 0.98 (0.34, 2.82) AdjHR: 0.59 (0.28, 1.22)
	Other-treatments-only	6	13,251	0.05/100 PYs	HR 1.00 (reference)
Kopylov et al., 2015	No use of TH/BIO/MTX	92	NA	NA	RR: 1.00
	TP and no BIO/MTX	26	NA	NA	RR: 0.87 (0.53–1.41)
	BIO and no TP/MTX	0	NA	NA	RR: 0
	TP and BIO and no MTX	3	NA	NA	RR: 3.10 (0.72–13.48)
Liu et al., 2015	Infliximab	3	NA	3.3/1,000 PYs	NA
	Adalimumab or certolizumab pegol	1	NA	1.1/1,000 PYs	NA
Lemaitre et al., 2017	Combination Therapy vs. Anti-TNF Monotherapy	14	14,753	0.95/1,000 PYs (0.45–1.45)	AdjHR: 2.35 (1.31–4.22)
	Combination Therapy vs. Thiopurine Monotherapy	14	14,753	0.95/1,000 PYs (0.45–1.45)	AdjHR: 2.53 (1.35–4.77)
	Anti-TNF Monotherapy vs. Unexposed to Thiopurines or Anti-TNF Agents	32	77,229	0.41/1,000 PYs (0.27–0.55)	AdjHR: 2.41 (1.60–3.64)
	Anti-TNF Monotherapy vs. Thiopurine Monotherapy	32	77,229	0.41/1,000 PYs (0.27–0.55)	AdjHR: 0.93 (0.60–1.44)
D'Haens et al., 2018	Adalimumab	10	16.680,4	0,060/100 PYs	NA

SIR, standardized incident rate ratio; NA, not available; OR, odds ratio; TP, thiopurines; MTX, methotrexate; BIO, biologics; AdjHR, adjusted hazard ratio; RR, relative risk.

Lymphoma as Composite Outcome

Among the selected studies, seven (Biancone et al., 2006, 2016; Fidder et al., 2009; Haynes et al., 2013; Beigel et al., 2014; Nyboe Andersen et al., 2014; D'Haens et al., 2017) reported data on lymphoma outcome included in a broader definition of malignancies (**Table 2B**).

Two studies (Biancone et al., 2016; D'Haens et al., 2017) provided results supporting an association between the investigated drugs and malignancies including lymphoma. Biancone et al. (2006) investigated the risk of extra-colonic cancer (two lymphoma cases out of 27 events of malignancy) in a population of patients taking any anti-TNF for IBD (Biancone et al., 2016). These authors found a positive association only in the subgroup of CD patients and not in those with UC. This study included prevalent patients, did not consider a lag period and the results are possibly affected by a time-window bias. D'Haens et al. (2017) investigated the risk of lymphoproliferative disorders and malignancies (nine lymphoma cases out of 49 malignancies) associated with infliximab in patients with CD (D'Haens et al., 2017). This study did not consider a lag period and likely included prevalent patients.

Five studies (Biancone et al., 2006; Fidder et al., 2009; Haynes et al., 2013; Beigel et al., 2014; Nyboe Andersen et al., 2014) did not demonstrate an association of anti-TNF drug exposure with the risk of malignancies including lymphoma. Three studies investigated the risk of malignancies in IBD patients exposed to any anti-TNF drug. Andersen et al. assessed the risk of hematopoietic and lymphoid tissue malignancies (six cases of lymphoma out of eight hematological malignancies). The definition of "exposure" might have biased the results due to the inclusion of immortal time (Nyboe Andersen et al., 2014). Beigel et al. investigated the risk of malignancies (one lymphoma out of eight malignancies) in patients receiving both thiopurines and anti-TNF inhibitors. This study included prevalent patients, did not consider a lag period and lacked sufficient power to assess the risk of malignancies. Moreover, results were likely affected by immortal-time bias (Beigel et al., 2014). Haynes et al. assessed the risk of any lymphoma or leukemia in patients exposed to anti-TNF drugs (<5 lymphoma events). In this study the author did not consider a lag period (Haynes et al., 2013).

The remaining two studies investigated the risk of neoplasia (Biancone et al., 2006) (no lymphoma cases in the exposed group and one out of seven in the control group) and of any cancer or dysplasia (two lymphoma cases out of 23 malignancies) (Fidder et al., 2009). Biancone et al. did not observe any risk in CD patients receiving infliximab (Biancone et al., 2006). However, this study included prevalent patients, did not consider a lag period, it could have matching issues and it was likely not

TABLE 2B | Studies with lymphoma included in a composite outcome.

References	Exposure	Events (*n*)	Person year (PY)	Incident rate (95% CI)	Risk (95%)
Biancone et al., 2006	Infliximab	9 (0 lymphoma)	NA	0	NA
	Immunosuppressant not further specified	7 (1 lymphoma)	NA	NA	1
Fidder et al., 2009	Infliximab	23 (2 lymphoma)	3,775	NA	OR: 0.97 (0.56–1.65, $p = 0.91$)
Haynes et al., 2013	Anti-TNF (96.8% of infliximab, 3.2% adalimumab) vs. other immunosuppressant drugs	<5	2,865.3	0.08/100 PYs	HR: 0.41 (0.07–2.35)
Nyboe Andersen et al., 2014	Anti-TNF	8 (6 lymphoma)	18,440	4.34/10,000 PYs	AdjRR: 0.90 (0.42–1.91)
	Not exposed to anti-TNF	260 (NA)	469,874	5.53/10,000 PYs	1
Beigel et al., 2014	TP monotherapy	20 (4 lymphoma)	NA	NA	HR: 4.15 (1.82–9.44)
	TP + Anti-TNF	8 (1 lymphoma)	NA	NA	NA
Biancone et al., 2016	Anti-TNF monotherapy	14 (0 lymphoma)	NA	NA	NA
	Anti-TNF and TP	27 (2 lymphoma)	NA	NA	OR: 2.15 (1.16–4.10) (CD) OR: 0.68 (0.20–2.8) (UC)
	No anti-TNF, No TP	61 (3 lymphoma)	NA	NA	NA
	TP monotherapy	28 (1 lymphoma)	NA	NA	NA
D'Haens et al., 2017	Infliximab vs. conventional therapy	49 (9 lymphoma)	7,362	7.6/1,000 PYs (5.7–9.9)	HR = 1.44; (0.86–2.42, $p = 0.163$)

TP, thiopurines; OR, odds ratio; HR, hazard ratio; NA, not available; AdjRR, adjusted rate ratio.

powered enough to detect the risk of lymphoma. Fidder et al. investigated IBD patients receiving infliximab (Fidder et al., 2009). In this study, prevalent patients were included, and a lag period was not considered. The results were likely affected by immortal-time bias (**Table 3**).

DISCUSSION

Observational studies are usually conducted in an attempt of overcoming the limitations of clinical trials by assessing the long-term effects of medications on infrequent outcomes or in specific sub-populations (Suissa, 2008), such as the risk of lymphoma in IBD patients receiving anti-TNF drugs. The widespread implementation of computer-based health databases, containing routinely collected administrative or clinical data, has encouraged the conduction of observational studies. However, no cautions for managing adequately the methodological underlying such investigations are usually taken. As a consequence, over the last decade, this superficial approach has led to an explosion of the publication of a high number of poorly conceived studies and analytic designs that have generated incorrect or unreliable conclusions on the safety of exposure to drugs (Sherman et al., 2016).

The results of the present systematic review are fully in line with the mentioned above trend. Indeed, very important methodologic issues, such as the inclusion of prevalent patients (11 out of 14 studies) and the lack of an adequate latency period in the definition of exposure (11 out of 14 studies) turned out to be very frequent among the selected studies. The results of seven selected studies were influenced also by important time-related biases, such as time-window bias (Biancone et al., 2006; Afif et al., 2013; Kopylov et al., 2015) and immortal-time bias (Fidder et al., 2009; Beigel et al., 2014; Lichtenstein et al., 2014; Nyboe Andersen et al., 2014). Thus, due to the above limitation, the overall evidence, either supporting the association or not, is strongly conditioned by the methodologic shortcomings of the available studies.

Among the 14 observational studies, only two (Herrinton et al., 2011; Lemaitre et al., 2017) reported data supporting an increased risk of lymphoma in IBD patients treated with anti-TNF, and both have important methodologic shortcomings. Lemaitre et al. estimated a significant relative risk of lymphoma in all treatment groups (thiopurines monotherapy, anti-TNF monotherapy and the combination of thiopurines plus anti-TNF) as compared with unexposed patients (adjusted hazard ratio [aHR]: 2.60; 95% CI, 1.96–3.44; $P < 0.001$; aHR: 2.41; 95% CI, 1.60–3.64; $P < 0.001$; aHR: 6.11; 95% CI, 3.46–10.8; $P < 0.001$, respectively). Of note, the findings of this study are biased at least in part, by the definition of "exposure." In the main analysis, a lag period was not considered. This means for instance that, if a diagnosis of lymphoma was made few days after the initiation of a treatment with an anti-TNF drug, the adverse event was attributed to the anti-TNF group, despite this outcome is not biologically plausible. In a correct time-dependent analysis, this event would have been attributed to the control group of unexposed patients or to the thiopurine treatment group, depending on whether the treatment with anti-TNF drugs had been a first line or a second line therapy, respectively. With the current analysis, we do not know how many events were attributed to the wrong group of treatment. However, it is likely that the as a ultimate consequence, this bias concentrated most of the event of lymphoma in the treatment groups while diluting the number of these events within the control group, thus leading to an apparent increased risk for all treatments. Of note, in an attempt of controlling for this issue, the authors performed a sensitivity analysis, where they introduced a lag period of 3 and

TABLE 3 | Methodologic features of the elected studies.

References	Adjustment/Matching	Prevalent patients (yes, no)	Lag period (yes-length, no)	Bias assessment
Biancone et al., 2006	◦ Matching ◦ Age (± 5 years) ◦ Sex ◦ Follow up period in the same center (±5 years) ◦ Immunosuppressant use (yes/no; type; duration) ◦ CD site (ileum, ileum-colon, colon, other) ◦ CD duration (±5 years)	Yes	No	Matching is inadequate to control for confounding (i.e., patients receiving infliximab have an average treatment with immunosuppressant drugs of 3 years compared with 2 years in the non-exposed group). The study has not likely the sufficient power to estimate rare endpoints like cancer (specific cancers in particular). It is not clear whether the exposed and not exposed patients are from the same cohort and the possibility of a selection bias is high
Fidder et al., 2009	◦ Adjustment ◦ Gender ◦ Age ◦ Weight ◦ Disease duration ◦ Concomitant immunosuppressive therapy	Yes	No	Possible immortal time bias (patients apparently did not contribute with person time to both exposed and unexposed group).
Herrinton et al., 2011	Not available	Yes	No	Person-time in patients receiving thiopurines and anti-TNF was attributed from the beginning of the treatment to the end of the coverage. The subsequent time was attributed to non-treatment despite the past exposure. This diluted the risk observed in non-exposed patients and concentrated the risk in exposed ones.
Afif et al., 2013	◦ Matching ◦ IBD patients ◦ ± 5 years at the Mayo clinic ◦ Subtype of IBD ◦ Geographic area ◦ Duration of follow-up at Mayo Clinic	Yes	No	Possible time-window bias (follow-up is not from the actual initial IBD diagnosis).
Haynes et al., 2013	◦ Matching ◦ Propensity score	No	No	–
Nyboe Andersen et al., 2014	◦ Adjustment ◦ Propensity score matching ◦ Year of birth ◦ Calendar year of cohort entry ◦ Sex ◦ Socioeconomic status ◦ Degree of urbanization ◦ Co-medications (not-IBD)	No	Yes-−3 months (1 year sensitivity analysis)	Cohort entry no clearly stated (likely IBD diagnosis). Not clear whether patients contributed with person time to both exposed and unexposed group. Lag period time was not included in the person-time of unexposed but considered in an unspecified "distinct category" (possible immortal time).
Beigel et al., 2014	◦ Adjustment ◦ Age ◦ Sex	Yes	No	The definition of cohort entry is not clear and we cannot exclude the inclusion of prevalent patients cannot be excluded. The study has not likely the sufficient power to estimate rare endpoints like cancer (specific cancers in particular). Time-fixed analysis with probable immortal time bias.
Lichtenstein et al., 2014	◦ Adjustment ◦ Age ◦ Sex ◦ Race	No	No	Immortal time bias: time fixed analysis in which person-time of patients receiving infliximab was classified as exposed to infliximab even before the starting of infliximab treatment
Kopylov et al., 2015	◦ Matching ◦ Age ◦ Sex ◦ Duration of disease	Yes	Yes-−1 year (6 months and 2 years in the sensitivity analysis)	Cohort entry definition may expose to the risk of time-window bias. The study has not likely the sufficient power to estimate rare endpoints like cancer (specific cancers in particular).
Liu et al., 2015	◦ Adjustment ◦ Age ◦ Sex	Yes	No	The study has not likely the sufficient power to estimate the risk of lymphoma across groups Despite cohort entry is established at the first drug prescription, we cannot exclude the assumption administration of the drug in over the 6 months preceding the index date (some patients could be prevalent)

(Continued)

TABLE 3 | Continued

References	Adjustment/Matching	Prevalent patients (yes, no)	Lag period (yes-length, no)	Bias assessment
Biancone et al., 2016	◦ Matching ◦ IBD center, ◦ IBD type [CD vs. UC] ◦ Sex ◦ Age [± 5 years]	Yes	No	Possible time-window bias (matching was not performed considering the duration of follow-up)
D'Haens et al., 2017	◦ Adjustment ◦ Age ◦ Disease duration	Yes	No	The definition of cohort entry is not clear and we cannot exclude the inclusion of prevalent patients cannot be excluded.
Lemaitre et al., 2017	◦ Adjustment ◦ Baseline ◦ Time-dependent covariates	Yes	Yes—(3/6 months)	In the sensitivity analysis, the exclusion of person-time associated with lag period did not resolve the problem of the lack of the lag period.
D'Haens et al., 2018	◦ Adjustment ◦ Exposure	Yes	No	Lack of a comparison group (the authors compared the incidence of lymphoma with an estimated background lymphoma rate in the general population, adjusted for thiopurines use).

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

6 months. Unfortunately, with this approach, they introduced a further bias that apparently confirmed the results of the main analysis. Indeed, instead of attributing the person-time of this lag period to the unexposed group or to the patients exposed to the first-line treatment, they eliminated this person-time from the analysis, including potential cases of lymphoma that should have been attributed to the unexposed group, thus amplifying artificially the risk for all treatment groups. Even assuming that such a loss of person-time did not delete any case of lymphoma, the exposure wrongly attributed to the treatments in the main analysis was likely to be depleted, at least in part. Indeed, if the exposure time in the denominator of an exposed group is reduced, the consequence will be an artificial magnification of the frequency of adverse events (i.e., lymphoma). Therefore, the ultimate effect of such a person-time loss in the exposed groups is likely a confirmation (or even an amplification) of the risk estimated in the main analysis. The second study supporting an association was that by Herrinton et al. (2011), which apparently made a similar mistake in the definition of the exposure. The correct way to define the exposure would be from the cohort entry (first intake of the drug) up to the censoring point (outcome of interest, death, end of the study period, loss to follow-up). The person-time included in this period should be attributed to the exposed group. Herrinton et al. attributed the person-time elapsed after the discontinuation of the anti-TNF treatment to the unexposed group. Consequently, the person-time attributed to the exposed group (denominator) strongly concentrated, thus increasing the frequency of lymphoma and resulting in an apparent increase of the risk. In support of this hypothesis, it is easy to verify that the person-time attributed to the exposed group in this study was the 0.9% of the overall person-time of the cohort.

The choice of investigating the risk of lymphoma within a broad composite endpoint including different cancers is biologically questionable. Indeed, the pathophysiological mechanisms supporting the development of cancer are extremely variable across the different types of cancers and it is therefore unlikely that a single drug can trigger all these mechanisms. Furthermore, several studies postulated, even though without any supportive evidence, that lymphoma could be a class effect of anti-TNF therapy. Such a clustering of endpoints and exposures seems to be often a choice driven by the need of increasing the power of the sample (especially in monocentric studies performed on small databases), disregarding any scientific rationale. Of note, in the two studies (Biancone et al., 2016; D'Haens et al., 2017) supporting an association of anti-TNF drugs with a group of cancers including lymphoma, the net contribution of lymphoma to the overall risk was not assessable and likely negligible [7% (Biancone et al., 2016) and 18% (D'Haens et al., 2017) of the all number of cancers]. Furthermore, the results of both studies are poorly reliable since they included prevalent patients and did not consider a lag period.

Notably, the association of anti-TNF drugs with lymphoma should be considered in light of the biological plausibility. TNF is a cytokine involved in systemic inflammation and modulation of immune system, and its role in the inhibition of carcinogenesis is well-known. Therefore, the inhibition of TNF would be expected to favor neoplastic processes (Aggarwal et al., 2012). Despite this, the risk of lymphoma associated with anti-TNF treatments has not been yet conclusively demonstrated in RA, mainly due to the intrinsic risk of lymphoma associated with this disease (Dias and Isenberg, 2011; Baecklund et al., 2014; Mercer et al., 2017). Since the evidence supporting a risk of lymphoma in IBDs is scarce (Baecklund et al., 2014), in these patients, it should be easier to demonstrate an association, if any, between anti-TNF drugs and lymphoma. However, even in IBD patients, such a risk remains undetermined. Based on this consideration, one might speculate that the association of anti-TNF treatment with lymphoma is unlikely. Nevertheless, differences in the pathophysiological patterns of RA and IBDs might likely play a role in the development of lymphoma and therefore we cannot exclude that this could be the case even under anti-TNF inhibition. On the other hands, we cannot exclude also that TNF

inhibition might promote lymphoma in RA but not in IBD, since differences among their background inflammatory conditions could play a role in determining a differential risk of lymphoma.

The present systematic review has some limitations. First, we did not include unpublished studies that could have provided good evidence of an association between anti-TNF drugs and lymphoma. Second, the assessment of methodological limitations was not based on validated tools, but only on the judgment of experts. A standardized evaluation of the quality of the studies could have provided interesting information. However, we do not believe that the above limitations may affect significantly the conclusions of the present review.

CONCLUSIONS

At present, the available observational studies, considering those supporting an association and those not, are biased by methodologic shortcomings and their results are not reliable. Thus, current evidence from observational studies does not allow excluding or confirming an association of lymphoma with the exposure to anti-TNF treatments in IBD patients. Additional well-designed observational studies are warranted to provide a conclusive answer to this relevant question. Moreover, it would be important also to stimulate meta-research studies, intended as critical appraisals of available evidence, particularly that coming from observational studies, to avoid overemphasis on biased results.

AUTHOR CONTRIBUTIONS

SF, LL, and IC reviewed the articles and wrote the manuscript. CB and MT reviewed the manuscript and served as supervisors of the reviewing process.

REFERENCES

Afif, W., Sandborn, W. J., Faubion, W. A., Rahman, M., Harmsen, S. W., Zinsmeister, A. R., et al. (2013). Risk factors for lymphoma in patients with inflammatory bowel disease: a case-control study. *Inflamm. Bowel Dis.* 19, 1384–1389. doi: 10.1097/MIB.0b013e318281325e

Aggarwal, B. B., Gupta, S. C., and Kim, J. H. (2012). Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood* 119, 651–665. doi: 10.1182/blood-2011-04-325225

Baecklund, E., Smedby, K. E., Sutton, L. A., Askling, J., and Rosenquist, R. (2014). Lymphoma development in patients with autoimmune and inflammatory disorders–what are the driving forcesα. *Semin. Cancer Biol.* 24, 61–70. doi: 10.1016/j.semcancer.2013.12.001

Beigel, F., Steinborn, A., Schnitzler, F., Tillack, C., Breiteneicher, S., John, J. M., et al. (2014). Risk of malignancies in patients with inflammatory bowel disease treated with thiopurines or anti-TNF alpha antibodies. *Pharmacoepidemiol. Drug Saf.* 23, 735–744. doi: 10.1002/pds.3621

Biancone, L., Armuzzi, A., Scribano, M. L., D'Inca, R., Castiglione, F., Papi, C., et al. (2016). Inflammatory bowel disease phenotype as risk factor for cancer in a prospective multicentre nested case-control IG-IBD study. *J. Crohns Colitis* 10, 913–924. doi: 10.1093/ecco-jcc/jjw048

Biancone, L., Orlando, A., Kohn, A., Colombo, E., Sostegni, R., Angelucci, E., et al. (2006). Infliximab and newly diagnosed neoplasia in Crohn's disease: a multicentre matched pair study. *Gut* 55, 228–233. doi: 10.1136/gut.2005.075937

Chen, Y., Sun, J., Yang, Y., Huang, Y., and Liu, G. (2016). Malignancy risk of anti-tumor necrosis factor alpha blockers: an overview of systematic reviews and meta-analyses. *Clin. Rheumatol.* 35, 1–18. doi: 10.1007/s10067-015-3115-7

Deepak, P., Sifuentes, H., Sherid, M., Stobaugh, D., Sadozai, Y., and Ehrenpreis, E. D. (2013). T-cell non-Hodgkin's lymphomas reported to the FDA AERS with tumor necrosis factor-alpha (TNF-α) inhibitors: results of the REFURBISH study. *Am. J. Gastroenterol.* 108, 99–105. doi: 10.1038/ajg.2012.334

D'Haens, G., Reinisch, W., Colombel, J. F., Panes, J., Ghosh, S., Prantera, C., et al. (2017). Five-year safety data from ENCORE, a European observational safety registry for adults with Crohn's disease treated with infliximab [Remicade®] or conventional therapy. *J. Crohns Colitis* 2017, 680–689. doi: 10.1093/ecco-jcc/jjw221

D'Haens, G., Reinisch, W., Panaccione, R., Satsangi, J., Petersson, J., Bereswill, M., et al. (2018). Lymphoma risk and overall safety profile of adalimumab in patients with Crohn's disease with up to 6 years of follow-up in the pyramid registry. *Am. J. Gastroenterol.* 113, 872–882. doi: 10.1038/s41395-018-0098-4

Dias, C., and Isenberg, D. A. (2011). Susceptibility of patients with rheumatic diseases to B-cell non-Hodgkin lymphoma. *Nat. Rev. Rheumatol.* 7, 360–368. doi: 10.1038/nrrheum.2011.62

Ekström Smedby, K., Vajdic, C. M., Falster, M., Engels, E. A., Martínez-Maza, O., Turner, J., et al. (2008). Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: a pooled analysis within the interlymph consortium. *Blood* 111, 4029–4038. doi: 10.1182/blood-2007-10-119974

Fidder, H., Schnitzler, F., Ferrante, M., Noman, M., Katsanos, K., Segaert, S., et al. (2009). Long-term safety of infliximab for the treatment of inflammatory bowel disease: a single-centre cohort study. *Gut* 58, 501–508. doi: 10.1136/gut.2008.163642

Haynes, K., Beukelman, T., Curtis, J. R., Newcomb, C., Herrinton, L. J., Graham, D. J., et al. (2013). Tumor necrosis factor α inhibitor therapy and cancer risk in chronic immune-mediated diseases. *Arthritis Rheum.* 65, 48–58. doi: 10.1002/art.37740

Herrinton, L. J., Liu, L., Weng, X., Lewis, J. D., Hutfless, S., and Allison, J. E. (2011). Role of thiopurine and anti-TNF therapy in lymphoma in inflammatory bowel disease. *Am. J. Gastroenterol.* 106, 2146–2153. doi: 10.1038/ajg.2011.283

Khor, B., Gardet, A., and Xavier, R. J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature* 474, 307–317. doi: 10.1038/nature10209

Kopylov, U., Vutcovici, M., Kezouh, A., Seidman, E., Bitton, A., and Afif, W. (2015). Risk of lymphoma, colorectal and skin cancer in patients with IBD treated with immunomodulators and biologics: a Quebec claims database study. *Inflamm. Bowel Dis.* 21, 1847–1853. doi: 10.1097/MIB.0000000000000457

Kotlyar, D. S., Lewis, J. D., Beaugerie, L., Tierney, A., Brensinger, C. M., Gisbert, J. P., et al. (2015). Risk of lymphoma in patients with inflammatory bowel disease treated with azathioprine and 6-mercaptopurine: a meta-analysis. *Clin. Gastroenterol. Hepatol.* 13, 847–858.e4. doi: 10.1016/j.cgh.2014.05.015

Lemaitre, M., Kirchgesner, J., Rudnichi, A., Carrat, F., Zureik, M., Carbonnel, F., et al. (2017). Association between use of thiopurines or tumor necrosis factor antagonists alone or in combination and risk of lymphoma in patients with inflammatory bowel disease. *JAMA* 318, 1679–1686. doi: 10.1001/jama.2017.16071

Lichtenstein, G. R., Feagan, B. G., Cohen, R. D., Salzberg, B. A., Diamond, R. H., Langholff, W., et al. (2014). Drug therapies and the risk of malignancy in Crohn's disease: results from the TREAT^{TM} registry. *Am. J. Gastroenterol.* 109, 212–223. doi: 10.1038/ajg.2013.441

Lichtenstein, G. R., Rutgeerts, P., Sandborn, W. J., Sands, B. E., Diamond, R. H., Blank, M., et al. (2012). A pooled analysis of infections, malignancy, and mortality in infliximab- and immunomodulator-treated adult patients with inflammatory bowel disease. *Am. J. Gastroenterol.* 107, 1051–1063. doi: 10.1038/ajg.2012.89

Liu, J., Sylwestrzak, G., Ruggieri, A. P., and DeVries, A. (2015). Intravenous versus subcutaneous anti-TNF-alpha agents for Crohn's disease: a comparison of effectiveness and safety. *J. Manag. Care Spec. Pharm.* 21, 559–566. doi: 10.18553/jmcp.2015.21.7.559

Mercer, L. K., Galloway, J. B., Lunt, M., Davies, R., Low, A. L., Dixon, W. G., et al. (2017). Risk of lymphoma in patients exposed to antitumour necrosis factor therapy: results from the British society for rheumatology biologics register for rheumatoid arthritis. *Ann. Rheum. Dis.* 76, 497–503. doi: 10.1136/annrheumdis-2016-209389

Nyboe Andersen, N., Pasternak, B., Basit, S., Andersson, M., Svanström, H., Caspersen, S., et al. (2014). Association between tumor necrosis factor-α antagonists and risk of cancer in patients with inflammatory bowel disease. *JAMA* 311, 2406–2413. doi: 10.1001/jama.2014.5613

Parakkal, D., Sifuentes, H., Semer, R., and Ehrenpreis, E. D. (2011). Hepatosplenic T-cell lymphoma in patients receiving TNF-alpha inhibitor therapy: expanding the groups at risk. *Eur. J. Gastroenterol. Hepatol.* 23, 1150–1156. doi: 10.1097/MEG.0b013e32834bb90a

Rothfuss, K. S., Stange, E. F., and Herrlinger, K. R. (2006). Extraintestinal manifestations and complications in inflammatory bowel diseases. *World J. Gastroenterol.* 12, 4819–4831. doi: 10.3748/wjg.v12.i30.4819

Shamseer, L., Moher, D., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., et al. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. *BMJ* 349:g7647. doi: 10.1136/bmj.g7647

Sherman, R. E., Anderson, S. A., Dal Pan, G. J., Gray, G. W., Gross, T., Hunter, N. L., et al. (2016). Real-world evidence — what is it and what can it tell us? *N. Engl. J. Med.* 375, 2293–2297. doi: 10.1056/NEJMsb1609216

Simon, T. A., Thompson, A., Gandhi, K. K., Hochberg, M. C., and Suissa, S. (2015). Incidence of malignancy in adult patients with rheumatoid arthritis: a meta-analysis. *Arthritis Res. Ther.* 17:212. doi: 10.1186/s13075-015-0728-9

Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., et al. (2000). Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 283, 2008–2012. doi: 10.1001/jama.283.15.2008

Suissa, S. (2008). Immortal time bias in pharmacoepidemiology. *Am. J. Epidemiol.* 167, 492–499. doi: 10.1093/aje/kwm324

Suissa, S., Dell'Aniello, S., Vahey, S., and Renoux, C. (2011). Time-window bias in case-control studies. *Epidemiology* 22, 228–231. doi: 10.1097/EDE.0b013e3182093a0f

Targownik, L. E., and Suissa, S. (2015). Understanding and avoiding immortal-time bias in gastrointestinal observational research. *Am. J. Gastroenterol.* 110, 1647–1650. doi: 10.1038/ajg.2015.210

Williams, C. J. M., Peyrin-Biroulet, L., and Ford, A. C. (2014). Systematic review with meta-analysis: malignancies with anti-tumour necrosis factor-α therapy in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 39, 447–458. doi: 10.1111/apt.12624

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation, though no other collaboration with the author MT at time of review.

frontiers in Pharmacology

MINI REVIEW
published: 29 March 2019
doi: 10.3389/fphar.2019.00305

Antisense Oligonucleotide: Basic Concepts and Therapeutic Application in Inflammatory Bowel Disease

Davide Di Fusco[1†], Vincenzo Dinallo[1†], Irene Marafini[1], Michele M. Figliuzzi[1], Barbara Romano[2] and Giovanni Monteleone[1]*

[1]Department of Systems Medicine, Gastroenterology, University of Tor Vergata, Rome, Italy, [2]Department of Pharmacy, School of Medicine and Surgery, University of Naples "Federico II", Naples, Italy

OPEN ACCESS

Edited by:
Ruixin Zhu,
Tongji University, China

Reviewed by:
Zhanju Liu,
Tongji University, China
Jose A. Uranga,
Universidad Rey Juan Carlos, Spain
Anthony C. Johnson,
Oklahoma City VA Health Care System, United States
Oksana Zayachkivska,
Danylo Halytsky Lviv National Medical University, Ukraine

***Correspondence:**
Davide Di Fusco
davidedifusco@gmail.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 03 December 2018
Accepted: 12 March 2019
Published: 29 March 2019

Citation:
Di Fusco D, Dinallo V, Marafini I, Figliuzzi MM, Romano B and Monteleone G (2019) Antisense Oligonucleotide: Basic Concepts and Therapeutic Application in Inflammatory Bowel Disease. Front. Pharmacol. 10:305. doi: 10.3389/fphar.2019.00305

Several molecular technologies aimed at regulating gene expression that have been recently developed as a strategy to combat inflammatory and neoplastic diseases. Among these, antisense technology is a specific, rapid, and potentially high-throughput approach for inhibiting gene expression through recognition of cellular RNAs. Advances in the understanding of the molecular mechanisms that drive tissue damage in different inflammatory diseases, including Crohn's disease (CD) and ulcerative colitis (UC), the two major inflammatory bowel diseases (IBDs) in humans, have facilitated the identification of novel druggable targets and offered interesting therapeutic perspectives for the treatment of patients. This short review provides a comprehensive understanding of the basic concepts underlying the mechanism of action of the oligonucleotide therapeutics, and summarizes the available pre-clinical and clinical data for oligonucleotide-based therapy in IBD.

Keywords: inflammatory bowel disease, ulcerative colitis, Crohn's disease, antisense oligonucleotide, RNA interference

INTRODUCTION

The central dogma of molecular biology states that DNA encodes RNA, which is then translated into proteins. In recent years, the use of compounds that are able to bind messenger RNAs (mRNAs) has gained increasing interest as inhibition of protein expression can be helpful for controlling the course of inflammatory and neoplastic diseases. The two major therapeutic approaches in this field are the antisense oligonucleotides (ASOs) that inhibit mRNA translation and the oligonucleotides, which function *via* RNA interference (RNAi) pathway (Chan et al., 2006; Chery, 2016). Synthetic oligonucleotides are negatively charged molecules with different chemical properties based on the technology used for their design. In order to regulate target gene expression, these compounds have to reach disease-associated tissues and cross cell membranes. This is in part facilitated by the manipulation of their chemical structure, which makes oligonucleotides also more powerful and less toxic with a lower chance to have off-target effects and to activate the host immune system (Sharma and Watts, 2015).

In the last decades, the advent of new techniques of molecular and cellular biology has advanced our understanding of the factors/mechanisms that promote tissue damage in several chronic inflammatory diseases, including Crohn's disease (CD) and ulcerative colitis (UC), the two major inflammatory bowel diseases (IBDs) in humans (Neurath, 2017). This has contributed in identifying novel druggable targets, thus offering interesting therapeutic perspectives for the treatment of these patients. We here shortly review the basic concepts underlying the mechanism of action of the ASOs and summarize the available data for ASO-based therapy in IBD.

ANTISENSE OLIGONUCLEOTIDE STRATEGY AND MOLECULE DESIGN

An antisense oligonucleotide (ASO) is a single-stranded deoxyribonucleotide, which is complementary to the mRNA target. The goal of the antisense approach is the downregulation of a molecular target, usually achieved by induction of RNase H endonuclease activity that cleaves the RNA-DNA heteroduplex with a significant reduction of the target gene translation (**Figure 1**). Other ASO-driven mechanisms include inhibition of 5′ cap formation, alteration of splicing process (splice-switching), and steric hindrance of ribosomal activity (Chan et al., 2006; Bennett et al., 2017; Crooke, 2017).

The recent developments in the human genome sequencing, the possibility of a rational design of oligonucleotides and the theoretical simplicity, and relatively cheap costs of these compounds led to their use as either therapeutic agents or tools for assessing gene function. Although, the researchers usually select the ASO candidate by testing the activity of few oligonucleotides that specifically regulate the target gene expression, it would be recommendable to identify the ideal ASO through an accurate evaluation of a panel of putative oligomers (Tu et al., 1998; Stein, 2001). It is crucial that the ASOs do not bind, even partially, to a nontarget mRNA. In this context, it is noteworthy that 6–7 base pairs between the ASO and nontarget mRNA are sufficient to initiate RNase activity, leading to cleavage of the wrong target. The secondary and tertiary structure of the RNA must be taken into account to minimize the possibility that the selected sequence is inaccessible to binding (Ho et al., 1998; Vickers et al., 2000; Andronescu et al., 2005). To this end, the use of software with a robust RNA folding program (e.g., Sfold or mfold) can help select the optimal candidate (Zuker, 2003; Ding et al., 2004). Generally, the length of an ASO is approximately 20 nucleotides and the ASO is selected to target either the methionine (AUG) initiation codon (to block translation) or splice sites (to block splicing) (Chan et al., 2006; Chery, 2016). The effective knockdown of the target is usually demonstrated at the protein level, but analysis of RNA expression should be made in order to exclude that the

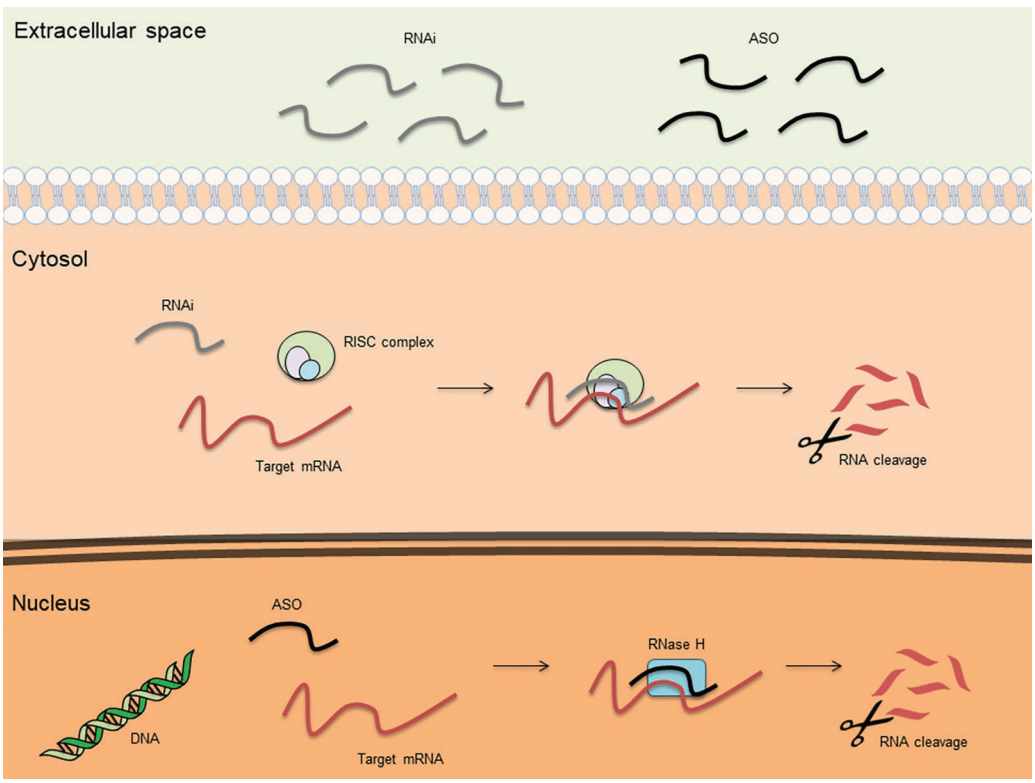

FIGURE 1 | Basic mechanisms of action for therapeutic antisense oligonucleotides (ASOs) and RNA interference (RNAi).

target protein is down-regulated through a non-sequence specific mechanism. To maximize sequence specificity, ASOs should not be designed into polymorphic/mutated regions of the genome and selection should exclude oligonucleotides targeting four contiguous guanosine residues in order to avoid generation of tetraplexes via Hoogsteen base-pair formation (Sen and Gilbert, 1992; Benimetskaya et al., 1997; Crooke, 2004).

Since in their "naïve" form, ASOs could be rapidly digested, thus limiting their bioavailability (Eder et al., 1991), most of ASOs are phosphorothioated (Eckstein, 2014). This modification facilitates binding of ASOs to plasma proteins, thereby reducing their renal loss and improving uptake to several organs (e.g. liver, bone marrow, and lymph nodes). The chemical modification influences neither RNase H activity nor ASO solubility, thus allowing administration by different routes (e.g. subcutaneous, intravenous, topical, oral, or rectal). However, phosphorothioate oligonucleotides containing one or more CpG motifs can bind the Toll-like receptor (TLR) 9 and trigger innate immune responses. This issue can be overcome by either selecting oligonucleotides containing no CG or replacing the C with 5methylC, which does not stimulate the immune system (Stein, 2001).

Increased ASO binding affinity and biostability have also been obtained using oligonucleotides with ribose modifications [i.e. substitution of the hydrogen at the 2-position by an O-alkyl group and locked nucleic acid technology (LNA)] that reduce conformational plasticity (Wahlestedt et al., 2000; Prakash, 2011; Hagedorn et al., 2018). However, LNA can accumulate in the liver and promote hepatotoxicity, mainly due to an off-target RNase H dependent RNA degradation (Burel et al., 2016).

ASOs have been already used in various human pathologies. For instance, in 1998, FDA approved the use of fomivirsen, a compound that inhibits the translation of the mRNA encoding for the major immediate early region proteins of cytomegalovirus, for the treatment of cytomegalovirus-induced retinitis (Jabs and Griffiths, 2002). In 2013, FDA approved the use of mipomersen, a compound targeting apolipoprotein B100, for the treatment of familial hypercholesterolemia (Duell et al., 2016), while later on, eteplirsen was introduced to treat Duchenne muscular dystrophy, and nusinersen was approved for spinal muscular atrophy treatment (Khorkova and Wahlestedt, 2017; Goyal and Narayanaswami, 2018). Eteplirsen binds to the disease-related-exon 51 of dystrophin RNA and allows the splicing of exon 52 to exon 51, thus generating a shortened but partly functional protein (exon skipping strategy) (Khorkova and Wahlestedt, 2017). Differently, nusinersen uses an exon switching strategy to increase the amount of functional full-length survival motor neuron-2 protein. After hybridization to its target, this oligonucleotide forces the inclusion of exon 7 into the mRNA and prevents the generation of short-lived/non-functional proteins (Goyal and Narayanaswami, 2018). Clinical trials employing ASOs in amyotrophic lateral sclerosis and familial amyloid polyneuropathy are also ongoing (Goyal and Narayanaswami, 2018).

ANTISENSE OLIGONUCLEOTIDE-BASED THERAPIES FOR INFLAMMATORY BOWEL DISEASE

Alicaforsen: Intercellular Adhesion Molecule-1 Antisense Oligonucleotide

IBD are chronic, immune-mediated diseases of the gastrointestinal tract, which are characterized by tissue damage and development of local and extra-intestinal lesions (Abraham and Cho, 2009; Neurath, 2017). One of the mechanisms sustaining the inflammatory process in IBD is the recruitment of immune cells from the peripheral blood to the intestine. Once activated in secondary lymphoid organs, such as Peyer's patches and isolated follicles, leukocytes enter the circulation, and through a process named gut homing, eventually go back to the intestinal wall. This process is triggered mainly by chemoattractants produced within the inflamed tissue and favored by interaction between integrins expressed on leukocyte surface and proteins expressed on endothelial cells, such members of immunoglobulin superfamily [i.e. intercellular adhesion molecule (ICAM)-1, ICAM-2, and vascular cell adhesion molecule (VCAM)-1] (Hart et al., 2010).

In inflamed gut of CD patients and UC patients, there is an enhanced expression of ICAM-1, a transmembrane glycoprotein constitutively expressed on the surface of intestinal epithelial cells and vascular endothelial cells (Vainer and Nielsen, 2000). Knockdown of ICAM-1 with specific ASO in mouse models of colitis reduced leukocyte trafficking to the gut and attenuated mucosal inflammation (Bennett et al., 1997). In a proof of concept study, Alicaforsen (ISIS 2302), a 20 base-long phosphorothioate ASO inhibiting ICAM1 production, was intravenously administered to 20 active CD patients for 26 days. The drug was well tolerated and superior to placebo in inducing clinical remission (Yacyshyn et al., 1998). However, steroid-dependent or resistant CD patients treated with intravenous or subcutaneous alicaforsen in two subsequent clinical trials showed no clinical benefit (Schreiber et al., 2001; Yacyshyn et al., 2002). Similar negative results were also obtained in two subsequent placebo-controlled phase III trials, in which alicaforsen was given to moderate-to-severe active CD patients (Yacyshyn et al., 2007). Therefore, the therapeutic development of alicaforsen in CD was discontinued.

An alicaforsen-containing enema formulation was developed for patients with UC or patients with pouchitis, an inflammatory condition of the ileal pouch reservoir, which can develop in UC patients undergoing colectomy and ileal pouch-anal anastomosis. In mild to moderate left-sided UC patients, alicaforsen enema had no significant effect on the course of the disease (Miner et al., 2006; Van Deventer et al., 2006). Afterwards, a retrospective analysis evaluating the efficacy of alicaforsen enema (240 mg/day for 6 weeks) showed clinical benefits in patients with left-sided and distal UC and in patients with chronic pouchitis (Greuter et al., 2018). However, treatment was not sufficient to stably control the inflammation as more than 2/3 of the patients relapsed within 16 weeks (Greuter et al., 2016). A phase III, multicenter, double-blind, placebo-controlled trial

(NCT02525523) assessing the safety and efficacy of topical alicaforsen enema (240 mg/day for 6 weeks) has been recently completed in subjects with antibiotic refractory pouchitis but results are not yet available.

NF-κB Antisense Oligonucleotide

NF-κB is a transcription factor composed of two proteins (p50 and p65) regulating the expression of many inflammatory and anti-inflammatory genes (Rogler et al., 1998; Schreiber et al., 1998). It was shown that either intravenous or intra-rectal ASO targeting the p65 subunit of NF-κB inhibited production of inflammatory cytokines and signs of colitis induced in mice by trinitrobenzene sulfonic acid (TNBS) or IL-10 deficiency (Neurath et al., 1996). Consistently, the specific p65 ASO reduced production of inflammatory cytokines in macrophages and endothelial cells isolated from the gut of CD patients (Neurath et al., 1998). These data were in line with the demonstration that downregulation of NF-κBp65 with a specific ASO attenuated dextran sodium sulfate (DSS)-induced colitis (Murano et al., 2000) and intestinal fibrogenic processes in mice (Lawrance et al., 2003). Despite these encouraging data in IBD-like murine models, no data are currently available on the use of NF-κB ASO in IBD.

Smad7 Antisense Oligonucleotide

IBD is believed to be triggered by complex interactions among host genetic susceptibility and many environmental factors, which lead to a sustained activation of inflammatory pathways and defects in counter-regulatory mechanisms in the gut (Gorelik and Flavell, 2002; MacDonald et al., 2011). In intestinal immunity, transforming growth factor (TGF)-β1, a pleiotropic cytokine produced by many cell types suppresses inflammatory responses to luminal antigens, thus contributing to immune tolerance induction. The anti-inflammatory mechanism of TGF-β1 relies mainly on the intracellular phosphorylation and subsequent activation of TGF-β1 receptor-associated Smad2/3 proteins (Heldin et al., 1997; Shi and Massague, 2003). In IBD patients, phosphorylated-Smad2/3 expression is reduced thus underlying the inability of TGF-β1 to adequately control inflammatory signals (Babyatsky et al., 1996). Such a defect has been associated with increased levels of Smad7, a cytosolic protein that inhibits TGF-β1/Smad-associated pathway (Monteleone et al., 2001; Sedda et al., 2015). Responsiveness of IBD mucosal cells to TGF-β1 is restored by downregulation of Smad7 with a specific ASO (Monteleone et al., 2001). Oral administration of Smad7 ASO to mice with TNBS and oxazolone-induced colitis restores TGF-β1-associated Smad signaling and mitigates intestinal inflammation (Boirivant et al., 2006).

Later on, a pharmaceutical compound, which contains a Smad7 ASO targeting the RNA encoding by the 107–128 DNA region, was developed for CD therapy. The drug, named mongersen (previously called GED-0301), (Monteleone et al., 2012; Laudisi et al., 2016), was formulated in order to maximize the active compound release into the lumen of the terminal ileum and right colon, the intestinal regions mainly involved in CD. A phase I clinical, open-label, dose-escalating study in patients with active, steroid-dependent/resistant CD showed that mongersen was safe and well-tolerated and treatment was associated with a clear clinical benefit (Monteleone et al., 2012). Although TGF-β1 is known to be pro-fibrogenic (Leask and Abraham, 2004; Vallance et al., 2005), no patient recruited into the trial developed strictures (Zorzi et al., 2012). This later result was consistent with data generated in mice with TNBS-mediated colitis-driven intestinal fibrosis, in which knockdown of Smad7 with the specific ASO reduced intestinal inflammation and fibrosis (Izzo et al., 2018). A double blind, placebo controlled, phase II trial was conducted in 166 active, steroid-dependent/resistant CD patients (Monteleone et al., 2015). Patients were allocated to receive one of three doses of mongersen (10, 40, or 160 mg per day) or placebo daily for 2 weeks. Patients receiving the 40 and 160 mg of mongersen reached significant higher rates of remission (55 and 65%, respectively) than those treated with 10 mg or placebo (12 and 10%, respectively). At the end of follow-up, the percentage of patients who had a steroid-free remission was significantly greater in the 160-mg group than in the placebo group. The study confirmed the safety profile of the drug (Monteleone et al., 2015). These data were confirmed by a subsequent multicenter, randomized study, which evaluated the effect of mongersen on endoscopic outcomes (Feagan et al., 2018). Sixty-three active CD patients were randomized (1:1:1) to 4, 8, or 12 weeks of oral mongersen (160 mg daily). Endoscopic improvement was observed in 37% of participants. All three mongersen regimens induced rapid, clinically meaningful decreases in Crohn's disease activity index scores. Moreover, reductions in high-sensitivity C-reactive protein levels and fecal calprotectin were observed in patients with increased values at baseline (Feagan et al., 2018). A phase III clinical trial has been recently suspended due to an interim analysis documenting the lack of efficacy of mongersen. The reasons for this unexpected result are still to be clarified.

RNA INTERFERENCE STRATEGY AND ITS THERAPEUTIC APPLICATION

Another strategy to inhibit the expression of mRNA is represented by RNAi. RNAi is mediated by many endogenous RNAs [e.g. piwi-interacting RNA (piRNA), microRNA (miRNA), and small interfering RNA (siRNA) (Li and Rana, 2012)]. Once incorporated into the RNA-induced silencing complex (RISC), these RNAs cause translational repression/degradation of the targeted mRNA through the partial or complete base paring of the guide strand (**Figure 1**). This result can be obtained by using single stranded RNAs (ssRNAs), which can be directly incorporated into RISC, or double stranded RNAs (dsRNAs), which require cleavage by the cytoplasmic endoribonuclease Dicer prior to be incorporated into RISC (Li and Rana, 2012). Due to their chemical characteristics, synthetic silencing RNAs do not efficiently enter into cells and are highly susceptible to nuclease degradation. To overcome these limitations, silencing RNAs can be complexed with nanoparticles, typically as polymer- or lipid-based formulations (Tatiparti et al., 2017). It is, however,

noteworthy that nanoparticles can increase the toxicity of the compound or alter pharmacokinetics and biodistribution of the silencing RNAs. Patisiran is the first RNAi-based drug approved by FDA for the treatment of polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR). It consists of a dsRNA encapsulated in a nanoparticle that allows the active molecule to reach the liver, where it specifically inhibits the hepatic synthesis of transthyretin (Adams et al., 2018). It remains to be clarified whether this strategy can be effective in organs other than liver, where delivery could be more difficult.

Another strategy is to conjugate the silencing RNAs with ligands of the target molecule. An example is the addition of N-acetylgalactosamine (GalNAc) to the RNA, thus enhancing asialoglycoprotein receptor (ASGR)-mediated uptake into liver hepatocytes (Nair et al., 2014). The major limitation of this strategy could be the rate of receptor recycling. GalNAc delivery is actually involved in several clinical and pre-clinical studies with exciting results (Tanowitz et al., 2017).

SMALL INTERFERING RNA-BASED THERAPIES FOR INFLAMMATORY BOWEL DISEASE

STNM01: Small Interfering RNA Targeting Carbohydrate Sulfotransferase 15

The late stage of inflammation in IBD is characterized by the fibrotic process, which derives from an altered balance between matrix deposition and degradation (Rieder et al., 2017). Carbohydrate sulfotransferase 15 (CHST15) is a sulfotransferase responsible for biosynthesis of chondroitin sulfate E-type (CS-E), which binds to pro-inflammatory and pro-fibrotic mediators, adhesion molecules, receptor for advanced glycation end-product (RAGE), and pathogenic microorganisms, all of them involved in fibrogenesis. CHST15 is increased in the colon of active CD patients (Belmiro et al., 2005; Suzuki et al., 2016, 2017). STNM01, a synthetic, double-stranded RNA oligonucleotide directed against CHST15, ameliorated acute and chronic DSS induced-colitis and reduced colonic deposition of collagen in mice (Suzuki et al., 2016). A phase 1, randomized, double blind, placebo-controlled, clinical trial evaluated the safety of STNM01 in patients with CD (Suzuki et al., 2017). Eighteen CD patients with mucosal lesions refractory to conventional therapies received a single-dose, endoscopic, submucosal injection of 2.5, 25, or 250 nM STNM01 (three patients per group) or placebo (nine patients). The drug was well tolerated, CHST15 expression was reduced 1 month after the injection, and the drug attenuated intestinal inflammation and fibrosis.

CONCLUSION

The rationale for the use of antisense-based therapies in IBD is supported by the benefit seen in preclinical models and initial clinical studies, together with the safety profiles of the compounds. Unfortunately, however, large clinical trials have not confirmed the promising results obtained with ASOs in preclinical models. Although, it is unclear why these treatments failed in patients, it is conceivable that some factors either related to the target or route of administration may have contributed to these negative results. For example, the negative results of alicaforsen can, in part, rely on the fact that ICAM-1 is just one of the various molecules involved in leukocytes trafficking, and therefore, even in the absence of ICAM-1, other integrins could promote recruitment of activated leukocytes in the gut. Another possibility is that systemic administration of ASO could be not ideal for allowing optimal concentration of the drug within the gut tissue, where there is the main expression of the target. This hypothesis is supported by the demonstration that rectal administration of alicaforsen is of benefit in patients with distal UC and in patients with pouchitis. While STNM01 is the only siRNA currently tested in IBD, there is sufficient evidence to believe that RNAi technology can represent a new and valid approach to regulate the expression of disease-related genes. Some issues in the design and development of these compounds, such as correct identification of target mRNAs, stability, and delivery to the site of interest remain to be solved.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

DF and VD wrote the paper. MF, IM and BR contributed to supervise parts of the paper. GM designed and drafted the paper.

REFERENCES

Abraham, C., and Cho, J. H. (2009). Inflammatory bowel disease. *N. Engl. J. Med.* 361, 2066–2078. doi: 10.1056/NEJMra0804647

Adams, D., Gonzalez-Duarte, A., O'riordan, W. D., Yang, C. C., Ueda, M., Kristen, A. V., et al. (2018). Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* 379, 11–21. doi: 10.1056/NEJMoa1716153

Andronescu, M., Zhang, Z. C., and Condon, A. (2005). Secondary structure prediction of interacting RNA molecules. *J. Mol. Biol.* 345, 987–1001. doi: 10.1016/j.jmb.2004.10.082

Babyatsky, M. W., Rossiter, G., and Podolsky, D. K. (1996). Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology* 110, 975–984. doi: 10.1053/gast.1996.v110.pm8613031

Belmiro, C. L. R., Souza, H. S. P., Elia, C. C. S., Castelo-Branco, M. T. L., Silva, F. R., Machado, R. L., et al. (2005). Biochemical and immunohistochemical analysis of glycosaminoglycans in inflamed and non-inflamed intestinal mucosa of patients with Crohn's disease. *Int. J. Color. Dis.* 20, 295–304. doi: 10.1007/s00384-004-0677-2

Benimetskaya, L., Berton, M., Kolbanovsky, A., Benimetsky, S., and Stein, C. A. (1997). Formation of a G-tetrad and higher order structures correlates with

biological activity of the RelA (NF-kappaB p65) 'antisense' oligodeoxynucleotide. *Nucleic Acids Res.* 25, 2648–2656. doi: 10.1093/nar/25.13.2648

Bennett, C. F., Baker, B. F., Pham, N., Swayze, E., and Geary, R. S. (2017). Pharmacology of antisense drugs. *Annu. Rev. Pharmacol. Toxicol.* 57, 81–105. doi: 10.1146/annurev-pharmtox-010716-104846

Bennett, C. F., Kornbrust, D., Henry, S., Stecker, K., Howard, R., Cooper, S., et al. (1997). An ICAM-1 antisense oligonucleotide prevents and reverses dextran sulfate sodium-induced colitis in mice. *J. Pharmacol. Exp. Ther.* 280, 988–1000.

Boirivant, M., Pallone, F., Di Giacinto, C., Fina, D., Monteleone, I., Marinaro, M., et al. (2006). Inhibition of Smad7 with a specific antisense oligonucleotide facilitates TGF-beta1-mediated suppression of colitis. *Gastroenterology* 131, 1786–1798. doi: 10.1053/j.gastro.2006.09.016

Burel, S. A., Hart, C. E., Cauntay, P., Hsiao, J., Machemer, T., Katz, M., et al. (2016). Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res.* 44, 2093–2109. doi: 10.1093/nar/gkv1210

Chan, J. H., Lim, S., and Wong, W. S. (2006). Antisense oligonucleotides: from design to therapeutic application. *Clin. Exp. Pharmacol. Physiol.* 33, 533–540. doi: 10.1111/j.1440-1681.2006.04403.x

Chery, J. (2016). RNA therapeutics: RNAi and antisense mechanisms and clinical applications. *Postdoc J.* 4, 35–50.

Crooke, S. T. (2004). Progress in antisense technology. *Annu. Rev. Med.* 55, 61–95. doi: 10.1146/annurev.med.55.091902.104408

Crooke, S. T. (2017). Molecular mechanisms of antisense oligonucleotides. *Nucleic Acid Ther.* 27, 70–77. doi: 10.1089/nat.2016.0656

Ding, Y., Chan, C. Y., and Lawrence, C. E. (2004). Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res.* 32, W135–W141. doi: 10.1093/nar/gkh449

Duell, P. B., Santos, R. D., Kirwan, B. A., Witztum, J. L., Tsimikas, S., and Kastelein, J. J. P. (2016). Long-term mipomersen treatment is associated with a reduction in cardiovascular events in patients with familial hypercholesterolemia. *J. Clin. Lipidol.* 10, 1011–1021. doi: 10.1016/j.jacl.2016.04.013

Eckstein, F. (2014). Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acid Ther.* 24, 374–387. doi: 10.1089/nat.2014.0506

Eder, P. S., Devine, R. J., Dagle, J. M., and Walder, J. A. (1991). Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3′ exonuclease in plasma. *Antisense Res. Dev.* 1, 141–151. doi: 10.1089/ard.1991.1.141

Feagan, B. G., Sands, B. E., Rossiter, G., Li, X., Usiskin, K., Zhan, X., et al. (2018). Effects of mongersen (GED-0301) on endoscopic and clinical outcomes in patients with active Crohn's disease. *Gastroenterology* 154, 61–64.e66. doi: 10.1053/j.gastro.2017.08.035

Gorelik, L., and Flavell, R. A. (2002). Transforming growth factor-beta in T-cell biology. *Nat. Rev. Immunol.* 2, 46–53. doi: 10.1038/nri704

Goyal, N., and Narayanaswami, P. (2018). Making sense of antisense oligonucleotides: a narrative review. *Muscle Nerve* 57, 356–370. doi: 10.1002/mus.26001

Greuter, T., Biedermann, L., Rogler, G., Sauter, B., and Seibold, F. (2016). Alicaforsen, an antisense inhibitor of ICAM-1, as treatment for chronic refractory pouchitis after proctocolectomy: a case series. *United European Gastroenterol. J.* 4, 97–104.

Greuter, T., Vavricka, S. R., Biedermann, L., Pilz, J., Borovicka, J., Seibold, F., et al. (2018). Alicaforsen, an antisense inhibitor of intercellular adhesion molecule-1, in the treatment for left-sided ulcerative colitis and ulcerative proctitis. *Dig. Dis.* 36, 123–129. doi: 10.1159/000484979

Hagedorn, P. H., Persson, R., Funder, E. D., Albaek, N., Diemer, S. L., Hansen, D. J., et al. (2018). Locked nucleic acid: modality, diversity, and drug discovery. *Drug Discov. Today* 23, 101–114. doi: 10.1016/j.drudis.2017.09.018

Hart, A. L., Ng, S. C., Mann, E., Al-Hassi, H. O., Bernardo, D., and Knight, S. C. (2010). Homing of immune cells: role in homeostasis and intestinal inflammation. *Inflamm. Bowel Dis.* 16, 1969–1977. doi: 10.1002/ibd.21304

Heldin, C. H., Miyazono, K., and Ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390, 465–471. doi: 10.1038/37284

Ho, S. P., Bao, Y., Lesher, T., Malhotra, R., Ma, L. Y., Fluharty, S. J., et al. (1998). Mapping of RNA accessible sites for antisense experiments with oligonucleotide libraries. *Nat. Biotechnol.* 16, 59–63. doi: 10.1038/nbt0198-59

Izzo, R., Bevivino, G., De Simone, V., Sedda, S., Monteleone, I., Marafini, I., et al. (2018). Knockdown of Smad7 with a specific antisense oligonucleotide attenuates colitis and colitis-driven colonic fibrosis in mice. *Inflamm. Bowel Dis.* 24, 1213–1224. doi: 10.1093/ibd/izy062

Jabs, D. A., and Griffiths, P. D. (2002). Fomivirsen for the treatment of cytomegalovirus retinitis. *Am J. Ophthalmol.* 133, 552–556. doi: 10.1016/S0002-9394(02)01325-9

Khorkova, O., and Wahlestedt, C. (2017). Oligonucleotide therapies for disorders of the nervous system. *Nat. Biotechnol.* 35, 249–263. doi: 10.1038/nbt.3784

Laudisi, F., Dinallo, V., Di Fusco, D., and Monteleone, G. (2016). Smad7 and its potential as therapeutic target in inflammatory bowel diseases. *Curr. Drug Metab.* 17, 303–306. doi: 10.2174/1389200217666151210130103

Lawrance, I. C., Wu, F., Leite, A. Z., Willis, J., West, G. A., Fiocchi, C., et al. (2003). A murine model of chronic inflammation-induced intestinal fibrosis down-regulated by antisense NF-kappa B. *Gastroenterology* 125, 1750–1761. doi: 10.1053/j.gastro.2003.08.027

Leask, A., and Abraham, D. J. (2004). TGF-beta signaling and the fibrotic response. *FASEB J.* 18, 816–827. doi: 10.1096/fj.03-1273rev

Li, Z., and Rana, T. M. (2012). Molecular mechanisms of RNA-triggered gene silencing machineries. *Acc. Chem. Res.* 45, 1122–1131. doi: 10.1021/ar200253u

Macdonald, T. T., Monteleone, I., Fantini, M. C., and Monteleone, G. (2011). Regulation of homeostasis and inflammation in the intestine. *Gastroenterology* 140, 1768–1775. doi: 10.1053/j.gastro.2011.02.047

Miner, P. B. Jr., Wedel, M. K., Xia, S., and Baker, B. F. (2006). Safety and efficacy of two dose formulations of alicaforsen enema compared with mesalazine enema for treatment of mild to moderate left-sided ulcerative colitis: a randomized, double-blind, active-controlled trial. *Aliment. Pharmacol. Ther.* 23, 1403–1413. doi: 10.1111/j.1365-2036.2006.02837.x

Monteleone, G., Fantini, M. C., Onali, S., Zorzi, F., Sancesario, G., Bernardini, S., et al. (2012). Phase I clinical trial of Smad7 knockdown using antisense oligonucleotide in patients with active Crohn's disease. *Mol. Ther.* 20, 870–876. doi: 10.1038/mt.2011.290

Monteleone, G., Kumberova, A., Croft, N. M., Mckenzie, C., Steer, H. W., and Macdonald, T. T. (2001). Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest.* 108, 601–609. doi: 10.1172/JCI12821

Monteleone, G., Neurath, M. F., Ardizzone, S., Di Sabatino, A., Fantini, M. C., Castiglione, F., et al. (2015). Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *N. Engl. J. Med.* 372, 1104–1113. doi: 10.1056/NEJMoa1407250

Murano, M., Maemura, K., Hirata, I., Toshina, K., Nishikawa, T., Hamamoto, N., et al. (2000). Therapeutic effect of intracolonically administered nuclear factor kappa B (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis. *Clin. Exp. Immunol.* 120, 51–58. doi: 10.1046/j.1365-2249.2000.01183.x

Nair, J. K., Willoughby, J. L., Chan, A., Charisse, K., Alam, M. R., Wang, Q., et al. (2014). Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc.* 136, 16958–16961. doi: 10.1021/ja505986a

Neurath, M. (2017). Current and emerging therapeutic targets for IBD. *Nat. Rev. Gastroenterol. Hepatol.* 14:688. doi: 10.1038/nrgastro.2017.138

Neurath, M. F., Fuss, I., Schurmann, G., Pettersson, S., Arnold, K., Muller-Lobeck, H., et al. (1998). Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease. *Ann. N. Y. Acad. Sci.* 859, 149–159. doi: 10.1111/j.1749-6632.1998.tb11119.x

Neurath, M. F., Pettersson, S., Meyer Zum Buschenfelde, K. H., and Strober, W. (1996). Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat. Med.* 2, 998–1004. doi: 10.1038/nm0996-998

Prakash, T. P. (2011). An overview of sugar-modified oligonucleotides for antisense therapeutics. *Chem. Biodivers.* 8, 1616–1641. doi: 10.1002/cbdv.201100081

Rieder, F., Fiocchi, C., and Rogler, G. (2017). Mechanisms, management, and treatment of fibrosis in patients with inflammatory bowel diseases. *Gastroenterology* 152, 340–350.e346. doi: 10.1053/j.gastro.2016.09.047

Rogler, G., Brand, K., Vogl, D., Page, S., Hofmeister, R., Andus, T., et al. (1998). Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 115, 357–369. doi: 10.1016/S0016-5085(98)70202-1

Schreiber, S., Nikolaus, S., and Hampe, J. (1998). Activation of nuclear factor kappa B inflammatory bowel disease. *Gut* 42, 477–484. doi: 10.1136/gut.42.4.477

Schreiber, S., Nikolaus, S., Malchow, H., Kruis, W., Lochs, H., Raedler, A., et al. (2001). Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. *Gastroenterology* 120, 1339–1346. doi: 10.1053/gast.2001.24015

Sedda, S., Marafini, I., Dinallo, V., Di Fusco, D., and Monteleone, G. (2015). The TGF-beta/Smad system in IBD pathogenesis. *Inflamm. Bowel Dis.* 21, 2921–2925. doi: 10.1097/MIB.0000000000000542

Sen, D., and Gilbert, W. (1992). Novel DNA superstructures formed by telomere-like oligomers. *Biochemistry* 31, 65–70.

Sharma, V. K., and Watts, J. K. (2015). Oligonucleotide therapeutics: chemistry, delivery and clinical progress. *Future Med. Chem.* 7, 2221–2242. doi: 10.4155/fmc.15.144

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113, 685–700. doi: 10.1016/S0092-8674(03)00432-X

Stein, C. A. (2001). The experimental use of antisense oligonucleotides: a guide for the perplexed. *J. Clin. Invest.* 108, 641–644. doi: 10.1172/JCI13885

Suzuki, K., Arumugam, S., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., et al. (2016). Pivotal role of carbohydrate sulfotransferase 15 in fibrosis and mucosal healing in mouse colitis. *PLoS One* 11:e0158967. doi: 10.1371/journal.pone.0158967

Suzuki, K., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., Aoyagi, Y., et al. (2017). Phase 1 clinical study of siRNA targeting carbohydrate sulphotransferase 15 in Crohn's disease patients with active mucosal lesions. *J. Crohns Colitis* 11, 221–228.

Tanowitz, M., Hettrick, L., Revenko, A., Kinberger, G. A., Prakash, T. P., and Seth, P. P. (2017). Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes. *Nucleic Acids Res.* 45, 12388–12400. doi: 10.1093/nar/gkx960

Tatiparti, K., Sau, S., Kashaw, S. K., and Iyer, A. K. (2017). siRNA delivery strategies: a comprehensive review of recent developments. *Nanomaterials* 7:77. doi: 10.3390/nano7040077

Tu, G. C., Cao, Q. N., Zhou, F., and Israel, Y. (1998). Tetranucleotide GGGA motif in primary RNA transcripts. Novel target site for antisense design. *J. Biol. Chem.* 273, 25125–25131. doi: 10.1074/jbc.273.39.25125

Vainer, B., and Nielsen, O. H. (2000). Changed colonic profile of P-selectin, platelet-endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and ICAM-3 in inflammatory bowel disease. *Clin. Exp. Immunol.* 121, 242–247. doi: 10.1046/j.1365-2249.2000.01296.x

Vallance, B. A., Gunawan, M. I., Hewlett, B., Bercik, P., Van Kampen, C., Galeazzi, F., et al. (2005). TGF-beta1 gene transfer to the mouse colon leads to intestinal fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 289, G116–G128. doi: 10.1152/ajpgi.00051.2005

Van Deventer, S. J., Wedel, M. K., Baker, B. F., Xia, S., Chuang, E., and Miner, P. B. Jr. (2006). A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment. Pharmacol. Ther.* 23, 1415–1425. doi: 10.1111/j.1365-2036.2006.02910.x

Vickers, T. A., Wyatt, J. R., and Freier, S. M. (2000). Effects of RNA secondary structure on cellular antisense activity. *Nucleic Acids Res.* 28, 1340–1347. doi: 10.1093/nar/28.6.1340

Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hokfelt, T., et al. (2000). Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *Proc. Natl. Acad. Sci. USA* 97, 5633–5638.

Yacyshyn, B. R., Bowen-Yacyshyn, M. B., Jewell, L., Tami, J. A., Bennett, C. F., Kisner, D. L., et al. (1998). A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 114, 1133–1142. doi: 10.1016/S0016-5085(98)70418-4

Yacyshyn, B., Chey, W. Y., Wedel, M. K., Yu, R. Z., Paul, D., and Chuang, E. (2007). A randomized, double-masked, placebo-controlled study of alicaforsen, an antisense inhibitor of intercellular adhesion molecule 1, for the treatment of subjects with active Crohn's disease. *Clin. Gastroenterol. Hepatol.* 5, 215–220. doi: 10.1016/j.cgh.2006.11.001

Yacyshyn, B. R., Chey, W. Y., Goff, J., Salzberg, B., Baerg, R., Buchman, A. L., et al. (2002). Double blind, placebo controlled trial of the remission inducing and steroid sparing properties of an ICAM-1 antisense oligodeoxynucleotide, alicaforsen (ISIS 2302), in active steroid dependent Crohn's disease. *Gut* 51, 30–36. doi: 10.1136/gut.51.1.30

Zorzi, F., Calabrese, E., Monteleone, I., Fantini, M., Onali, S., Biancone, L., et al. (2012). A phase 1 open-label trial shows that smad7 antisense oligonucleotide (GED0301) does not increase the risk of small bowel strictures in Crohn's disease. *Aliment. Pharmacol. Ther.* 36, 850–857. doi: 10.1111/apt.12051

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31, 3406–3415. doi: 10.1093/nar/gkg595

Conflict of Interest Statement: GM has filed a patent related to the treatment of IBD with Smad7 antisense oligonucleotides.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 24 April 2019
doi: 10.3389/fphar.2019.00314

Oligonucleotides—A Novel Promising Therapeutic Option for IBD

Patrizio Scarozza[1,2]*, *Heike Schmitt*[2], *Giovanni Monteleone*[1], *Markus F. Neurath*[2] and *Raja Atreya*[2]*

[1] Department of Systems Medicine, Gastroenterology, University of Tor Vergata, Rome, Italy, [2] Department of Medicine 1, Friedrich-Alexander-University of Erlangen-Nürnberg, Erlangen, Germany

Inflammatory Bowel Diseases (IBD), whose denomination comprehends Crohn's Disease (CD) and Ulcerative Colitis (UC), are intestinal chronic diseases that often require lifelong medical therapy. In the last two decades monoclonal antibodies against the cytokine TNF have become integral parts in the treatment of IBD patients, however there are unwanted side-effects and one third of patients show primary non-response while another subgroup loses response over time. Finding novel drugs which could act as therapies against precise pro-inflammatory molecular targets to avoid unwanted systemic side effects and additionally the process of immunization, represents an important aim for subsequent therapeutic approaches. Oligonucleotide based therapies represent a promising novel concept for the treatment of IBD. The molecular action of oligonucleotides ranges from inhibition of the translational process of mRNA transcripts of pro-inflammatory molecules, to mimicking bacterial DNA which can activate cellular targets for immunomodulation. Alicaforsen, selectively targets ICAM-1 mRNA. ICAM-1 is an adhesion molecule which is upregulated on endothelial cells during IBD, thereby mediating the adhesion and migration of leucocytes from blood to sites of active inflammation. In CD parenteral application of alicaforsen did not show therapeutic efficacy in phase II trials, but it demonstrated an improved efficacy as a topical enema in distal UC. Topical application of alicaforsen might represent a therapeutic perspective for refractory pouchitis as well. SMAD7 is a protein that inhibits the signaling of TGFβ, which is the mainstay of a regulatory counterpart in cellular immune responses. An antisense oligonucleotide against SMAD7 mRNA (mongersen) demonstrated pre-clinical and phase II efficacy in CD, but a phase III clinical trial was stopped due to lack of efficacy. Cobitolimod is a single strand oligonucleotide, which mimics bacterial DNA as its CpG dinucleotide sequences can be recognized by the Toll-like receptor 9 on different immune cells thereby causing induction of different cytokines, for example IL10 and IFNα. Topical application of cobitolimod was studied in UC patients. We will also discuss two other novel oligonucleotides which act on the GATA3 transcription factor (SB012) and on carbohydrate sulfotransferase 15 (STNM01), which could both represent novel promising therapeutic options for the treatment of UC.

Keywords: Crohn disease, ulcerative colitis, IBD, antisense oligonucleotide (ASO), target therapies

OPEN ACCESS

Edited by:
Luca Antonioli,
University of Pisa, Italy

Reviewed by:
Maria Cecilia Giron,
University of Padova, Italy
Antonio Di Sabatino,
University of Pavia, Italy

***Correspondence:**
Patrizio Scarozza
scarozzapatrizio@gmail.com
Raja Atreya
raja.atreya@uk-erlangen.de

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *15 October 2018*
Accepted: *14 March 2019*
Published: *24 April 2019*

Citation:
Scarozza P, Schmitt H, Monteleone G, Neurath MF and Atreya R (2019) Oligonucleotides—A Novel Promising Therapeutic Option for IBD. Front. Pharmacol. 10:314. doi: 10.3389/fphar.2019.00314

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the intestine whose phenotypic spectrum mainly comprehends Crohn's disease (CD) and ulcerative colitis (UC). IBD are characterized by a chronic disease course with the alternation of active and quiescent phases, thereby requiring continuous follow up and management by health care systems (Maaser et al., 2019). In the last 20 years, there has been an increase in the prevalence of IBD from a percentage of 0.3–0.5% in North America and similarly in Northern Europe, Western Europe, and Oceania (Kaplan and Ng, 2017). Moreover, a profound rise in incidence was described in Asia and Latin America (Ng et al., 2018).

IBD patients often require treatment with immunomodulatory drugs such as thiopurines, methotrexate; biological drugs, like anti-Tumor Necrosing Factor (anti-TNF), anti-adhesion, or anti-interleukin 12/23p40 (IL12/23p40) antibodies and lately also Janus Kinase Inhibitors (JAK-inhibitors). These different substance classes have become integral parts in the treatment of IBD patients; however there are unwanted, sometimes even severe side effects and over one third of patients show primary non-response while another subgroup of patients loses response over time (Roda et al., 2016). There is therefore the dire need for novel efficacious therapies with a favorable safety profile. Oligonucleotides represent an emerging substance class of targeted therapies with only limited systemic exposure to the drug, giving hope for a novel and rather safe treatment modality in IBD.

The molecular mechanism of action of oligonucleotides ranges from inhibition of the translational process of messenger ribonucleic acid (mRNA) transcripts of pro-inflammatory molecules, to mimicking bacterial deoxyribonucleic acid (DNA) which can activate cellular targets for immunomodulation.

Antisense technology is based on two principal different modes of action: activation of Ribonuclease H (RNase H) or double-strand RNases. The activation of RNase H is mediated by the recognition of the sense-antisense heterodimer, after the binding of the single-strand oligonucleotide to the complementary target mRNA (Crooke, 2004). The heterodimer induces the activation of the RNase H enzyme, which inhibits the expression of the target mRNA (Aboul-Fadl, 2005). The second antisense mechanism is linked to the action of double-strand RNases which are activated by silence RNA (siRNA) molecules that are characterized by a double-strand oligonucleotide formed by a sense and an antisense RNA strand. After entering the cell, the antisense RNA recognizes the target cytoplasmic mRNA, forming a sense-antisense duplex, that is finally degraded by endogenous double-strand RNases (Crooke, 2004).

For the last 20 years, antisense technology has been improved in various fields of medical research. In 1999 the European Agency for the Evaluation of Medical Products (EMEA) approved Fomivirsen, as the first therapeutically used antisense oligonucleotide, developed for the treatment of Cytomegalovirus (CMV) retinitis. In the meantime many more oligonucleotide based substances have been tested in respective trials or already entered clinical practice: Macugen to treat age-related macular degeneration; Mipomersen for homozygous familial hypercholesterolemia; Eteplirsen for Duchenne muscular dystrophy; Defibrotide for severe hepatic veno-occlusive disease; and Nusinersen for the treatment of infants with types 1, 2, and 3 spinal muscular atrophy (Stein and Castanotto, 2017).

This review focuses on pharmacodynamics and clinical perspectives of oligonucleotide based therapeutics so far studied for the treatment of IBD. (**Supplementary Material**).

ALICAFORSEN

Active inflammation in IBD is characterized by overexpression of adhesion molecules on endothelial cells that are centrally involved in mediating the gut homing of leukocytes. Inhibition of migration has become one of the established therapeutic approaches in IBD, with approval of the alpha4beta7 antibody vedolizumab for treatment of both IBD entities and ongoing clinical trials for many more therapeutic targets of the homing process (Sands, 2017; Zundler and Neurath, 2017). Intercellular Cell Adhesion Molecule-1 (ICAM1) is an adhesion molecule derived from the immunoglobulin superfamily acting as a ligand for the integrins expressed by leukocytes and represents one of the novel targets of antisense oligonucleotide (ASO) based therapeutic strategies (Mosli et al., 2014; Sands, 2017).

ICAM1 is overexpressed by endothelial cells during active disease in CD patients and its presence is thus diminished during remission. It was furthermore shown, that in UC patients the expression of ICAM1 in colonic tissue is higher than in respective controls (Jones et al., 1995; Vainer and Nielsen, 2000; Rivera-Nieves et al., 2008).

Preclinical studies have figured out the efficacy of a monoclonal antibody against ICAM1 administered topically in the experimental dextran sulfate sodium (DSS) murine model of colitis, which led to the reduction of mucosal inflammation (Hamamoto et al., 1999). When combined with anti-Vascular Cell Adhesion Molecule-1 (anti-VCAM1) or anti-alpha4 integrin antibodies, the ICAM1 antibody similarly exerted a positive effect on reducing the inflammatory response in a murine model of ileitis (Burns et al., 2001).

Alicaforsen is a 20-base phosphorothioate ASO that hybridizes the mRNA of ICAM1 and induces the hydrolysis of the DNA-RNA complex by an RNAse enzyme (Gewirtz and Sitaraman, 2001). A randomized trial of ISIS 2302, an ASO to ICAM1, was conducted in patients affected by severe rheumatoid arthritis, without evidence of significant efficacy, but with a good safety profile (Maksymowych et al., 2002). A pivotal phase I dose ranging trial conducted on healthy volunteers who intravenously received the ICAM1 ASO, showed good tolerance to the drug, with reproducible plasma levels and limited side effects (dose-related increase in activated partial thromboplastin time, clinical insignificant increases in complement factor C3a) at the time of the peak plasma concentration, without occurrence of associated clinical symptoms (Glover et al., 1997).

A dose ranging pharmacokinetic trial of high-dose alicaforsen randomized 22 patients with active CD to three alicaforsen treatment groups: 250-, 300-, or 350-mg doses, infused intravenously three times a week for 4 weeks. The authors

evidenced a possible effectiveness of alicaforsen therapy in CD. Clinical remission was evidenced in 41% of all treated subjects and in 53% of those treated with more than three of the 12 infusions planned (Yacyshyn et al., 2002a).

A randomized trial of ISIS 2302, conducted in twenty CD patients, allocated to receive one of three different doses of this drug (0.5, 1, or 2 mg/kg) or saline placebo, evidenced a therapeutic success of the experimental drug, which was infused intravenously in 13 doses over 26 days (Yacyshyn et al., 1998; Reinisch et al., 2018). The remission rate after 1 month of therapy was 47% (7/15) in patients treated with the investigated substance and 20% (1/5) in patients treated with placebo. At the end of the 6 months follow up period, 5 of the 7 patients treated with the investigated substance maintained clinical remission and 33% (5 of 15) successfully tapered concomitant corticosteroid treatment (Yacyshyn et al., 1998).

These positive perspectives about the steroid sparing effect of ISIS 2302 were not confirmed in a subsequent trial, which primary endpoint was steroid-free remission at week 14. Here, 75 steroid refractory CD patients were randomized to four groups of treatment with ISIS 2302 or to placebo. The study drug was administered subcutaneously at four different dose-interval regimens (0.5 mg/kg once daily for 2 days; 0.5 mg/kg five times a week for 1 week; 0.5 mg/kg five times a week for 2 weeks or 0.5 mg/kg five times a week for 4 weeks) (Schreiber et al., 2001; Lobaton et al., 2014). Steroid-free remission at week 14 was registered in only 3.3% (2/60) of patients treated with ISIS 2302 and in none of the placebo patients (Schreiber et al., 2001; Lobaton et al., 2014). This negative property to induce steroid-free remission was also confirmed by another study (Yacyshyn et al., 2002b). Here, 299 steroid-dependent CD patients were randomized into three treatment arms: ISIS 2302 (2 mg/kg) given intravenously three times a week for two weeks, vs. ISIS 2302 (2 mg/kg) given intravenously three times a week for 4 weeks, compared to placebo application. Steroid free remission at week 14 was defined as the primary endpoint. It was reached by 20.2 and 21.2%, respectively in the ISIS 2302 arm with treatment lasting 2 or 4 weeks compared to 18.8% in the placebo arm. Therefore, no statistical significant difference regarding clinical efficacy could be shown (Yacyshyn et al., 2002b).

The last clinical trial conducted in CD patients, which results were published in 2007, confirmed the inefficacy of alicaforsen in inducing clinical remission in CD patients. In this study, 331 CD patients were randomized to two treatment groups: alicaforsen given intravenously, at a dosage of 100 mg at the time of the first infusion, followed by 300 mg given three times a week for 4 weeks ($n = 221$) compared to placebo administration ($n = 110$). The primary endpoint was clinical remission at week 12. No statistical differences regarding clinical remission at week 12 were evidenced between the two treatment groups (33.9% in the group treated with alicaforsen vs. 34.5% in the placebo group; $p = 0.89$) (Yacyshyn et al., 2007). These results have led to the halt of further clinical studies of this compound in CD patients.

In UC, some clinical studies demonstrated efficacy of alicaforsen in inducing clinical response and remission via topical application. First, an effective induction of clinical response by topical application of alicaforsen was evidenced by a randomized multicenter trial conducted in 40 UC patients affected by mild to moderate distal colitis, who were randomized to four dosing cohorts of an alicaforsen enema (0.1, 0.5, 2, or 4 mg/ml) or placebo, given once daily for 28 consecutive days (van Deventer et al., 2004). This therapeutic procedure resulted in the induction of clinical response in a dose-dependent way, with induction of response in 70% of alicaforsen 4 mg/ml treated patients compared to a placebo response of 28% at week 4, which was statistically significant ($p = 0.004$). In the group treated with alicaforsen at a dosage of 2 mg/ml, clinical response was evidenced in 45% of treated patients ($p = 0.201$). During the 6 months clinical follow up period, half of the patients in the placebo arm (4/8) required another medication or surgical intervention, whereas none of the patients treated with the highest dose of alicaforsen and two patients in the 2 mg/ml group required treatment escalation (van Deventer et al., 2004).

A randomized controlled trial conducted in active UC patients affected by mild to moderate left-sided colitis did not lead to a significantly different clinical outcome between the groups treated with topical application of the alicaforsen enema compared to placebo administration. The patients were randomized to five treatment arms: alicaforsen enema at a dosage of 120 mg daily for the first 10 days of 6 weeks of treatment and then every other day thereafter; 240 mg every other day for 6 weeks; 240 mg daily for the first 10 days of 6 weeks of treatment and then every other day thereafter or 240 mg daily for 6 weeks or placebo application. Primary endpoint was the Disease Activity Index (DAI) score at week 6. No significant differences were evidenced between the treatment arms and placebo (van Deventer et al., 2006). All groups demonstrated a decrease in the DAI score, but the best cohort response was identified in the group treated with daily application of 240 mg alicaforsen enema. Clinical response at week 6 was 47% in the 240 mg alicaforsen arm and 33% in the placebo arm ($p =$ N.S.). Despite no significant differences identified for the primary endpoint clinical response, this study demonstrated that patients treated with an alicaforsen enema maintained an improved DAI score from week 18 to week 30 after commencement of therapy, compared to patients treated with placebo. The decrease in the mean DAI score compared to baseline was 51% at week 18 and 50% at week 30 in the group treated with daily application of 240 mg alicaforsen enema, compared to 18% ($p = 0.04$) and 11% in the placebo arm ($p = 0.03$) (van Deventer et al., 2006).

A subsequent randomized study was conducted in UC patients affected by mild to moderate distal UC, which were randomized to three treatment arms: 120 or 240 mg alicaforsen or 4 g mesalazine enema, given once daily for a total of 6 weeks (Miner et al., 2006). The aim of the study was to compare the effects of alicaforsen enema administration to standard mesalazine enema therapy. The primary endpoint was DAI reduction at week 6. No significant differences were highlighted between the treatment arms. The mean percentage reduction of the DAI score from baseline was 50% for the group treated with the mesalazine enema and 40% and 41% for the group treated with 120 and 240 mg of the alicaforsen enema ($p = 0.27$ and 0.32, respectively). Despite the failed difference regarding the primary endpoint, in the therapeutic arms of the alicaforsen enema the

maintenance of clinical response was longer (128 and 146 days, respectively) in comparison to the mesalazine arm (54 days) (Miner et al., 2006). Moreover, the highest rate of mucosal healing in the 240 mg alicaforsen group was evidenced at week 10 and was achieved by 24% of patients; in the mesalazine arm it was achieved by 17% of patients and was evidenced at week 3 and 6 ($p = 0.06$) (Miner et al., 2006). These two studies indicated a possible modest durable effect of alicaforsen enema application and sustained maintenance of clinical response, but nevertheless non-significance regarding the primary endpoint.

A *post-hoc* meta-analysis focused on the results of four phase 2 clinical studies and investigated the overall efficacy of alicaforsen enema administration in the treatment of patients with active left-sided UC and pancolitis. The authors considered for the meta-analysis three different subgroups of patients: the subgroup with distal UC with active inflammation and disease extension from 5–40 cm; the subgroup with moderate or severe disease regardless of the extension of disease and the subgroup with both distal colitis and moderate/severe inflammation. The authors considered for the analysis the following treatment patterns: 240 mg alicaforsen enema once daily for a duration of 6 weeks; 4 g mesalazine enema once daily for 6 weeks or placebo. The higher efficacy (measured as mean percentage reduction in the DAI score) of alicaforsen over placebo was statistically significant at week 6 (51.4 vs. 27.1%; $p = 0.004$) and week 10 (51 vs. 24.7%; $p = 0.014$) in patients with distal disease. At week 30, alicaforsen enema application did not demonstrate a significant effect of maintenance of clinical response in comparison to placebo therapy (Vegter et al., 2013).

In the meta-analysis, a similar clinical efficacy (measured as mean percentage reduction in the DAI score) at week 6 and 10 was evidenced between alicaforsen and mesalazine enema therapy (51.4 vs. 51.3% at week 6 and 51 vs. 41.6% at week 10). However, the efficacy of alicaforsen enema was more durable than that of mesalazine, after 6 weeks of treatment. At week 30 the efficacy (mean percentage reduction in the DAI score) of alicaforsen enema application resulted to be 40.1%, while the efficacy of mesalazine enema was 20.6% ($p = 0.05$) in the treated patients. Similar results as for the subgroup with distal UC were found for the remaining two other subgroups of patients (Vegter et al., 2013).

Despite the profound improvement in UC management with biological drugs, a substantial proportion of patients require surgical treatment with proctocolectomy and ileal-pouch-anal anastomosis (IPAA) in the course of disease, in particular for severe flares of active disease, refractory to available medical therapies or due to the diagnosis of non-resectable colonic dysplasia. Onset of acute or chronic pouchitis is one of the most frequently occurring post-operative complications that patients experience and almost half of them present with this kind of problem within 5 years after the time of surgery (Tiainen and Matikainen, 1999). Alicaforsen could represent a novel therapeutic option for this hard to treat clinical problem, as indicated by conducted studies.

A first open-label uncontrolled study conducted in 12 patients affected by chronic pouchitis figured out a statistical significant reduction of the Pouch-Disease-Activity-Index (PDAI), which was 11.42 at baseline and 6.83 at week 6 ($p = 0.001$) and also of the PDAI endoscopic sub-score at week 6, which was 3.75 at baseline and 2.25 at week 6 ($p = 0.0117$) after daily topical administration of alicaforsen at a dosage of 240 mg for 6 weeks (Miner et al., 2004; McConnell et al., 2009). A phase III randomized trial conducted in patients affected by refractory pouchitis has been recently completed (Fiorino et al., 2011; EPG Health, 2018).

The main findings regarding the above mentioned clinical trials can be found in **Table 1**.

MONGERSEN

Immune homeostasis in the intestinal mucosa plays a fundamental role in preventing inflammatory immune responses in IBD (MacDonald and Monteleone, 2005; MacDonald et al., 2011). One of the most important cytokines that mediate a regulatory mucosal immune response is Transforming Growth Factor β (TGFβ). This cytokine has been implicated in the function of regulatory T (T reg) cells that mediate suppression of inflammatory responses, as evidenced in mice models of colitis (Powrie et al., 1996). Different studies figured out a greater level of regulatory T cells in the inflamed mucosa of IBD patients than in healthy controls, thereby inducing subsequent studies for the analysis of T reg cells in the tissue of IBD patients (Monteleone et al., 2001).

A study conducted in mice models of colitis demonstrated increased TGFβ levels in the inflamed tissue of colitic mice, indicating that a blockade of TGFβ signaling transduction by counter-regulatory proteins overexpressed during the active state of inflammation might be involved to suppress its anti-inflammatory function (Boirivant et al., 2006). This finding has been the first step toward the discovery of Mothers against decapentaplegic homolog 7 (Smad7) that has an inhibitory role within the TGFβ signaling cascade. This intracellular signaling molecule is an inhibitor of TGFβ signaling, as it can bind to the TGFβ receptor thereby inhibiting phosphorylation of downstream signaling molecules such as Smad2 and Smad3, whose activation mediates TGFβ signal transduction.

First studies figured out a heightened expression of Smad7 in the mucosal intestinal compartment of IBD patients (Monteleone et al., 2001). In contrast, high expression of phosphorylated Smad3 was seen in the normal intestinal mucosa (Monteleone et al., 2001). The same study showed an increased response of intestinal mononuclear cells to TGFβ after the administration of a Smad7 ASO, which restored the phosphorylation of Smad3. These findings demonstrate that Smad7 blockade by a Smad7 ASO enabled TGFβ to inhibit the function of pro-inflammatory intestinal cells.

A subsequent phase I open-label study of an ASO against Smad7 (GED0301) was conducted in CD patients with steroid-dependent/resistant disease. Fifteen CD patients were randomized to receive one of the three treatment dosages of oral GED0301 (40, 80, or 160 mg) once daily for 7 consecutive days. The drug was well-tolerated. Oral administration of GED0301 (mongersen) led to the reduction of pro-inflammatory C-C Chemokine Receptor 9 (CCR9)-positive T cells in the bloodstream. This study showed a good safety and tolerability profile of the studied substance (Monteleone et al., 2012). Since TGFβ has been described to induce the

TABLE 1 | Overview of clinical studies with alicaforsen.

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
ISIS 2302	Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302) (Glover et al., 1997)	Phase 1	Healthy male volunteers	Double-blind, placebo-controlled study	44 male volunteers	Four healthy male volunteers recruited to each of seven single-dose groups (0.06, 0.12, 0.24, 0.5, 1.0, 1.5, and 2.0 mg/kg) and each of four multiple-dose groups (0.2, 0.5, 1.0, and 2.0 mg/kg). One subject in each group was allocated to placebo arm. The drug was infused intravenously on day 1 in the single dose-group and on days 1, 3, 5, and 7 in the multiple-dose group	Safety and pharmacokinetic profile of the drug	Good tolerability to the drug, with reproducibility of plasma pharmacokinetics
ISIS 2302	Dose ranging pharmacokinetic trial of high-dose alicaforsen (intercellular adhesion molecule-1 antisense oligodeoxynucleotide) (ISIS 2302) in active Crohn's disease (Yacyshyn et al., 2002a)	/	Patients with active CD (CDAI ≥220)	Double-blind, randomized study	22 CD patients	10 patients were randomized to the 300 mg arm, 10 to the 350 mg arm and 2 patients to the 250 mg arm. The drug was infused intravenously three times a week for 4 weeks	Clinical remission (CDAI ≤150) at week 8, 12, and after 6 months	In the intention to treat (ITT) population 14% (3/22) of the patients at week 8 and 23% (5/22) of the patients at week 12 were in clinical remission. After 6 months, 18% (4/22) of the ITT patients were in clinical remission. The efficacy was equivalent with all the three doses used
ISIS 2302	A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease (Yacyshyn et al., 1998)	/	Patients with active moderate CD (CDAI 220–350)	Double-blind, placebo-controlled study	20 CD patients	The patients were randomized (3:1, ISIS 2302 to placebo). The drug was infused intravenously at a dosage of 0.5, 1, or 2 mg/kg and was administered in 13 doses over 26 days	Assessment of tolerability, pharmacology and efficacy of the drug in steroid-dependent CD	Clinical remission was figured out in 47% (7/15) of the patients treated with the drug and in 20% (1/5) of those treated with placebo after 26 days from the start of the therapy. After 6 months 5 of the 7 patients treated with the drug maintained remission. The drug was correlated to a good profile of safety
ISIS 2302	Efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease (Schreiber et al., 2001)	/	Patients with steroid-refractory CD (CDAI 200–400)	Double-blind, placebo-controlled study	75 CD patients	75 patients were randomized to 4 treatment arms and a placebo group. 14 patients received treatment for 2 days, 17 patients for 1 week, 15 patients for 2 weeks and 14 patients for 4 weeks. 15 patients received placebo. The drug was administered subcutaneously at a dosage of 0.5 mg/kg	Complete clinical remission (CDAI <150 with discontinuation of steroids) at week 14	Clinical remission was reached by 18.3% of treated patients and by 20% of those treated with placebo at the week 14. No difference in clinical remission between the drug and placebo was evidenced

(Continued)

TABLE 1 | Continued

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
ISIS 2302	Double blind, placebo controlled trial of the remission inducing and steroid sparing properties of an ICAM-1 antisense oligodeoxynucleotide, alicaforsen (ISIS 2302), in active steroid dependent Crohn's disease (Yacyshyn et al., 2002b)	/	Steroid dependent CD patients with active disease (CDAI 200–350)	Double-blind, placebo-controlled study	299 CD patients	The patients were randomized into three groups: ISIS 2302 at a dosage of 2 mg/kg intravenously three times a week for 2 weeks, ISIS 2302 at the same dosage and with the same frequency but for 4 weeks and placebo	Steroid free remission with a CDAI <150 at the end of week 14	Steroid free remission at week 14 was 20.2% in the group treated for 2 weeks, 21.2% in the group treated for 4 weeks and 18.8% in placebo group. No difference in clinical remission between the drug and placebo was evidenced
ISIS 2302 (Alicaforsen)	A randomized, double-masked, placebo-controlled study of alicaforsen, an antisense inhibitor of intercellular adhesion molecule 1, for the treatment of subjects with active Crohn's disease (Yacyshyn et al., 2007)	Phase 3	Patients with active CD (CDAI 220–400)	Double-blind, placebo-controlled study	331 CD patients	221 patients were randomized to the study drug at a dosage of 100 mg at the first infusion and for the other 11 infusions at a dosage of 300 mg (patients weighing more than 50 kg) or 200 mg (patients weighing 36–50 kg). The drug was administered intravenously 3 times a week for 4 weeks. 110 patients were enrolled in placebo arm	Clinical remission at week 12	No statistical difference between the study drug and placebo for clinical remission at week 12 (33.9% in the drug and 34.5% in the placebo arm)
ISIS 2302 (Alicaforsen)	A randomized, controlled, double blind, escalating dose study of alicaforsen enema in active ulcerative colitis (van Deventer et al., 2004)	/	Patients with active distal UC (DAI 4–10)	Double-blind, placebo-controlled study	40 UC patients	The patients were randomized to the alicaforsen enema administration at four different dosages (0.1, 0.5, 2, or 4 mg/ml) or to placebo. Each patient received alicaforsen enema once daily for 28 days	Safety and efficacy of the drug enema after 1, 3, and 6 months	At 1 month alicaforsen enema (4 mg/ml) was correlated with 70% of improved disease activity index compared to 28% with placebo, with statistical significant difference. This positive outcome was evidenced also after 3 and 6 months
ISIS 2302 (Alicaforsen)	A Phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis (van Deventer et al., 2006)	Phase 2	Patients with active distal UC (DAI 4–10) with disease flare	Double-blind, placebo-controlled study	120 UC patients	The patients were randomized to five treatment arms: placebo, alicaforsen enema at a dosage of 120 mg daily for 10 days and then every other day, 240 mg every other day, 240 mg daily for 10 days and then every other day, 240 mg daily	Disease Activity Index at week 6	No significant difference between alicaforsen and placebo enema was evidenced at week 6, but the arm of alicaforsen (240 mg with daily administration) evidenced a statistical benefit over placebo for prolonged reduction of DAI from the week 18 to week 30

(Continued)

TABLE 1 | Continued

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
ISIS 2302 (Alicaforsen)	Safety and efficacy of two dose formulations of alicaforsen enema compared with mesalazine enema for treatment of mild to moderate left-sided ulcerative colitis: a randomized, double-blind, active-controlled trial (Miner et al., 2006)	/	Patients with active UC (DAI 4–10)	Double-blind, active-controlled study	159 UC patients	The patients were randomized to alicaforsen enema at a dosage of 120 mg or 240 mg or to mesalazine enema at a dosage of 4 g. The enema was administered daily for 6 weeks	DAI at week 6 relative to baseline	No difference in reducing clinical activity was evidenced between the treatment strategies at week 6, but alicaforsen enema evidenced more prolonged response than mesalazine enema
ISIS 2302 (Alicaforsen)	An enema formulation of alicaforsen, an antisense inhibitor of intercellular adhesion molecule-1, in the treatment of chronic, unremitting pouchitis (Miner et al., 2004)	/	Patients with chronic, unremitting pouchitis with a Pouchitis Disease Activity Index (PDAI) score of ≥7	Open-label, uncontrolled study	12 patients affected by chronic unremitting pouchitis	The patients received daily administration of alicaforsen enema at a dosage of 240 mg for 6 weeks	Efficacy of alicaforsen enema in the treatment of chronic unremitting pouchitis	A significant benefit of alicaforsen enema was evidenced at week 6. The PDAI score resulted to be 6.83 at week 6 compared to 11.42 at baseline. This benefit was evidenced also for improvement of endoscopic activity

CD, Crohn's disease; UC, ulcerative colitis; CDAI, Crohn's disease Activity Index; DAI, Disease Activity Index; PDAI, Pouchitis Disease Activity Index; ICAM1, Intercellular Cell Adhesion Molecule-1; /, phase indication not found.

profibrogenetic response of fibroblasts, patients were monitored for 6 months with intestinal ultrasonography, not showing signs of intestinal stenosis. Moreover, half of the patients maintained remission during the 6 months follow up period (Zorzi et al., 2012).

Afterwards, a randomized, double-blind, phase 2 study was conducted in CD patients with inflammatory lesions in the terminal ileum and/or right colon, with steroid-dependence/resistance, randomized to three different daily oral drug doses (10, 40, or 160 mg of mongersen or placebo) for 2 weeks. The primary outcome was clinical remission at day 15, with maintenance of remission for at least 2 weeks. Clinical remission was not significantly different between the patients in the 10 mg mongersen group and placebo (12 vs. 10%; p = N.S.); however clinical remission was reached by 55% and 65% of patients in the 40 and 160 mg mongersen group, as compared to 10% in the placebo group ($p < 0.001$). The drug showed a good safety and tolerability profile (Monteleone et al., 2015).

A following study demonstrated efficacy of GED0301 in inducing both clinical and endoscopic response in a study group of 63 CD patients. The patients were randomized to three treatment groups (4, 8, or 12 weeks of treatment with oral GED-0301 at a dose of 160 mg/day). 37% of patients had an endoscopic response, defined as ≥25% reduction in Simple Endoscopic Score for Crohn's disease (SES-CD), at week 12, with a substantial proportion of patients in clinical remission at the same time point (32% in the group of 4 weeks of treatment, 35 and 48%, respectively in the group of 8 and 12 weeks of treatment) (Feagan et al., 2018). This study however lacked a placebo control group.

Despite these promising results, a recently conducted phase 3 study was stopped following the finding of non-effectiveness of the studied drug. No safety findings were reported (Bevivino et al., 2018; White et al., 2018).

The main findings regarding the above mentioned clinical trials can be found in **Table 2**.

NFkB ASO

Preclinical studies, conducted in murine models of colitis, demonstrated the efficacy of an ASO against the p65 subunit of the transcription factor Nuclear Factor Kappa B (NFkB) (Neurath et al., 1996; Murano et al., 2000). This transcription factor mediates a key role in the pro-inflammatory response of immune-mediated disorders (Zhang et al., 2017).

A preclinical study evidenced an upregulation of p65 expression in macrophages located in the Lamina propria of patients affected by CD. The authors demonstrated the efficacy of a human p65 ASO to reduce the production of pro-inflammatory cytokines by these cells (Neurath et al., 1996). Another study conducted in a murine model of colitis demonstrated a down-regulation of chronic inflammation- induced intestinal fibrosis by the p65 ASO (Lawrance et al., 2003).

Despite these promising results, no clinical study has been published so far regarding the use of this therapeutic strategy in IBD patients (Marafini and Monteleone, 2018).

TABLE 2 | Overview of clinical studies with mongersen.

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
GED0301	Phase I clinical trial of Smad7 knockdown using antisense oligonucleotide in patients with active Crohn's disease (Monteleone et al., 2012)	Phase 1	CD patients with moderate to severe active disease (CDAI>220)	open-label study	15 CD patients	The patients were randomized to three treatment groups: GED0301 at a dosage of 40, 80, or 160 mg. The drug was administered orally once daily for 7 days	Safety and tolerability of the drug	The study evidenced good tolerability and safety of the drug
GED0301	A phase 1 open-label trial shows that smad7 antisense oligonucleotide (GED0301) does not increase the risk of small bowel strictures in Crohn's disease (Zorzi et al., 2012)	Phase 1	CD patients with an inflammatory phenotype and dependence and/or resistance to steroids	open-label study	15 CD patients	The patients were randomized to three treatment groups: GED0301 at a dosage of 40, 80, or 160 mg. The drug was administered orally once daily for 7 days	To evaluate whether GED0301 is associated with the formation of small bowel strictures	The treatment with GED0301 is not associated with the development of small bowel stricture as assessed by intestinal sonography
Mongersen (GED0301)	Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease (Monteleone et al., 2015)	Phase 2	CD patients with ileitis and/or right-sided colonic disease and moderate to severe active disease (CDAI 220–400)	Double-blind, placebo-controlled study	166 CD patients	The patients were randomized to four treatment arms: mongersen at a dosage of 10 mg per day, 40 mg per day, 160 mg per day or placebo. The drug was administered orally once daily for 2 weeks	Clinical remission (CDAI < 150) at day 15 and maintenance of remission for at least 2 weeks	Clinical remission at day 15 and maintenance for 2 weeks was evidenced in 55% of the patients treated with 40 mg of the drug, 65% of those treated with 160 mg and in 10% of placebo patients. This difference was statistically significant. Clinical remission at days 28 and 84 was significantly higher in the patients treated with 40 or 160 mg of the drug than in the group treated with 10 mg of the drug or with placebo
Mongersen (GED0301)	Effects of mongersen (GED-0301) on endoscopic and clinical outcomes in patients with active Crohn's disease (Feagan et al., 2018)	Phase 1	CD patients with active disease (CDAI≥220 to ≤450)	Double-blind study	63 CD patients	The patients were randomized to three treatment groups: GED0301 at a dosage of 160 mg per day for 4 weeks, 160 mg per day for 8 weeks and 160 mg per day for 12 weeks. The drug was administered orally	To evaluate endoscopic outcomes at week 12	The study evidenced endoscopic improvement (25% reduction SES-CD) in 37% of the patients with evaluable endoscopy at week 12. No significant difference was evidenced between treatment groups

CDAI, Crohn's disease Activity Index; Smad7, Mothers against decapentaplegic homolog 7; CD, Crohn's disease.

COBITOLIMOD

Cobitolimod (formerly referred to as DIMS0150) is a single-stranded oligonucleotide that is recognized by the Toll-like Receptor 9 (TLR9) and represents a possible promising novel therapy for UC patients. The altered response of intestinal immune cells to the commensal bacterial flora has been considered to be one of the pivotal pathogenic mechanisms in UC (Strober et al., 2007). The protective immune cell response plays the counterpart action against the over activation of the mucosal immune system (Neurath, 2014). Toll-like receptors (TLRs) belong to pattern recognition receptors (PRRs), which mediate the recognition process of self-antigens from foreign-antigens by the host. These receptors can recognize the pathogen associated molecular patterns (PAMPs) and are implicated in the first defensive innate immune response against luminal pathogens, but can also induce mechanisms of cellular and mucosal repair (Lee et al., 2006). TLR9 is a member of the TLR family and recognizes bacterial DNA, but also viral DNA (e.g., Cytomegalovirus DNA). This receptor in particular recognizes bacterial Cytosine Guanine (CpG) dinucleotides motifs (Hemmi et al., 2000).

A study in the murine model of Salmonella typhimurium infection figured out that the activation of TLR9 maintained the homeostasis of intestinal mucosal cells, demonstrating that disruption of TLR9 signaling could result in augmented inflammation and worsening of intestinal injury (Li et al., 2017).

Cobitolimod is a 19 base single-strand oligonucleotide which mimics bacterial DNA as its CpG dinucleotide sequences are recognized by the TLR9 expressed by different immune cells, thereby mediating anti-inflammatory effects. In preclinical studies in murine models of colitis, administration of the TLR9 oligonucleotide agonist suppressed the severity of colitis in RAG-/- mice. The type I Interferon (IFN α/β) was induced by TLR9 activation and mediated anti-inflammatory responses (Katakura et al., 2005). In another preclinical study conducted in the experimental DSS model and spontaneous colitis mice, administration of CpG oligonucleotides improved clinical, biochemical and histologic activity of colonic inflammation (Rachmilewitz et al., 2002).

A study published in 2013 considered the possible efficacy of DIMS0150 in treating UC patients who had been considered as treatment failures with indication for colectomy. Seven patients received a single dose of topical DIMS0150 (30 mg) and one patient three doses with an interval of 4 weeks in between. The study drug was applied during colonoscopy with a spray catheter in the upper descending colon or the transverse colon. Efficacy was recorded as an improvement of the DAI score and of endoscopic and histological activity at week 12. Clinical response was found in all patients 1 week after single endoscopic administration of the drug. After 12 weeks, 82% of patients had a clinical response while 71% were in clinical remission. In the 2-year follow up one patient needed colectomy (Musch et al., 2013).

A non-interventional pilot study analyzed the transcripts of 34 steroid response genes by polymerase chain reaction (PCR) in the blood derived peripheral mononuclear cells (PBMCs) of steroid refractory UC patients, after their incubation with DIMS0150 (25 or 100 μM) in the presence of Dexamethasone. The authors evidenced that addition of DIMS0150 to steroid treatment produced a significant enhancement of mRNA levels of three steroid response genes (CD163, TSP-1, and IL-1RII) and suggested to consider these genes as potential biomarkers of response to the study drug. This study highlighted the potential effect of DIMS0150 to restore steroid sensitivity in steroid refractory UC patients (Kuznetsov et al., 2014).

After these findings a randomized, double-blind, placebo-controlled trial (COLLECT study) was conducted in 131 patients affected by moderate to severe UC. Patients were randomized to receive two topical endoscopic administrations of cobitolimod (DIMS0150) at a dosage of 30 mg at baseline and week 4 or respective placebo application. The administration was done proximally to the site of mucosal inflammation, or in the transverse colon in the event of pancolitis, using a spray catheter during endoscopy (Atreya et al., 2016). There was no statistical difference in the primary endpoint clinical remission, defined as a CAI score of $\leq$4 at week 12, which was recorded in 44.4% of cobitolimod treated patients and in 46.5% of those treated with placebo (Atreya et al., 2016). However, symptomatic remission, which was defined as the absence of blood in stool and a weekly frequency of <35 stools, was shown in a larger proportion of patients treated with the drug compared to those treated with placebo (32.1% in the cobitolimod group vs. 14% in the placebo group at week 4 ($p = 0.02$) and 44.4 vs. 27.9%, respectively at week 8 ($p = 0.06$). Mucosal healing at week 4 was also achieved by more cobitolimod treated patients compared to the placebo group, but missed statistical significance (34.6 vs. 18.6%; $p = 0.09$). Histological improvement, defined as a Geboes score of 0–2, was evident in 30.9% of the cobitolimod treated vs. 9.3% of the placebo treated patients ($p = 0.007$). The combination of clinical and endoscopic remission could be observed in 21% of cobitolimod treated patients vs. 4.7% of patients on placebo ($p = 0.021$) at week 4 (Atreya et al., 2016).

A recent *post-hoc* analysis, based on data from the COLLECT study, has shown that the clinical efficacy of the drug is significant if patient-reported-outcomes (PRO) were considered, as the investigated outcome measure. Symptomatic remission was studied in 104 patients based on their e-diary records and was defined as the absence of blood in stool and a mean daily stool frequency <4 (Atreya et al., 2018). Symptomatic remission was found at week 4 in 17.1% of patients treated with cobitolimod compared to 5.9% of patients treated with placebo ($p = 0.13$) and, respectively, in 35.7 and 17.6% ($p = 0.07$) at week 8 and in 38.6 and 17.6% ($p = 0.04$) at week 12 (Atreya et al., 2018).

BL7040 (Monarsen) is an orally applied oligonucleotide that modulates the TLR9 and it was investigated in a prospective multicenter phase 2 study in patients with moderately active UC at doses of 12 mg once daily for 3 weeks followed by 40 mg once daily for 2 weeks. The primary endpoint was the proportion of patients with clinical response at the end of 5 weeks of treatment. Clinical response was defined by a decrease of at least three points and 30 % reduction of the Mayo score compared to baseline. Half of the patients (8/16) achieved clinical response at the end of the

course of treatment, with a good tolerability profile of the drug (Dotan et al., 2016).

Altogether, agonists of the Toll-like receptor-9 appeared safe and well-tolerated in moderate to severe UC patients and could represent a novel promising therapeutic option for the management of UC patients.

Currently, a phase 2 study (CONDUCT) of different doses and different administration intervals of cobitolimod in an enema formulation is tested in patients affected by moderate to severe UC[1].

The main findings regarding the above mentioned clinical trials can be found in **Table 3**.

GATA3 DNAzyme

The adaptive immune response and inappropriate production of pro-inflammatory cytokines have an important pathogenic role in IBD pathogenesis. CD is characterized by a prevalence of T helper 1 (Th1) cytokines such as Interferon gamma (IFNγ) and TNF, whereas UC is marked by a of more Th2 oriented cytokine profile, characterized by IL4, IL5, and IL13 (Fuss et al., 1996, 2004). However, these subsets of T cells are not the only responsible cells for the inflammatory response in IBD. Th17 cells are another lymphocyte subpopulation which has a major role in intestinal inflammation in both CD and UC (Giuffrida et al., 2018). A recent published review revealed also the important role of other lymphocyte subsets in mucosal inflammation (Giuffrida et al., 2018). Importantly, Th9 cells and IL9 have also been described to play a pivotal role in the pathogenesis of UC (Nalleweg et al., 2015; Weigmann and Neurath, 2017).

The GATA3 specific DNAzyme (SB010) is an oligonucleotide which can mediate the cleavage of the mRNA of the transcription factor GATA3 and its possible therapeutic usage in human diseases was first investigated in patients affected by allergic asthma. A double-blind placebo-controlled study randomized 40 patients, affected by allergic asthma, to receive 10 mg of SB010 or placebo. The drug was administered by inhalation once daily for 28 days. Treatment with SB010 was found to be effective in attenuating the late and early asthmatic responses to allergen provocation; thereby the drug induced also a significant reduction of IL5 blood levels (Krug et al., 2015).

The Th2 response is mediated by the transcription factor GATA3, which has proven to be over-expressed in the colonic mucosa of UC patients (Ho et al., 2009; Ohtani et al., 2010). This transcription factor is not only linked to Th2 cell responses, but also plays an important role in regulating the differentiation of Th9 lymphocytes (Wan, 2014).

A study conducted with intestinal biopsies of UC patients as well as murine models of colitis showed a correlation between the expression of the transcription factor GATA3 and the production of inflammatory Th2 and Th9 related cytokines (Popp et al., 2016). Conditional GATA3 deficiency in T cells prevented experimental colitis in mice. Intrarectal administration of a GATA3 specific DNAzyme (hgd40) significantly ameliorated colitis in oxazolone and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-mediated colitis models (Popp et al., 2016). The authors also demonstrated that the inhibition of GATA3 transcription by hgd40 was able to ameliorate colitis also in TNF receptor (TNFR) double-knockout mice. This result evidenced the efficacy of the experimental drug in reducing colitis in mice, independently of the presence of TNF (Popp et al., 2016).

These results underline the important role of GATA3 in the inflammatory response in UC and suggest a possible future role of the GATA3 DNAzyme in the therapy of UC patients.

A study of this novel drug as an enema formulation (SB012, SECURE study) in patients with moderate to severe UC has recently been completed (NCT02129439)[2].

STNM01: AN OLIGONUCLEOTIDE AGAINST CARBOHYDRATE SULFOTRANSFERASE 15

IBD pathogenesis is not only connected to an active acute and chronic inflammatory response but also to structural damage, with matrix remodeling and tissue fibrosis, which can take place independently of the inflammatory activity (Suzuki and Yoneyama, 2017). Approximately one third of patients with CD and 5% of those with UC are diagnosed with fibrotic stenosis in the clinical course (Rieder et al., 2013; de Bruyn et al., 2015). There are no specific therapies for the management of fibrotic intestinal remodeling, induced by the over activation of stromal cells during chronic inflammation. Invasive treatment (endoscopic dilation and/or surgical treatment) represent the inevitable final management step of this complication.

Carbohydrate sulfotransferase 15 (CHST15) is an intracellular enzyme that mediates the biosynthesis of sulfated matrix glycosaminoglycans (GAG) which can induce fibrotic reactions in IBD patients.

STNM01 is a novel double-strand RNA oligonucleotide that selectively blocks the expression of CHST15 mRNA and can inhibit the excessive production of glycosaminoglycans in the colon by fibroblasts (Suzuki et al., 2016).

For this reason an endoscopic approach of drug delivery through submucosal injection, to directly target the gut lesion, was established in mice in 2016 (Suzuki et al., 2016). CHST15 siRNA or negative control siRNA were endoscopically administered by submucosal injection at day 6 in DSS-mediated colitis models. The mice were sacrificed at day 19 to evaluate the effect of the experimental drug. CHST15 siRNA reduced CHST15 mRNA in the colon of treated mice and significantly ameliorated endoscopic and histologic disease activity (Suzuki et al., 2016).

A subsequent published randomized placebo-controlled trial evaluated the safety of STNM01 in CD patients whose mucosal ulcerative lesions were refractory to conventional therapy. Eighteen CD patients were randomized to receive a single endoscopic submucosal injection of STNM01 (2.5,

[1] ClinicalTrials.gov Identifier: NCT03178669.

[2] https://www.clinicaltrials.gov/ct2/show/NCT02129439?term=NCT02129439&rank=1

TABLE 3 | Overview of clinical studies with cobitolimod.

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
DIMS0150	Topical treatment with the Toll-like receptor agonist DIMS0150 has potential for lasting relief of symptoms in patients with chronic active ulcerative colitis by restoring glucocorticoid sensitivity (Musch et al., 2013)	Phase 1	UC patients with chronic active disease, with indication for for colectomy	Open-label study	8 UC patients	7 patients were treated with single topical administration of 30 mg of DIMS0150 and one patient with three doses of 30 mg of DIMS0150 with an interval of 4 weeks between doses	Efficacy of the drug at week 12	82% of clinical response and 71% of clinical remission were evidenced at week 12. After two years all but one of treated patients avoided colectomy
DIMS0150 (cobitolimod)	Clinical effects of a topically applied Toll-like receptor 9 agonist in active moderate-to-severe Ulcerative Colitis (Atreya et al., 2016)	Phase 3, COLLECT study	UC patients with moderate to severe disease (CAI≥9 with an endoscopic Mayo score ≥2)	Double-blind, placebo-controlled study	131 UC patients	The patients were randomized to the treatment arm with DIMS0150 at a dosage of 30 mg or to placebo arm. The drug was administered topically during lower gastrointestinal endoscopy at baseline and at week 4	Clinical remission (CAI ≤ 4) at week 12	Clinical remission at week 12 was 44.4% in the group treated with DIMS0150 and 46.5% in the group treated with placebo. No significant difference in clinical remission was evidenced between the groups
DIMS0150 (cobitolimod)	Clinical efficacy of the Toll-like receptor 9 agonist cobitolimod using patient-reported-outcomes defined clinical endpoints in patients with ulcerative colitis (Atreya et al., 2018)	Post-hoc analysis of the COLLECT study (Atreya et al., 2016)	UC patients with moderate to severe disease (CAI≥9 with an endoscopic Mayo score ≥2) with available e-diary data at baseline	Retrospective analysis of the COLLECT study	104 UC patients	The patients were randomized to the treatment arm with DIMS0150 at a dosage of 30 mg or to placebo arm. The drug was administered topically during lower gastrointestinal endoscopy at baseline and at week 4	Post hoc analysis of Symptomatic remission (absence of blood in stool and a mean daily stool frequency<4) at week 4, 8, and 12	Symptomatic remission at week 4 was evidenced in 17.1% of the patients treated with cobitolimod and in 5.9% of those treated with placebo (p=0.13). At week 8 it was 35.7% vs. 17.6% (p = 0.07). At week 12 it was 38.6% vs. 17.6% (p = 0.04).
BL7040	Ameliorating active ulcerative colitis via an orally available Toll-like receptor-9 modifier: A prospective open-label, multicenter phase II trial (Dotan et al., 2016)	Phase 2	UC patients with moderate disease (Mayo score ≥5 and ≤9, endoscopic sub-score ≥2)	Open-label study	22 UC patients	BL7040 was administered orally at a dosage of 12 mg per day for 19–21 days, followed by 40 mg of BL7040 per day for 14 days	To evaluate the efficacy and safety of BL7040 in patients affected by UC	The study evidenced efficacy and safety of the drug

CAI, Clinical Activity Index; UC, ulcerative colitis.

TABLE 4 | Overview of clinical studies with STNM01.

Study drug	Study	Phase	Study population	Study design	Cohort size	Drug dosage and frequency of doses	Primary endpoint	Results
STNM01	Phase 1 clinical study of siRNA targeting carbohydrate sulfotransferase 15 in Crohn's disease patients with active mucosal lesions (Suzuki et al., 2017)	Phase 1	CD patients with active endoscopic disease (SES-CD: from 1 to 11)	Double-blind, placebo-controlled study	18 CD patients	The patients were randomized to four different treatment arms: STNM01 at a dosage of 2.5 nM, STNM01 at a dosage of 25 nM, STNM01 at a dosage of 250 nM or placebo arm. The drug was administered endoscopically with a single dose submucosal injection	To evaluate the safety of STNM01	The study evidenced the safety of the drug and reduced the segmental SES-CD score

SES-CD, Simple Endoscopic Score for Crohn's disease; siRNA, silenceRNA; CD, Crohn's disease.

25, or 250 nM) or placebo. Each dose was administered to eight sites directly surrounding the biggest ulcerative lesion evidenced by total colonoscopy. STNM01 was able to reduce the segmental SES-CD score at day 30 when compared to the placebo arm and was also able to induce a reduced extension of fibrosis, documented by histological analyses. The experimental drug also showed a good safety profile (Suzuki et al., 2017).

A phase 2 clinical trial in UC patients affected by refractory left-sided UC has recently been completed. The patients were randomized to submucosal injections of STNM01 or placebo in the last 35 cm of the colon.

The main findings regarding the above mentioned clinical trials can be found in **Table 4**.

CONCLUSIONS

In this review, we focused on recent and past approaches to test the therapeutic efficacy of oligonucleotide based therapies in IBD.

The combining mechanistic mode of oligonucleotide based therapeutics is a targeted action on specific pro-inflammatory molecules, which are over activated in IBD patients and contribute significantly to disease pathogenesis. The proposed high selectivity of the agents is derived from its mode of action, that aims to specifically block certain inflammatory molecular patterns, without a general systemic effect on other molecular targets. It would be important for each oligonucleotide to present more data regarding its specific mode of molecular action. Ideally, interaction with the proposed molecular target should be presented. Overall, the good tolerance and safety profile has allowed increasing development of this therapeutic approach in IBD clinical research and trials.

This new treatment strategy has the potential to address the main concern of a safe long-term therapy for IBD patients. Moreover, efficacious therapy could be prolonged as lasting maintenance therapy, as there is no risk for secondary loss-of-response due to the formation of anti-drug antibodies, which is one of the main reasons for limited long-term efficacy of biological drugs (Atreya and Neurath, 2018).

The above-mentioned properties and selectivity of oligonucleotides could respond to the dire need for a safe and efficacious long-term therapy. The numerous clinical trials conducted so far have shown the possibility of oligonucleotide treatment to match the target of safety and efficacy, but till now no oligonucleotide based therapy has been approved for clinical practice in the treatment of IBD patients and we still await successful late-stage clinical studies that demonstrate their efficaciousness.

AUTHOR CONTRIBUTIONS

PS wrote the manuscript. HS, GM, and MN assessed the articles and their relevance to the above topics. RA supervised and drafted the manuscript and is the corresponding author.

FUNDING

Funding by the German Research Foundation: DFG-CRC1181 Project C02 (RA) and DFG-SFB/TRR241 Project No. C02 (RA). The Heisenberg Professorship of RA is funded by the DFG.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00314/full#supplementary-material

REFERENCES

Aboul-Fadl, T. (2005). Antisense oligonucleotides: the state of the art. *Curr. Med. Chem.* 12, 2193–2214. doi: 10.2174/0929867054864859

Atreya, R., Bloom, S., Scaldaferri, F., Gerardi, V., Admyre, C., Karlsson, Å., et al. (2016). Clinical effects of a topically applied Toll-like receptor 9 agonist in active moderate-to-severe Ulcerative Colitis. *J. Crohn Colitis* 10, 1294–1302. doi: 10.1093/ecco-jcc/jjw103

Atreya, R., and Neurath, M. F. (2018). Mechanisms of molecular resistance and predictors of response to biological therapy in inflammatory bowel disease. *Lancet Gastroenterol. Hepatol.* 3, 790–802. doi: 10.1016/S2468-1253(18)30265-6

Atreya, R., Reinisch, W., Peyrin-Biroulet, L., Scaldaferri, F., Admyre, C., Knittel, T., et al. (2018). Clinical efficacy of the Toll-like receptor 9 agonist cobitolimod using patient-reported-outcomes defined clinical endpoints in patients with ulcerative colitis. Dig. Liver Dis. 50, 1019–1029. doi: 10.1016/j.dld.2018.06.010

Bevivino, G., Sedda, S., Marafini, I., and Monteleone, G. (2018). Oligonucleotide-based therapies for inflammatory bowel disease. *BioDrugs* 32, 331–338. doi: 10.1007/s40259-018-0286-1

Boirivant, M., Pallone, F., Di Giacinto, C., Fina, D., Monteleone, I., Marinaro, M., et al. (2006). Inhibition of Smad7 with a specific antisense oligonucleotide facilitates TGF-β1–mediated suppression of colitis. *Gastroenterology* 131, 1786–1798. doi: 10.1053/j.gastro.2006.09.016

Burns, R. C., Rivera-Nieves, J., Moskaluk, C. A., Matsumoto, S., Cominelli, F., and Ley, K. (2001). Antibody blockade of ICAM-1 and VCAM-1 ameliorates inflammation in the SAMP-1/Yit adoptive transfer model of Crohn's disease in mice. *Gastroenterology* 121, 1428–1436. doi: 10.1053/gast.2001.29568

Crooke, S. T. (2004). Progress in antisense technology. *Annu. Rev. Med.* 55, 61–95. doi: 10.1146/annurev.med.55.091902.104408

de Bruyn, J. R., Meijer, S. L., Wildenberg, M. E., Bemelman, W. A., van den Brink, G. R., and D' Haens, G. R. (2015). Development of fibrosis in acute and longstanding ulcerative colitis. *J. Crohn Colitis* 9, 966–972. doi: 10.1093/ecco-jcc/jjv133

Dotan, I., Levy-Nissenbaum, E., Chowers, Y., Fich, A., Israeli, E., Adar, T., et al. (2016). Ameliorating active ulcerative colitis via an orally available toll-like receptor-9 modifier: a prospective open-label, multicenter phase II trial. *Dig. Dis. Sci.* 61, 3246–3254. doi: 10.1007/s10620-016-4276-1

EPG Health. (2018). *Randomized Study of Topical Alicaforsen Enema in Antibiotic Refractory Pouchitis*. Available online at: https://clinicaltrials.gov/ct2/show/record/NCT02525523?term=alicaforsen&rank=1 (accessed November 1, 2014).

Feagan, B. G., Sands, B. E., Rossiter, G., Li, X., Usiskin, K., Zhan, X., et al. (2018). Effects of mongersen (GED-0301) on endoscopic and clinical outcomes in patients with active Crohn's disease. *Gastroenterology* 154, 61–64.e6. doi: 10.1053/j.gastro.2017.08.035

Fiorino, G., Cesarini, M., and Danese, S. (2011). Biological therapy for ulcerative colitis: what is after anti-TNF. *Current Drug Targets* 12, 1433–1439. doi: 10.2174/138945011796818225

Fuss, I. J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Fichtner-Feigl, S., et al. (2004). Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J. Clin. Invest.* 113, 1490–1497. doi: 10.1172/JCI19836

Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., et al. (1996). Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol.* 157, 1261–1270.

Gewirtz, A. T., and Sitaraman, S. (2001). Alicaforsen. Isis pharmaceuticals. *Curr. Opin. Invest. Drugs* 2, 1401–1406.

Giuffrida, P., Corazza, G. R., and Di Sabatino, A. (2018). Old and new lymphocyte players in inflammatory bowel disease. *Dig. Dis. Sci.* 63, 277–288. doi: 10.1007/s10620-017-4892-4

Glover, J. M., Leeds, J. M., Mant, T. G., Amin, D., Kisner, D. L., Zuckerman, J. E., et al. (1997). Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302). *J. Pharmacol. Exp. Ther.* 282, 1173–1180.

Hamamoto, N., Maemura, K., Hirata, I., Murano, M., Sasaki, S., and Katsu, K. (1999). Inhibition of dextran sulphate sodium (DSS)-induced colitis in mice by intracolonically administered antibodies against adhesion molecules endothelial leucocyte adhesion molecule-1 (ELAM-1) or intercellular adhesion molecule-1 (ICAM-1). *Clin. Exp. Immunol.* 117, 462–468. doi: 10.1046/j.1365-2249.1999.00985.x

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., et al. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745. doi: 10.1038/35047123

Ho, I. C., Tai, T. S., and Pai, S. Y. (2009). GATA3 and the T-cell lineage: essential functions before and after Thelper-2-cell differentiation. *Nat. Rev. Immunol.* 9, 125–135. doi: 10.1038/nri2476

Jones, S. C., Banks, R. E., Haidar, A., Gearing, A. J., Hemingway, I. K., Ibbotson, S. H., et al. (1995). Adhesion molecules in inflammatory bowel disease. *Gut* 36, 724–730. doi: 10.1136/gut.36.5.724

Kaplan, G. G., and Ng, S. C. (2017). Understanding and preventing the global increase of inflammatory bowel disease. *Gastroenterology* 152, 313–321. doi: 10.1053/j.gastro.2016.10.020

Katakura, K., Lee, J., Rachmilewitz, D., Li, G., Eckmann, L., and Raz, E. (2005). Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J. Clin. Invest.* 115, 695–702. doi: 10.1172/JCI22996

Krug, N., Hohlfeld, J. M., Kirsten, A. M., Kornmann, O., Beeh, K. M., Kappeler, D., et al. (2015). Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. *N. Engl. J. Med.* 372, 1987–1995. doi: 10.1056/NEJMoa1411776

Kuznetsov, N. V., Zargari, A., Gielen, A. W., von Stein, O. D., Musch, E., Befrits, R., et al. (2014). Biomarkers can predict potential clinical responders to DIMS0150 a toll-like receptor 9 agonist in ulcerative colitis patients. *BMC Gastroenterol.* 14:79. doi: 10.1186/1471-230X-14-79

Lawrance, I. C., Wu, F., Leite, A. Z., Willis, J., West, G. A., Fiocchi, C., et al. (2003). A murine model of chronic inflammation-induced intestinal fibrosis down-regulated by antisense NF-kappa B. *Gastroenterology* 125, 1750–1761. doi: 10.1053/j.gastro.2003.08.027

Lee, J., Mo, J. H., Katakura, K., Alkalay, I., Rucker, A. N., Liu, Y. T., et al. (2006). Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat. Cell. Biol.* 8, 1327–1336. doi: 10.1038/ncb1500

Li, Y., Liu, M., Zuo, Z., Liu, J., Yu, X., Guan, Y., et al. (2017). TLR9 regulates the NF-κB-NLRP3-IL-1β pathway negatively in *Salmonella*-induced NKG2D-mediated intestinal inflammation. *J. Immunol.* 199, 761–773. doi: 10.4049/jimmunol.1601416

Lobaton, T., Vermeire, S., Van Assche, G., and Rutgeerts, P. (2014). Anti-adhesion therapies for inflammatory bowel disease. Aliment. Pharmacol. Ther. 39, 579–594. doi: 10.1111/apt.12639

Maaser, C., Sturm, A., Vavricka, S. R., Kucharzik, T., Fiorino, G., Annese, V., et al. (2019). European Crohn's and Colitis Organisation [ECCO] and the European Society of Gastrointestinal and Abdominal Radiology [ESGAR]. ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications. *J Crohns Colitis.* 13, 144–164. doi: 10.1093/ecco-jcc/jjy113

MacDonald, T. T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science* 307, 1920–1925. doi: 10.1126/science.1106442

MacDonald, T. T., Monteleone, I., Fantini, M. C., and Monteleone, G. (2011). Regulation of homeostasis and inflammation in the intestine. *Gastroenterology* 140, 1768–1775. doi: 10.1053/j.gastro.2011.02.047

Maksymowych, W. P., Blackburn, W. D., Tami, J. A., and Shanahan, W. R. Jr. (2002). A randomized, placebo controlled trial of an antisense oligodeoxynucleotide to intercellular adhesion molecule-1 in the treatment of severe rheumatoid arthritis. *J. Rheumatol.* 29, 447–453.

Marafini, I., and Monteleone, G. (2018). Inflammatory bowel disease: new therapies from antisense oligonucleotides. *Ann. Med.* 16, 1–22. doi: 10.1080/07853890.2018.1490025

McConnell, E. L., Liu, F., and Basit, A. W. (2009). Colonic treatments and targets: issues and opportunities. *J. Drug Targeting* 5, 335–363. doi: 10.1080/10611860902839502

Miner, P., Wedel, M., Bane, B., and Bradley, J. (2004). An enema formulation of alicaforsen, an antisense inhibitor of intercellular adhesion molecule-1, in the treatment of chronic, unremitting pouchitis. *Aliment. Pharmacol. Ther.* 19, 281–286. doi: 10.1111/j.1365-2036.2004.01863.x

Miner, P. B. Jr, Wedel, M. K., Xia, S., and Baker, B. F. (2006). Safety and efficacy of two dose formulations of alicaforsen enema compared with mesalazine enema for treatment of mild to moderate left-sided ulcerative colitis: a randomized, double-blind, active-controlled trial. *Aliment. Pharmacol. Ther.* 23, 1403–1413. doi: 10.1111/j.1365-2036.2006.02837.x

Monteleone, G., Fantini, M. C., Onali, S., Zorzi, F., Sancesario, G., Bernardini, S., et al. (2012). Phase I clinical trial of Smad7 knockdown using antisense oligonucleotide in patients with active Crohn's disease. *Mol. Ther.* 20, 870–876. doi: 10.1038/mt.2011.290

Monteleone, G., Kumberova, A., Croft, N. M., McKenzie, C., Steer, H. W., and MacDonald, T. T. (2001). Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J. Clin. Investig.* 108, 601–609. doi: 10.1172/JCI12821

Monteleone, G., Neurath, M. F., Ardizzone, S., Di Sabatino, A., Fantini, M. C., Castiglione, F., et al. (2015). Mongersen, an oral Smad7 antisense oligonucleotide, and Crohn's disease. *N. Engl. J. Med.* 372:2461. doi: 10.1056/nejmc1504845

Mosli, M. H., Rivera-Nieves, J., and Feagan, B. G. (2014). T-cell trafficking and anti-adhesion strategies in inflammatory bowel disease: current and future prospects. *Drugs* 74, 297–311. doi: 10.1007/s40265-013-0176-2

Murano, M., Maemura, K., Hirata, I., Toshina, K., Nishikawa, T., Hamamoto, N., et al. (2000). Therapeutic effect of intracolonically administered nuclear factor kappa B (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis. *Clin. Exp. Immunol.* 120, 51–58. doi: 10.1046/j.1365-2249.2000.01183.x

Musch, E., Lutfi, T., von Stein, P., Zargari, A., Admyre, C., Malek, M., et al. (2013). Topical treatment with the Toll like receptor agonist DIMS0150 has potential for lasting relief of symptoms in patients with chronic active ulcerative colitis by restoring glucocorticoid sensitivity. *Inflamm. Bowel Dis.* 19, 283–292. doi: 10.1002/ibd.23019

Nalleweg, N., Chiriac, M. T., Podstawa, E., Lehmann, C., Rau, T. T., Atreya, R., et al. (2015). IL-9 and its receptor are predominantly involved in the pathogenesis of UC. *Gut* 64, 743–755. doi: 10.1136/gutjnl-2013-305947

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* 14, 329–342. doi: 10.1038/nri3661

Neurath, M. F., Pettersson, S., Meyer zum Buschenfelde, K. H., and Strober, W. (1996). Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat. Med.* 2, 998–1004. doi: 10.1038/nm0996-998

Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., et al. (2018). Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 390, 2769–2778. doi: 10.1016/S0140-6736(17)32448-0

Ohtani, K., Ohtsuka, Y., Ikuse, T., Baba, Y., Yamakawa, Y., Aoyagi, Y., et al. (2010). Increased mucosal expression of GATA-3 and STAT-4 in pediatric ulcerative colitis. *Pediatr. Int.* 52, 584–589. doi: 10.1111/j.1442-200X.2009.03019.x

Popp, V., Gerlach, K., Mott, S., Turowska, A., Garn, H., Atreya, R., et al. (2016). Rectal delivery of a DNAzyme that specifically blocks the transcription factor GATA3 reduces colitis in mice. *Gastroenterology* 152, 176–192.e5. doi: 10.1053/j.gastro.2016.09.005

Powrie, F., Carlino, J., Leach, M. W., Mauze, S., and Coffman, R. L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4 T cells. *J. Exp. Med.* 183, 2669–2674. doi: 10.1084/jem.183.6.2669

Rachmilewitz, D., Karmeli, F., Takabayashi, K., Hayashi, T., Leider-Trejo, L., Lee, J., et al. (2002). Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 122, 1428–1441. doi: 10.1053/gast.2002.32994

Reinisch, W., Hung, K., Hassan-Zahraee, M., and Cataldi, F. (2018). Targeting endothelial ligands: (ICAM-1/Alicaforsen, MAdCAM-1). *J. Crohn Colitis* 12, S669–S677. doi: 10.1093/ecco-jcc/jjy059

Rieder, F., Zimmermann, E. M., Remzi, F. H., and Sandborn, W. J. (2013). Crohn's disease complicated by strictures: a systematic review. *Gut* 62, 1072–1084. doi: 10.1136/gutjnl-2012-304353

Rivera-Nieves, J., Gorfu, G., and Ley, K. (2008). Leukocyte adhesion molecules in animal models of inflammatory bowel disease. *Inflamm. Bowel Dis.* 14, 1715–1735. doi: 10.1002/ibd.20501

Roda, G., Jharap, B., Neeraj, N., and Colombel, J. F. (2016). Loss of response to anti-TNFs: definition, epidemiology and management. *Clin. Translat. Gastroenterol.* 7:e135. doi: 10.1038/ctg.2015.63

Sands, B. E. (2017). Leukocyte anti-trafficking strategies: current status and future directions. *Dig. Dis.* 35, 13–20. doi: 10.1159/000449077

Schreiber, S., Nikolaus, S., Malchow, H., Kruis, W., Lochs, H., Raedler, A., et al. (2001). Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. *Gastroenterology* 120, 1339–1346. doi: 10.1053/gast.2001.24015

Stein, C. A., and Castanotto, D. (2017). FDA-approved oligonucleotide therapies in 2017. *Mol. Ther.* 25, 1069–1075. doi: 10.1016/j.ymthe.2017.03.023

Strober, W., Fuss, I., and Mannon, P. (2007). The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* 117, 514–521. doi: 10.1172/JCI30587

Suzuki, K., Arumugam, S., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., et al. (2016). Pivotal role of carbohydrate sulfotransferase 15 in fibrosis and mucosal healing in mouse colitis. *PLoS ONE* 11:e0158967. doi: 10.1371/journal.pone.0158967

Suzuki, K., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., Aoyagi, Y., et al. (2017). Phase 1 clinical study of siRNA targeting carbohydrate sulphotransferase 15 in Crohn's disease patients with active mucosal lesions. *J Crohn Colitis* 11, 221–228. doi: 10.1093/ecco-jcc/jjw143

Suzuki, K., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., Aoyagi, Y., et al. (2017). Phase 1 clinical study of siRNA targeting carbohydrate sulphotransferase 15 in Crohn's disease patients with active mucosal lesions. J. Crohns Colitis. 11, 221–228. doi: 10.1093/ecco-jcc/jjw143

Suzuki, K., and Yoneyama, H. (2017). New endoscopic approach of anti-fibrotic therapy for inflammatory bowel disease. *Ann. Transl. Med.* 5:191. doi: 10.21037/atm.2017.03.65

Tiainen, J., and Matikainen, M. (1999). Health-related quality of life after ileal J-pouch-anal anastomosis for ulcerative colitis: long-term results. *Scand. J. Gastroenterol.* 34, 601–605. doi: 10.1080/003655299750026065

Vainer, B., and Nielsen, O. H. (2000). Changed colonic profile of P-selectin, platelet-endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and ICAM-3 in inflammatory bowel disease. *Clin. Exp. Immunol.* 121, 242–247. doi: 10.1046/j.1365-2249.2000.01296.x

van Deventer, S. J., Tami, J. A., and Wedel, M. K. (2004). A randomised, controlled, double blind, escalating dose study of alicaforsen enema in active ulcerative colitis. *Gut* 53, 1646–1651. doi: 10.1136/gut.2003.036160

van Deventer, S. J., Wedel, M. K., Baker, B. F., Xia, S., Chuang, E., Miner, P. B., et al. (2006). A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment. Pharmacol. Ther.* 23, 1415–1425. doi: 10.1111/j.1365-2036.2006.02910.x

Vegter, S., Tolley, K., Wilson Waterworth, T., Jones, H., Jones, S., and Jewell, D. (2013). Meta-analysis using individual patient data: efficacy and durability of topical alicaforsen for the treatment of active ulcerative colitis. *Aliment. Pharmacol. Ther.* 38, 284–293. doi: 10.1111/apt.12369

Wan, Y. Y. (2014). GATA3: a master of many trades in immune regulation. *Trends Immunol.* 35, 233–242. doi: 10.1016/j.it.2014.04.002

Weigmann, B., and Neurath, M. F. (2017). Th9 cells in inflammatory bowel diseases. *Semin. Immunopathol.* 39, 89–95. doi: 10.1007/s00281-016-0603-z

White, J. R., Phillips, F., Monaghan, T., Fateen, W., Samuel, S., Ghosh, S., et al. (2018). Review article: novel oral-targeted therapies in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 19:14669. doi: 10.1111/apt.14669

Yacyshyn, B., Chey, W. Y., Wedel, M. K., Yu, R. Z., Paul, D., and Chuang, E. (2007). A randomized, double-masked, placebo-controlled study of alicaforsen, an antisense inhibitor of intercellular adhesion molecule 1, for the treatment of subjects with active Crohn's disease. *Clin. Gastroenterol. Hepatol.* 5, 215–220. doi: 10.1016/j.cgh.2006.11.001

Yacyshyn, B. R., Barish, C., Goff, J., Dalke, D., Gaspari, M., Yu, R., et al. (2002a). Dose ranging pharmacokinetic trial of high-dose alicaforsen (intercellular adhesion molecule-1 antisense oligodeoxynucleotide) (ISIS 2302) in active Crohn's disease. *Aliment. Pharmacol. Ther.* 16, 1761–1770. doi: 10.1046/j.1365-2036.2002.01341.x

Yacyshyn, B. R., Bowen-Yacyshyn, M. B., Jewell, L., Tami, J. A., Bennett, C. F., and Kisner, D. L. (1998). A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 114, 1133–1142. doi: 10.1016/S0016-5085(98)70418-4

Yacyshyn, B. R., Chey, W. Y., Goff, J., Salzberg, B., Baerg, R., Buchman, A. L., et al. (2002b). Double blind, placebo controlled trial of the remission inducing and steroid sparing properties of an ICAM-1 antisense oligodeoxynucleotide, alicaforsen (ISIS 2302), in active steroid dependent Crohn's disease. *Gut* 51, 30–36. doi: 10.1136/gut.51.1.30

Zhang, Q., Lenardo, M. J., and Baltimore, D. (2017). 30 years of NF-κB: a blossoming of relevance to human pathobiology. *Cell* 12, 37–57. doi: 10.1016/j.cell.2016.12.012

Zorzi, F., Calabrese, E., Monteleone, I., Fantini, M., Onali, S., Biancone, L., et al. (2012). A phase 1 open-label trial shows that Smad7 antisense oligonucleotide (GED0301) does not increase the risk of small bowel strictures in Crohn's disease. *Aliment. Pharmacol. Ther.* 36, 850–857. doi: 10.1111/apt.12051

Zundler, S., and Neurath, M. F. (2017). Novel insights into the mechanisms of gut homing and antiadhesion therapies in inflammatory bowel diseases. *Inflamm. Bowel Dis.* 23, 617–627. doi: 10.1097/MIB.0000000000001067

Conflict of Interest Statement: RA reports grants or personal fees from AbbVie, Biogen, Boehringer Ingelheim GmbH & Co. KG, Cellgene, DrFalk Pharma GmbH, Ferring GmbH, GlaxoSmithKline plc, InDex Pharmaceuticals AG, Janssen-Cilag GmbH, MSD Sharp & Dome GmbH, Philogen, Pfizer Inc., Roche Pharma, Samsung Bioepsis, Stelic Institute, Sterna Biologicals, Takeda Pharma GmbH & Co. KG, and Tillotts Pharma AG outside the submitted work. MN reports personal fees from MSD Sharp & Dohme GmbH, PPM Services S.A., Index Pharmaceuticals AB, Shire GmbH, Boehringer Ingelheim GmbH & Co. KG, Janssen Cilag GmbH, Pentax Europe GmbH, Tillotts Pharma AG, e.Bavarian Health GmbH, Takeda Pharma GmbH & Co. KG, and grants from German Research Council, German Cancer Aid, outside the submitted work. GM has served as an advisory board member for ABBVIE.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 18 June 2019
doi: 10.3389/fphar.2019.00671

Novel Pharmacological Therapy in Inflammatory Bowel Diseases: Beyond Anti-Tumor Necrosis Factor

*Cristiano Pagnini[1], Theresa T. Pizarro[2] and Fabio Cominelli[2]**

[1] Department of Gastroenterology and Digestive Endoscopy, S. Giovanni Addolorata Hospital, Rome, Italy, [2] Department of Medicine and Pathology, Case Western Reserve University, Digestive Health Institute, University Hospitals of Cleveland, Cleveland, OH, United States

Inflammatory bowel diseases (IBDs) are chronic conditions of the gastrointestinal tract in which dysregulated immune responses cause persistent inflammation of the gut mucosa. Biologic therapy with anti-TNF blockers has revolutionized the therapeutic management of IBD for their remarkable efficacy and potential impact on disease course and for many years has represented the sole treatment option for patients refractory or intolerant to conventional therapy. In recent years, more molecules, both biologically and chemically synthetized, have been developed as potential therapeutic options for IBD that target different molecular pathways aside from TNF blockade, and which have been proposed as targets for novel drugs. This is particularly relevant for the present, as well as future, management of IBD, considering that some patients are refractory to anti-TNF. This review will summarize the pharmacological options, either currently available or in the pipeline, for market approval to treat IBD, besides anti-TNF strategies, based on their mechanism(s) of action. We will also analyze the current evidence for effectiveness and safety, as well as offer perspective, regarding the potential implementation for such therapies in the future.

Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, therapy, biologics, oral small molecules

OPEN ACCESS

Edited by:
Luca Antonioli,
University of Pisa, Italy

Reviewed by:
Matteo Fornai,
University of Pisa, Italy
Davide Di Fusco,
University of Rome Tor Vergata, Italy
Raja Atreya,
University Hospital Erlangen, Germany

***Correspondence:**
Fabio Cominelli
fabio.cominelli@uhhospitals.org

Specialty section:
This article was submitted to Gastrointestinal and Hepatic Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 December 2018*
Accepted: *23 May 2019*
Published: *18 June 2019*

Citation:
Pagnini C, Pizarro TT and Cominelli F (2019) Novel Pharmacological Therapy in Inflammatory Bowel Diseases: Beyond Anti-Tumor Necrosis Factor. Front. Pharmacol. 10:671. doi: 10.3389/fphar.2019.00671

INTRODUCTION

Inflammatory bowel disease (IBD) is a condition involving the gastrointestinal tract that displays a chronic remittent clinical course, with alternating bouts of remission and flares of active inflammation. The etiology of IBD remains unknown, but its pathogenesis is associated with dysregulated immune responses that drives a persistent inflammatory state within the intestinal mucosa. Crohn's disease (CD) and ulcerative colitis (UC) are the two main entities of IBD, which each presents particular clinical and anatomo-pathological features (Abraham and Cho, 2009). Specifically, CD is characterized by transmural inflammation that typically involves all layers of the gut wall, is discontinuous and patchy in appearance with alternating affected and non-affected areas, and can affect the entire gastrointestinal tract, from mouth to anus (Baumgart and Sandborn, 2012). UC, on the other hand, affects the most superficial mucosal layer of the gut wall, which usually arises from the anus and continuously extends proximally to variable degrees throughout the whole colon (Ordás et al., 2012). Both CD and UC display specific differences, but both conditions represent challenges for patients and physicians, and are considered disabling diseases. As such, therapeutic strategies to treat IBD have changed throughout the years, shifting from solely resolving disease symptoms to profound healing of the intestine, with the end result of not only treating short-term complications, but also impacting the natural history of

disease by reducing important outcomes, including hospitalization and surgery (Neurath and Travis, 2012). Biologics have been at the forefront of this change. In fact, biologic (or biotechnologic) drugs are molecules that, differently from "classic" or chemical drugs, are produced by biologic systems and target specific molecules or pathways involved in the inflammatory cascade that is triggered during IBD. They are characterized by consistent efficacy, rigorously evaluated by pre-clinical and phase II/III clinical studies, and are generally indicated for moderate-to-severe IBD patients that are not responding or are intolerant to conventional therapies. Biologics are generally large molecules (e.g., monoclonal antibodies) that require parenteral administration and are characterized by a variable degree of immunization.

The prototypic biologic drug is infliximab, a chimeric anti-TNF antibody that appeared on the market for the treatment of IBD in the late 1990s. From that time until just a few years ago, the anti-TNF blockers (e.g., infliximab, adalimumab, certolizumab pegol, and golimumab) have been the only approved biologic drugs for the treatment of IBD, with the exception of natalizumab, which has been available only in the U.S., under specific restrictions due to its safety profile (Pagnini et al., 2017). These drugs, which remain the gold standard to treat moderate-to-severe IBD, have displayed a consistent response rate for the induction and maintenance of disease remission. Moreover, they have shown dependable efficacy for not only healing of the gut mucosa and relief of symptoms, but also reduced hospital admission rates and improved quality of life for IBD patients (Van Assche et al., 2010). Nonetheless, a consistent subset of patients (around 20%) do not respond to treatment, and a similar proportion of patients is likely to lose efficacy every year (Wong and Cross, 2017). Although these drugs are generally considered safe, adverse events are still not infrequent and some patients present contraindications (Pagnini et al., 2015). Thus, considering the aforementioned issues, and the fact that the proportion of patients who have already experienced anti-TNF therapy is constantly increasing, the development of different biologic drugs with alternative mechanism(s) of action has become an urgent need for the treatment of IBD. Besides biologic drugs, a new generation of chemically synthetized oral small molecules have also been recently developed. In fact, oral administration is generally better accepted by patients, does not confer costly and time-consuming infusions in a hospital setting, and generally guarantees a lower risk of immunization. On the other hand, serum concentrations of the drug are less tightly regulated, and the absorption of the compound may be more variable and affected by active inflammation or a resected bowel (Vetter and Neurath, 2017). At present, new biological and chemical drugs have recently been emerging on the market, and more molecules are yet under way for approval. The focus of this review is to summarize the present and upcoming new drugs, aside from TNF blockers, for the treatment of IBD according to their mechanism of action(s).

DRUGS INTERFERING WITH LEUKOCYTE TRAFFICKING

The first pathway to be investigated for therapeutic intervention, aside from TNF blockade, was to target leukocyte trafficking, which is a process that includes extravasation and priming of cells from the vasculature, migration and homing of activated cells into the intestinal tissues, and retention and egress of leukocytes within the gut mucosa (Zundler and Neurath, 2017). Specifically, the infiltration of lymphocytes (e.g., T-cells) into the intestinal mucosa represents a target for molecular intervention for therapeutic purposes in IBD. Leukocyte adhesion and extravasation is a multi-step process that includes the tethering/rolling, activation, adhesion, and extravasation/migration of leukocytes into the intestinal mucosa. This complex process is characterized by low-affinity bonds between integrins on the surface of circulating lymphocytes and their inducible ligands located in intestinal cells of the endothelium, interaction that induces a rolling and adhesion effect, with the result of a slowing down of the circulating leukocyte. Integrins are heterodimeric receptors with α and β subunit, with several forms of these subunits resulting from different combinations. Cellular adhesion molecules (CAMs), members of the immunoglobulin superfamily expressed on the surface of vascular endothelial cells, are the natural integrin ligands. Both vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is specifically expressed on vascular endothelial cells of the gastrointestinal tract, are receptors for the α4 family of integrins. Disruption of integrin/CAM interactions blocks the recruitment of leukocytes across the endothelium and into inflamed parenchymal tissues. In CD, the interaction between α4β7 integrin and its endothelial receptor, MAdCAM-1, has been demonstrated as a relevant factor for the development of chronic intestinal inflammation (Arseneau and Cominelli, 2015).

In fact, the first compound that was developed that interfered with lymphocyte homing was natalizumab, a recombinant humanized IgG4κ monoclonal antibody that binds to the α4 subunit of two different integrins expressed on the surface of T and B cells: α4β1, responsible for homing of leukocytes to several inflamed, yet non-intestinal, tissues, such as skin, lung, and central nervous system, and α4β7, which has a specific role in the gut homing. By means of the bound to the α4 subunit, natalizumab inhibits α4-mediated adhesion of leukocytes to their receptors. The clinical effect of natalizumab in CD is most likely mediated by the inhibition of engagement between the α4β7 integrin and MAdCAM-1, expressed on the vascular endothelium in the actively inflamed gut. However, parallel actions on the α4β1 integrin/VCAM-1 pathway may still have a role for the inhibitory effects since VCAM-1 expression can be induced in submucosal vessels of the intestinal inflamed mucosa (Pagnini et al., 2017). Clinical efficacy of natalizumab has been demonstrated by three large phase III clinical trials: ENACT-1, ENACT-2, and ENCORE. The intent of the first two studies was to investigate the efficacy of natalizumab for the induction (ENACT-1) and maintenance (ENACT-2) of remission in moderate-to-severe CD patients (CDAI ≥ 220 and ≤450) in 142 centers. In the ENACT-1 trial, 905 CD patients were randomized 4:1 to natalizumab 300 mg/kg at 0, 4, and 8 weeks, or placebo, and response at week 10 was set as the primary endpoint. The induction study showed a significant difference in response and remission rate, but only in the subset of patients with high CRP

levels (response = 58% vs. 45%, remission = 40% vs. 28%, $p < 0.05$ for both). In the ENACT-2 trial, patients from ENACT-1, who responded at week 10 and 12 (n = 339), were randomized 1:1 to maintenance therapy with natalizumab (300 mg/kg every 4 weeks) or placebo, and response (set as primary endpoint) and remission were evaluated at week 36. The results showed that in the treated group sustained clinical response and remission were significantly higher compared with patients in the placebo group (response = 61% vs. 28%, $p < 0.001$, and remission = 44% vs. 26%, $p = 0.003$) (Sandborn et al., 2005). Finally, the ENCORE trial confirmed the efficacy of natalizumab in the induction of remission in patients with high baseline CRP level (CDAI ≥ 220 and ≤450, and CRP > 2.87 mg/L) in a large study, which included 509 patients from 114 different centers. In the treatment group (three natalizumab infusions at week 0, 4, and 8) higher rates of response (48% vs. 32%, $p < 0.001$) and remission (26% vs. 16%, $p = 0.002$) at week 8 through week 12, compared to placebo, have been observed, with response and remission rates significantly higher in the treatment vs. placebo groups at every time point (4, 8, and 12 weeks) (Targan et al., 2007). Despite consistent positive results, the utilization of natalizumab has been strongly limited by safety issues. In fact, although rare, the occurrence of progressive multifocal leukoencephalopathy (PML), together with the development of a novel compound with a similar mechanism of action, but a more favorable safety profile, have contributed to the restricted use of natalizumab therapy for CD. Currently, natalizumab is approved for the treatment of multiple sclerosis worldwide, but is only approved for CD in the U.S. (and Switzerland), under a specific regulatory and distribution program (Pagnini et al., 2017).

Vedolizumab is a second-generation molecule that specifically interferes with leukocyte homing into the inflamed gut mucosa, and has consistently overcome safety issue of its predecessor, natalizumab. Vedolizumab has been approved for the treatment of IBD for the past few years, and represents the first biologic therapy specifically designed for a gastroenterological indication. Vedolizumab is a humanized IgG1 monoclonal antibody that specifically targets the α4β7 integrin, thereby preventing its binding to MAdCAM-1, exclusively expressed on the gut endothelium, with no effects on α4β1 integrin/VCAM-1 engagement, and consequently, no effect on leukocyte trafficking within the central nervous system. Such selectivity of action directly reflects on favorable drug safety profiles, with no cases of PML recorded thus far, and minimal adverse events confirmed in long-term follow-up studies (Colombel et al., 2017). The efficacy of vedolizumab to treat IBD patients has been demonstrated by large phase III studies, GEMINI I (for UC patients), and GEMINI II and III (for CD patients). In GEMINI I, 374 patients with active UC and unresponsive to treatment with either corticosteroids, immune modulators, or anti-TNF antibodies (nearly 40% of patients), were randomized (3:2) to receive vedolizumab (300 mg i.v. at weeks 0, 2, and 6) or placebo. The primary end point (clinical response defined by a decrease in Mayo score by at least three points from baseline, as well as no individual score greater than 1), evaluated at week 6, was achieved by 47% of patients when compared to those treated with vedolizumab vs. 26% of those patients receiving placebo ($p < 0.0001$). Secondary end-points, including clinical remission (17% vs. 5%, $p = 0.001$) and mucosal healing (41% vs. 25%, $p = 0.001$), showed significantly higher rates in vedolizumab- vs. placebo-treated patients. As follow-up, a total of 373 patients, including both patients who achieved clinical response in the randomized controlled trial and those who achieved clinical response during an open label trial, were re-randomized (1:1:1) to receive either placebo infusions or vedolizumab either every 4 or 8 weeks as maintenance therapy. The primary end-point (clinical remission at week 52) was achieved by 16%, 42%, and 45% of the placebo, as well as the 8- and 4-week vedolizumab groups, respectively ($p < 0.0001$ for both vedolizumab doses vs. placebo). Moreover, maintenance treatment with vedolizumab showed a durable clinical response (24%, 57%, and 52%, $p < 0.0001$), mucosal healing (20%, 52%, and 56%, $p < 0.0001$), durable clinical remission (9%, 20%, and 24%, $p < 0.05$ and $p < 0.001$, for 8- and 4-week vedolizumab groups vs. placebo, respectively), and corticosteroid-free remission (14%, 31%, and 45%, $p < 0.05$ and $p < 0.0001$), for 8- and 4-week vedolizumab groups vs. placebo, respectively, also through 52 weeks (Feagan et al., 2013). GEMINI II had a study design similar to that for GEMINI I: 368 patients with active CD, refractory to corticosteroids, immunomodulators, or anti-TNF antibodies (nearly 50% of patients), were randomized (3:2) to receive either vedolizumab (300 mg i.v. at weeks 0, 2, and 6) or placebo. Only one of the two primary end points for induction at week 6 was achieved; clinical remission rate (CDAI less than or equal to 150) was 15% in the treated group and 7% in the placebo group ($p < 0.05$), while no significant difference between groups was found for enhanced clinical response (CDAI decrease by 100 from baseline). Again, a total of 461 patients, including both patients who achieved clinical response in the randomized controlled trial of induction and patients who achieved clinical response during open label induction, were re-randomized (1:1:1) to receive placebo infusions, as well as vedolizumab, every 4 and every 8 weeks as maintenance therapy. The primary end point of clinical remission at week 52 was achieved in 39%, 36%, and 22% of patients assigned to maintenance vedolizumab infusions every 8 and 4 weeks, as well as those treated with placebo, respectively ($p < 0.001$ and $p < 0.05$ vs. placebo for 8- and 4-week groups). Moreover, enhanced clinical response (44%, 45%, and 30%, $p < 0.05$) for treatment groups vs. placebo, and corticosteroid-free remission (32%, 29% and 16%, $p < 0.05$) for treatment groups vs. placebo rates were significantly higher in maintenance vedolizumab compared to placebo (Sandborn et al., 2013). Finally, the GEMINI III study further explored the efficacy of vedolizumab for induction of remission in a particularly complicated set of patients, in which three quarters had previously failed anti-TNF therapy. In fact, 416 patients were randomized (1:1) to receive induction therapy with vedolizumab at 0, 2, and 6 weeks or placebo. The primary end point was clinical remission at week 6, but additional evaluation was performed at week 10. The results showed no statistically significant difference between treated and placebo groups; the week 10 analysis showed a trend for increased efficacy (15 vs. 12% at week 6 and 27 vs. 12% at week 10 in treated and placebo group, respectively) (Sands et al., 2014). Taken together, data from randomized control trials indicate that vedolizumab

confirms the important therapeutic effect of blocking leukocyte homing to the gut mucosa, as already shown by the efficacy of natalizumab in CD. The important data on efficacy (in particular in UC patients) and its favorable safety profile have definitely launched vedolizumab for its use in treating IBD patients, both for anti-TNF-experienced patients (particularly for primary non-responders) and for naïve patients, especially those with absolute or relative contraindication for anti-TNF blockade. In fact, pool safety data from six trials including 2,830 patients have shown that vedolizumab is not associated with an increased risk of malignancy and/or serious or opportunistic infections, with infusion-related reaction reported in <5% of patients, and no cases of PML found (Colombel et al., 2017). Moreover, the favorable safety profile was confirmed by the results at 5 years of the GEMINI open-label extension study (Loftus et al., 2017). Data from GEMINI III indicated that in CD, the onset of the effect may be slower, specifically in TNF-experienced patients. Therefore, co-treatment with steroids, and/or a supplementary infusion at week 10 in the induction phase, may increase remission/response rates. Efficacy and safety of vedolizumab therapy has been further confirmed in real-life studies and observational studies, where even in CD patients, efficacy was better than in the registrative studies (Baumgart et al., 2016; Dulai et al., 2016; Amiot et al., 2017; Kopylov et al., 2017).

Some novel anti-integrin molecules, not yet approved for market distribution, have demonstrated efficacy for the treatment of IBD in clinical studies and deserve confirmation in large phase III trials. Abrilumab, a fully humanized monoclonal IgG2 antibody against α4β7, has demonstrated efficacy in the induction of remission in UC and CD (particularly in TNF-experienced CD patents) in two phase II studies. PF-00547659 (fully humanized MAdCAM-1 IgGk2 blocking antibody), AJM300 (oral small molecule targeting α4-integrin), and vercirnon (oral CCR9 small molecule antagonist) showed preliminary promising results, but need further evaluation for efficacy and safety (Arseneau and Cominelli, 2013; Park and Jeen, 2018). Among forthcoming anti-integrin molecules, etrolizumab appears to be the closest to market approval. It is a monoclonal IgG1 antibody against β7-integrin, a subunit common to α4β7 and αEβ7, whose ligand is E-cadherin, mainly expressed on epithelial cells. Thus, blocking β7-integrin, both lymphocyte trafficking into the gut and their retention in the intraepithelial compartment are prevented, with potentially less gut selectivity than vedolizumab, but hopefully higher efficacy. Efficacy of etrolizumab in inducing remission in moderate to severe UC patients have been demonstrated by a multi-centric, double-blind, placebo-controlled, randomized, phase II study, in which a total of 124 patients were randomized (1:1:1) to two different doses of subcutaneous etrolizumab or placebo. Primary end-point of clinical remission at week 10 was achieved in 8/39 (21%) of patients in etrolizumab 100 mg group ($p = 0.004$), in 4/39 (10%) of patients in etrolizumab 300 mg group ($p = 0.048$), and in no patient in placebo group, with no significant difference among groups for clinical response rate, and with serious adverse events recorded in 12%, 5%, and 12% in etrolizumab 100 mg, 300 mg, and placebo group, respectively (Vermeire et al., 2014). Large phase III studies further investigating efficacy and safety of etrolizumab in UC and CD patients are currently ongoing.

Another side of the leukocyte trafficking process that has been recently investigated for therapeutic purposes in IBD is the homing and egress of activated lymphocytes in lymph nodes. In particular, sphingosine-1 phosphate (S1P) receptor appears to have a relevant role in controlling lymphocyte trafficking to lymphoid organs, and blockade of that receptor arrests activated lymphocytes within lymph nodes, preventing their egress towards the gut (Nielsen et al., 2017). Fingolimod was the first S1P modulator approved for therapeutic purposes (in multiple sclerosis), but due to its unselective blockade of S1P, was characterized by important cardiovascular and hepatic side effects (Pelletier and Hafler, 2012). Ozanimod, an oral small molecule, selectively modulates S1P subtype 1 and 5 and has a more favorable safety profile. The molecule has shown preliminary proven efficacy for induction and maintenance of remission in UC patients in the double blind, placebo-controlled phase II TOUCHSTONE trial, in which 197 moderate 1 to severe UC patients were randomized (1:1:1) to treatment with ozanimod 0.5 mg/day, 1 mg/day, or placebo up to 32 weeks. In fact, primary end-point of clinical remission at week 8 was achieved in 11/67 (16%) of patients in the ozanimod 1 mg group ($p = 0.048$), 9/65 (14%) of patients in ozanimod 0.5 mg group ($p = 0.14$), and 4/65 (6%) of patients in placebo group, and clinical remission at week 32 was observed in 26%, 21%, and 6% in ozanimod 0.5 mg, 1 mg, and placebo group, respectively ($p = 0.002$ and $p = 0.01$ vs. placebo). At week 8, absolute lymphocyte counts reduction of 49% and 32% from baseline was observed in the ozanimod 1 and 0.5 mg group, respectively (Sandborn et al., 2016). Phase II and phase III trials that will further assess efficacy and safety of ozanimod, both in UC and CD patients, are still ongoing.

INHIBITORS OF PRO-INFLAMMATORY CYTOKINES

The first biologics that were developed to target pathogenic disease mechanism(s) were designed to inhibit the action of pro-inflammatory cytokines (for instance, TNF). In line with this original concept, new drugs blocking other pro-inflammatory cytokines, aside from TNF, have been developed as a therapy for IBD patients. IL-12/IL-23 represents the pathway most intensely investigated thus far. IL-12 and IL-23 are two cytokines of the IL-12 family that play an important role in the transition from innate to adaptive immune activation. Both are expressed by macrophages and dendritic cells activated by microbial stimulation, and promote acquired immunity through differentiation of naïve $CD4^+$ cells into Th1 IFNγ-producing cells (IL-12) and Th17 cells (IL-23) that in turn activate a cascade of pro-inflammatory cytokines, such as IL-17, IL-6, and TNF (Iwakura and Ishigame, 2006). In particular, the activation of the IL-23/IL-17 axis appears of particular relevance to the pathogenesis of intestinal inflammation in IBD, as suggested from experimental observations (Becker et al., 2003; Tozawa et al., 2003). The first therapeutic agent of this class investigated in IBD is ustekinumab, a human IgG1k monoclonal Ab that binds the p40 subunit shared by both IL-12 and IL-23, thus preventing the interaction with their specific receptors on the surface of NK and

T cells (Trinchieri et al., 2003). Ustekinumab, formerly approved for psoriasis and psoriatric arthritis, has recently been approved in Europe and then the U.S. for the treatment of CD patients. The efficacy of the drugs has been clearly demonstrated by three large multi-center, placebo-controlled phase III clinical studies reported in a single paper: UNITI-1, UNITI-2, and IM-UNITI (Feagan et al., 2016). The first two trials evaluated the induction of remission/response: CD patients with moderate-to-severe CD who failed or were intolerant to anti-TNF (UNITI-1; n = 741 patients), or naïve to anti-TNF therapy (UNITI-2; n = 628 patients) were randomized (1:1:1) to receive either ustekinumab at 130 mg IV, ustekinumab at 6 mg/kg, or placebo, with a primary end-point of clinical response at week 6, and a secondary end-point of clinical remission at week 8. Both primary (UNITI-1: 34.3%, 33.7%, and 21.15%, $p < 0.003$ vs placebo; UNITI-2: 51.7%, 55.5%, and 28.4%, $p < 0.001$ vs. placebo) and secondary end points (UNITI-1: 15.9%, 20.9%, and 7.3%, $p = 0.003$ and $p < 0001$ vs placebo; UNITI-2: 30.6%, 40.2%, and 19.6%, $p = 0.009$ and $p < 0.0001$ vs. placebo, respectively) were achieved in the treated groups. In the IM-UNITI trial, a total of 1,281 patients who responded to ustekinumab induction at week 8 (397 from UNITI-1 and UNITI-2 and 884 from an open label study) were randomized (1:1:1) to maintenance with ustekinumab (90 mg subcutaneous every 8 weeks, 12 weeks) or placebo, with a primary end-point of clinical remission at week 44, achieved by both treatment groups (remission rate: 53.1% and 48.8% in 8- and 12-weeks treatment, 35.9% in placebo group, $p = 0.005$ and $p = 0.04$, respectively). Safety profiles were similar between groups, and ustekinumab showed very limited immunogenicity (anti-drug antibodies at week 44 were found in 2.3% of patients). For the consistent efficacy rate and favorable safety profile, ustekinumab appears to be an important resource in the treatment of CD patients, both in TNF naïve and experienced patients, and particularly in patients with extraintestinal manifestations. Since most of the data come from registrative trials, post-marketing and real-life studies will hopefully confirm efficacy and safety data for this drug.

Considering the more prominent role of IL-23 over IL-12 blockade, novel drugs selectively targeting the IL-23/IL-17 axis have been investigated. Among the latter, MEDI2070 and risankizumab, monoclonal Abs that bind the p19 subunit of IL-23, have shown promising results in phase II trials, particularly in difficult-to-treat CD patients. In fact, in an induction study, MEDI2070 (700 mg IV at week 0–4) induced a significantly higher response rate than placebo in anti-TNF-experienced CD patients (49.2% vs. 26.7%, $p = 0.01$) (Sands et al., 2017). In addition, patients treated with risankizumab (200 mg IV and 600 mg IV) showed a significantly higher response (39% vs. 20.5%) and remission (30.5% vs. 15.4%) rate compared to placebo (Feagan et al., 2017).

Other molecules targeted at blocking pro-inflammatory cytokines are also being investigated, but results are preliminary. Tocilizumab, a fully humanized monoclonal antibody that blocks both soluble and membrane-bound IL-6, already approved for rheumatoid and juvenile arthritis, has shown potential efficacy in CD patients in a small pilot study (Ito et al., 2004), but no further investigation has been done. Very recently, results of a phase II trial evaluating PF-04236921, a subcutaneous monoclonal antibody blocking IL-6, has been published (ANDANTE I and II trial). In the induction study, 249 moderate-to-severe CD patients who had inadequate response to anti-TNFα were randomized 1:1:1:1 to placebo or 10, 50, or 200 mg of subcutaneous administration of PF-04236921 at day 1 and 28 (enrollment in the 200 mg group was prematurely stopped due to safety issue emerged in a trial in patients with systemic lupus erythematosus), and primary end-point was CDAI-70 response at weeks 8 and 12. In the open-label extension study, 191 patients received, after induction, PF-04236921 50 mg every 8 weeks for 48 weeks followed by 28 weeks of follow-up, in order to evaluate the safety of the treatment. In the induction study, PF-04236921 50 mg showed significant CDAI-70 response rate than placebo, both at weeks 8 and 12 (49.3 vs. 30.6% and 47.4 vs. 28.6%, respectively, $p < 0.05$ for both), and remission rate at week 12 was 27.4% in 50 mg treated group and 10.9% in placebo group ($p < 0.05$). Regarding safety, SAEs were reported in 30.4% of patients in the open-label extension study, and 6 patients in the induction study and 10 in the open-label extension study experienced abdominal abscess or perforation, complications at higher risk for the mechanism of action of the drug (Danese et al., 2019). Considering the results of this study, PF-04236921 appears a potentially useful treatment for difficult CD patients, but the lack of endoscopic efficacy data and the safety issue require further investigation in phase III trials.

Two phase II RCT trials investigated the potential efficacy of monoclonal antibodies blocking IL-13 (tralokinumab and anrunkinzumab) in UC patients, but primary end-points were not achieved (Danese et al., 2015; Reinisch et al., 2015). Quite surprisingly, besides the high efficacy of blocking IL-17 in other chronic inflammatory conditions, such as psoriasis, psoriatic arthritis, and ankylosing spondylitis, as well as the relevant role of IL-17 in CD pathogenesis and severity (Jiang et al., 2014), the IL-17 blockers, secukinumab and brodalumab, further worsened disease activity in CD patients in RCT trials (Hueber et al., 2012; Targan et al., 2016). Aside from multiple biological explanations, the negative results of IL-17 blockade make one reflect on the pleiotropic and complex molecular pathways underlying IBD, in which the same cytokine potentially possesses both protective and pathogenic effects on mucosal inflammation in different phases of disease, or within different compartments of the innate/acquired immune systems (Pagnini et al., 2010). Although similarities can be found with other chronic inflammatory conditions, such as rheumatoid arthritis, IBD represents a unique and peculiar pathologic entity, particularly considering the critical impact of the gut microbiome on disease pathogenesis. Therefore, translation of therapies, protocols, and specifics of management from other diseases need to be very cautiously addressed.

BLOCKERS OF DOWNSTREAM CYTOKINE SIGNALING PATHWAYS

The Janus kinases (JAK), whose name derives from the double-faced Roman God, Janus, for the presence of two phosphate-transferring domains with opposite effects, are a group of

heterodimeric intracellular enzymes that transduce the signaling generated by interaction between several cytokines and their cell surface receptors. Those interactions determine the activation of the JAK-STAT phosphorylation pathway, with the final result of nuclear transcription of effector proteins. There are four members of the JAK family [JAK1, JAK2, JAK3, and thyrosine kinase (TYK) 2] (Yamaoka et al., 2004). Since important pro-inflammatory cytokines, such as IFNγ, IL-2, IL-6, IL-12, and IL-23, utilize the JAK-STAT signaling system, blockade of its downstream activity has demonstrated therapeutic efficacy in several chronic inflammatory conditions, such as psoriasis (Papp et al., 2012), rheumatoid arthritis (Fleischmann et al., 2012), and, more recently, IBD. In particular, tofacitinib, a non-selective oral small molecule JAK inhibitor (that preferentially inhibits JAK1 and 3), has been recently investigated in CD and UC patients. In CD, tofacitinib failed to demonstrate significant response and remission rate over placebo both in induction and in maintenance phase II studies, but nonetheless showed a certain anti-inflammatory effect with a consistent reduction of C-reactive protein levels (Sandborn et al., 2014; Panés et al., 2017). More solid data have been shown by tofacitinib in phase III trials as a therapeutic agent in UC patients (Sandborn et al., 2017), and it has been recently authorized for marketing by the FDA and EMA for this indication. In two large induction trials, anti-TNF naïve (OCTAVE induction 1, n = 598) and experienced (OCTAVE induction 2, n = 541) UC patients were randomized 4:1 to tofacitinib (10 mg twice-a-day) or placebo. The drug-induced clinical remission at week 8 (primary end-point) was achieved at a significantly higher rate compared with placebo (18.5% vs. 8.2%, $p = 0.007$, and 16.6% vs. 3.6%, $p < 0.001$, in OCTAVE induction 1 and 2, respectively). A key secondary end-point of mucosal healing was achieved in both induction trials (31.3% vs. 15.6%, and 28.4% vs. 11.6%, in OCTAVE induction 1 and 2, $p < 0.001$ for both). In a maintenance trial, 593 patients who had clinical response to induction therapy were randomized (1:1:1) to tofacitinib (10 mg twice daily, 5 mg twice daily) or placebo. At week 52, the primary end-point of clinical remission was achieved in 40.6%, 34.3%, and 11.1%, of 10 mg, 5 mg, and placebo group, respectively ($p < 0.001$). The secondary end-point of mucosal healing was significantly higher in both treated groups comparing with placebo (45.7%, 37.4%, and 13.1%, $p < 0.001$). Besides these consistent efficacious results, some concerns about safety were raised by the OCTAVE trials. In fact, in the induction trials, overall infections, specifically serious infections, were higher in treated vs. placebo groups (23.3% vs. 15.6% in OCTAVE induction 1 and 18.2% vs. 15,2% in OCTAVE induction 2), and in the maintenance trial, a higher rate of overall infections (39.8%, 35.9%, and 24.2% in 10 mg, 5 mg, and placebo group, respectively) and herpes zoster infections (5.1%, 1.5%, and 0.5%) were recorded in treated vs. placebo groups. Moreover, more cases of non-melanoma skin cancers, cardiovascular events, and increased serum lipid levels have been observed across the three trials in treated patients. In order to overcome these safety issues, selective JAK1 blockers are now being investigated. Among the latter, filgotinib has shown the most promising results in CD patients in a phase II trial (FITZROY). In fact, a total of 174 CD patients with active disease, confirmed by a centrally read endoscopy, were randomized 3:1 to filgotinib 200 mg once a day or placebo for 10 weeks, and then re-randomized to filgotinib 100 mg/die, 200 mg/die, or placebo for an observational period of further 10 weeks. The primary end-point of clinical remission at week 10 was achieved by 47% of treated and 23% of placebo group ($p < 0.01$), and treated patients had a significant improvement of quality of life, but not statistically significant improvement of endoscopic activity has been observed, despite a trend for higher SES-CD 50%, endoscopic response, endoscopic remission, and deep remission in the treated group. Concerning safety, combining together the 20 weeks of the study, treatment-emergent adverse event rate was similar in treated and placebo group (75% and 67% in treated and placebo group, respectively), and serious treatment-emergent adverse events rate was 9% in treated and 4% in placebo group, with 3% of serious infection in treated group and none in placebo group. Interestingly, filgotinib was effective in TNFα-naïve (60% of remission rate at week 10) and -experienced patients (37%), and no significant serum lipid alterations were recorded (Vermeire et al., 2017).

OTHER MECHANISM(S) OF ACTION

There are a few other drugs with mechanism(s) of action that are not applicable to the aforementioned categories, and that have been preliminary investigated as potential therapeutic options in IBD patients. For example, laquinimod is an oral small molecule that has demonstrated efficacy in the treatment of multiple sclerosis. Its mechanism of action has not yet been fully elucidated, but it is secondary to a shift in a regulatory phenotype of T-cells and reduction of pro-inflammatory cytokines (Varrin-Doyer et al., 2014). A phase II dose finding RCT showed that a lower dose of the drug (0.5 mg/day) has higher remission and response rates (48% and 55% respectively vs. 32% and 16% in the placebo group) in CD patients (D'Haens et al., 2015). Mongersen, an oral antisense oligonucleotide that binds SMAD7 mRNA, thus preventing the inhibition of TGFβ signaling, has previously shown consistent pre-clinical and clinical results in a phase II study, as well as effective impact on endoscopic activity in CD patients (Monteleone et al., 2015; Feagan et al., 2018). Unfortunately, the impressive results were not confirmed in a phase III trial, which was prematurely suspended after the interim analysis. As such, future development of drugs of this class is uncertain. A synthetic representation of the drugs evaluated in the current review is presented in **Table 1**.

DISCUSSION

Published studies and real-life experiences indicate that standard biologic therapy with anti-TNF blockers, which often remains the first-line therapy for moderate-to-severe IBD patients not responding to conventional therapy, has several drawbacks and the majority of patients either still do not respond or lose response over time. This issue has pushed the

TABLE 1 | Main non anti-TNF pharmacological drugs that show beneficial effects in IBD therapy in randomized clinical trials.

Class	Drug	Route	Mechanism of action	Indication	Studies
Leukocyte trafficking	Natalizumab	IV	Antibody to α4 subunit	CD	Phase III—approved in US
	Vedolizumab	IV	Antibody to α4β7-integrin	UC and CD	Phase III—approved
	Etrolizumab	IV	Antibody to β7-integrin	UC and CD	Phase II
	Ozanimod	oral	Small molecule S1P 1-5 inhibitor	UC	
Inhibitors of pro-inflammatory cytokines	Ustekinumab	IV/SC	Antibody to IL-12/IL-23 (p40)	CD	Phase III—approved
	MEDI2070	IV	Antibody to IL-23 (p19)	CD	Phase II
	Risankizumab	IV	Antibody to IL-23 (p19)	CD	Phase II
	PF-04236921	SC	Antibody to IL-6	CD	Phase II
Blockers of downstream cytokine signaling pathways	Tofacitinib	oral	Small molecule JAK blocker	UC	Phase III—approved
	Filgotinib	oral	Small molecule JAK1 blocker	CD	Phase II
Other pathways	Laquinimod	oral	Small molecule active on T-cells	CD	Phase II
	Morgensen	oral	Antisense nucleotide of SMAD7	CD	Phase II—phase III suspended

IV, intravenous; SC, subcutaneous; CD, Crohn's disease; UC, ulcerative colitis.

field towards research and characterization of novel drugs that are potentially useful for the treatment of IBD, with different mechanisms of action from TNF blockade. Among these drugs, three (i.e., natalizumab, vedolizumab, and ustekinumab) are already available for clinical use and one (tofacitinib) has received approval for market distribution, while the other molecules are still in the pipeline. As a consequence, the efficacy data are mostly preliminary and mainly coming from registrative or RCT trials, in which the clinical conditions are often not comparable to reality. Even more relevant, safety data are absolutely preliminary, since registry data and post-marketing surveillance, which are fundamental tools for the identification and evaluation of drug safety, are mostly lacking for novel molecules. Nonetheless, the expansion of the drug armamentarium for the treatment of IBD patients in recent years is remarkable, as well as the positive perspectives for the near future, considering the relative paucity of effective IBD drugs compared to other chronic inflammatory conditions.

At present, a number of relevant questions are raised, and several issues need to be addressed, including: are the new drugs really improving the efficacy of treating IBD patients? Did they offer a gain in remission/response rate in naïve and TNF-experienced IBD patients? Are the drugs better than the "established" anti-TNF blockers? Perhaps the more correct way to respond to these inquiries would be to evaluate head-to-head trials, directly comparing different drugs to each other. Unfortunately, results of these trials are not yet available. A phase III multicenter clinical trial directly comparing vedolizumab and adalimumab in UC patients has, to date, recruited 770 patients and results are pending for 2019 (ClinicalTrials.gov Identifier: NCT02497469). Furthermore, a phase III study comparing etrolizumab vs. adalimumab in UC patients (ClinicalTrials.gov Identifier: NCT02171429) and ustekinumab vs. adalimumab in CD patients (ClinicalTrials.gov Identifier: NCT03464136) are still in the process of recruiting patients. Since results of these studies are not yet available, the only surrogate data we have for drug comparison studies derive from indirect statistical evaluation of different trials, by means of network meta-analysis. To date, 10 studies have been published, among which 4 evaluated trials in UC patients (Danese et al., 2014; Vickers et al., 2016; Bonovas et al., 2018; Singh et al., 2018), 4 in CD patients (Singh et al., 2014; Stidham et al., 2014; Hazlewood et al., 2015; Pagnini et al., 2018), and 2 studies both CD and UC patients (Cholapranee et al., 2017; Mao et al., 2017) have been performed. Together, the results of these studies further confirm the efficacy of treatments vs. placebo, both in naïve and TNF-experienced patients, but failed to identify a drug clearly superior to the others. Moreover, such results need to be interpreted with great caution, since heterogeneity among studies may profoundly affect meta-analysis results.

A further speculation that one could make is: now that drugs with multiple mechanism(s) of action are competing in the market for IBD treatment, how can we improve the efficacy of such therapies? Probably the best approach is to attack from several fronts by improving the selection of patients by identification of pre-treatment features predictive of response to a specific drug, and evaluating the possibility of multi-drug co-treatments. In fact, the concept of personalized medicine for the treatment of IBD patients has been recently explored. Recognizing clinical and/or molecular markers predictive of response to specific drugs may be of paramount importance in the near future, when more drug options become available. This could be relevant for both the first drug of choice in naïve patients, when an effective therapeutic strategy is more likely to positively impact long-term outcome, and for patients who have already failed a first line of therapy, where a specific second-line drug may guarantee a higher response rate in this difficult-to-treat subset of patients. Most of the research in this field of investigation focuses on predictors of response to anti-TNF therapy. Considering clinical characteristics, some studies have demonstrated that, in CD patients, young age, isolated colitis, and elevated CRP levels are predictors of response to anti-TNF therapy, while smoking and disease duration more than 2 years are predictors of non-responders (Louis et al., 2002; Parsi et al., 2002; Arnott et al., 2003; Siegel and Melmed, 2009).

More recently, Barrè et al. performed a literature review on predictors of response to vedolizumab and ustekinumab. The authors found that severe disease, prior anti-TNF exposure, was a negative predictor of vedolizumab response, while ileocolonic disease, no prior surgery, and an uncomplicated phenotype were associated with a better response to ustekinumab in CD (Barre et al., 2018). Focusing on gene expression profiles, Arijs et al. (2010) identified a five gene set (i.e., TNFAIP6, S100A8, IL11, G0S2, S100A9) that discriminated with 100% accuracy patients with CD colitis as responders vs. non-responders to infliximab, while no predictive genes were found in CD ileitis. The same authors identified top five genes (i.e., osteoprotegerin, stanniocalcin-1, prostaglandin-endoperoxide synthase 2, IL-13 receptor α2, IL-11) differentially expressed in UC patient responders and non-responders to infliximab (Arijs et al., 2009). Moreover, utilization of molecular imaging, such as single photon emission computed tomography (SPECT) and positron emission tomography (PET), have been evaluated for prediction of response to anti-TNF therapy. Van den Brande et al. (2007) demonstrated that (99 m) technetium (Tc)-annexin V SPECT could discriminate infliximab responders vs. non-responders in a murine model and in CD patients. In another study, CD patients with high numbers of membrane-bound TNF immune cells, detected by topical antibody administration, showed significantly higher short-term response rates to anti-TNF therapy compared with patients with low cell counts (Atreya et al., 2014). Interestingly, two very recently published elegant studies investigated the occurrence of specific inflammatory pathways in IBD patients associated with a low response to anti-TNF therapy. West et al. demonstrated high tissue levels of oncostatin M, a member of the IL-6 cytokine family with potent pro-inflammatory activity, in an animal model of anti-TNF resistant intestinal inflammation and in anti-TNF non-responder IBD patients from two cohorts of phase III clinical trials. The authors propose OMS as a potential biomarker and therapeutic target in IBD that may have an important role in anti-TNF resistant patients (West et al., 2017). Gaujoux et al. observed a significant increase, pre-treatment, in plasma cells from biopsy samples of anti-TNF non-responder patients, which was coupled to an increase in triggering receptor expressed on myeloid cells 1 (TREM-1) and the chemokine receptor type 2 (CCR2)–chemokine ligand 7 (CCL7) axes. In addition, the authors showed that pre-treatment downregulation of TREM expression in peripheral blood of CD patients accurately predicts non response to anti-TNF therapy with an AUC of 94%, thus proposing systemic TREM-1 expression as a non-invasive diagnostic marker of non-response to anti-TNF therapy at baseline (Gaujoux et al., 2018). In addition, interesting data have been obtained from an etrolizumab phase II trial and from retrospective analysis. In fact, higher levels of granzyme A and integrin αE mRNA expression in colon tissues could discriminate patients who are more likely to respond to the drug (Tew et al., 2016). Moreover, serum pre-treatment concentration of IL-22 has been proposed as predictor of response to the IL-23 blocker MEDI2070, and cytokine level above 15.6 pg/ml has been associated with higher response rate, while clinical outcome similar to placebo has been observed in CD patients with IL-22 serum level below that threshold value (Sands et al., 2017). Recently, differences in microbiome composition at baseline have been preliminarily investigated as a potential biomarker predictor of response to infliximab (Shaw et al., 2016) and vedolizumab (Ananthakrishnan et al., 2017), but more data are needed at this time to properly evaluate its predictive value.

The availability of drugs interfering with different molecular inflammatory pathways may bear the attractive concept that contemporaneous block of multiple pathways results in high efficacy, but safety issues need to be considered. A recent systematic review showed limited benefit for combination biologic therapy in rheumatoid arthritis, while data in IBD patients, coming from a few case reports, suggest potential benefit (Hirten et al., 2015; Yzet et al., 2016; Bethge et al., 2017; Fischer et al., 2017). The only explorative study for combination therapy in CD is a multi-center randomized clinical trial primarily evaluating the safety and tolerability of three natalizumab infusions in 79 CD patients with active disease, despite infliximab therapy. Besides the small number of patients and the fact that the study was not designed for efficacy evaluation, a trend for higher response rates in the natalizumab + infliximab group was observed compared to placebo + infliximab, and safety evaluation was reassuring (Sands et al., 2007). An open label prospective study, EXPLORER (Clinical Trials.gov Identifier: NCT02764762), aimed at determining the effect of triple combination therapy with vedolizumab, adalimumab, and methotrexate on endoscopic remission in moderate-to-severe CD patients, stratified at higher risk for complications, is also currently underway.

In conclusion, in recent years, novel drugs with different mechanism(s) of action are likely to expand the physician's armamentarium to treat IBD patients. Such novel drugs should confirm the positive results from registrative trials in real-life settings, where difficult-to-treat patients are more frequent and ideal conditions of the trials are not considered. At the same time, the increased availability of therapeutic options represents a great opportunity and challenge for IBD specialists. In fact, the correct stance and implementation for use of available novel drugs, together with an increased ability in patients' selection and therapeutic tailoring, will hopefully lead to more effective therapies, as well as increased safety, for the treatment of IBD patients.

AUTHOR CONTRIBUTIONS

CP conceived and wrote the manuscript. TTP wrote and edited the manuscript. FC conceived, wrote, and edited the manuscript.

REFERENCES

Abraham, C., and Cho, J. H. (2009). Inflammatory bowel disease. *N. Engl. J. Med.* 361, 2066–2078. doi: 10.1056/NEJMra0804647

Amiot, A., Serrero, M., Peyrin-Biroulet, L., Filippi, J., Pariente, B., Roblin, X., et al. (2017). One-year effectiveness and safety of vedolizumab therapy for inflammatory bowel disease: a prospective multicentre cohort study. *Aliment. Pharmacol. Ther.* 46, 310–321. doi: 10.1111/apt.14167

Ananthakrishnan, A. N., Luo, C., Yajnik, V., Khalili, H., Garber, J. J., Stevens, B. W., et al. (2017). Gut microbiome function predicts response to anti-integrin biologic therapy in inflammatory bowel diseases. *Cell Host Microbe* 21, 603–610 e3. doi: 10.1016/j.chom.2017.04.010

Arijs, I., Li, K., Toedter, G., Quintens, R., Van Lommel, L., Van Steen, K., et al. (2009). Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis. *Gut* 58, 1612–1619. doi: 10.1136/gut.2009.178665

Arijs, I., Quintens, R., Van Lommel, L., Van Steen, K., De Hertogh, G., Lemaire, K., et al. (2010). Predictive value of epithelial gene expression profiles for response to infliximab in Crohn's disease. *Inflamm. Bowel Dis.* 16, 2090–2098. doi: 10.1002/ibd.21301

Arnott, I. D., McNeill, G., and Satsangi, J. (2003). An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease. *Aliment. Pharmacol. Ther.* 17, 1451–1457. doi: 10.1046/j.1365-2036.2003.01574.x

Arseneau, K. O., and Cominelli, F. (2013). Vercirnon for the treatment of Crohn's disease. *Expert Opin. Investig. Drugs* 22, 907–913. doi: 10.1517/13543784.2013.795946

Arseneau, K. O., and Cominelli, F. (2015). Targeting leukocyte trafficking for the treatment of inflammatory bowel disease. *Clin. Pharmacol. Ther.* 97, 22–28. doi: 10.1002/cpt.6

Atreya, R., Neumann, H., Neufert, C., Waldner, M. J., Billmeier, U., Zopf, Y., et al. (2014). In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat. Med.* 20, 313–318. doi: 10.1038/nm.3462

Barre, A., Colombel, J. F., and Ungaro, R. (2018). Review article: predictors of response to vedolizumab and ustekinumab in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 47, 896–905. doi: 10.1111/apt.14550

Baumgart, D. C., and Sandborn, W. J. (2012). Crohn's disease. *Lancet* 380, 1590–605. doi: 10.1016/S0140-6736(12)60026-9

Baumgart, D. C., Bokemeyer, B., Drabik, A., Stallmach, A., Schreiber, S.; Vedolizumab Germany Consortium. (2016). Vedolizumab induction therapy for inflammatory bowel disease in clinical practice–a nationwide consecutive German cohort study. *Aliment. Pharmacol. Ther.* 43, 1090–1102. doi: 10.1111/apt.13594

Becker, C., Wirtz, S., Blessing, M., Pirhonen, J., Strand, D., Bechthold, O., et al. (2003). Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J. Clin. Invest.* 112, 693–706. doi: 10.1172/JCI200317464

Bethge, J., Meffert, S., Ellrichmann, M., Conrad, C., Nikolaus, S., and Schreiber, S. (2017). Combination therapy with vedolizumab and etanercept in a patient with pouchitis and spondylarthritis. *BMJ Open Gastroenterol.* 4, e000127. doi: 10.1136/bmjgast-2016-000127

Bonovas, S., Lytras, T., Nikolopoulos, G., Peyrin-Biroulet, L., and Danese, S. (2018). Systematic review with network meta-analysis: comparative assessment of tofacitinib and biological therapies for moderate-to-severe ulcerative colitis. *Aliment. Pharmacol. Ther.* 47, 454–465. doi: 10.1111/apt.14449

Cholapranee, A., Hazlewood, G. S., Kaplan, G. G., Peyrin-Biroulet, L., and Ananthakrishnan, A. N. (2017). Systematic review with meta-analysis: comparative efficacy of biologics for induction and maintenance of mucosal healing in Crohn's disease and ulcerative colitis controlled trials. *Aliment. Pharmacol. Ther.* 45, 1291–1302. doi: 10.1111/apt.14030

Colombel, J. F., Sands, B. E., Rutgeerts, P., Sandborn, W., Danese, S., D'Haens, G., et al. (2017). The safety of vedolizumab for ulcerative colitis and Crohn's disease. *Gut* 66, 839–851. doi: 10.1136/gutjnl-2015-311079

D'Haens, G., Sandborn, W. J., Colombel, J. F., Rutgeerts, P., Brown, K., Barkay, H., et al. (2015). A phase II study of laquinimod in Crohn's disease. *Gut* 64, 1227–1235. doi: 10.1136/gutjnl-2014-307118

Danese, S., Fiorino, G., Peyrin-Biroulet, L., Lucenteforte, E., Virgili, G., Moja, L., et al. (2014). Biological agents for moderately to severely active ulcerative colitis: a systematic review and network meta-analysis. *Ann. Intern. Med.* 160, 704–711. doi: 10.7326/M13-2403

Danese, S., Rudziński, J., Brandt, W., Dupas, J. L., Peyrin-Biroulet, L., Bouhnik, Y., et al. (2015). Tralokinumab for moderate-to-severe UC: a randomised, double-blind, placebo-controlled, phase IIa study. *Gut* 64, 243–249. doi: 10.1136/gutjnl-2014-308004

Danese, S., Vermeire, S., Hellstern, P., Panaccione, R., Rogler, G., Fraser, G., et al. (2019). Randomised trial and open-label extension study of an anti-interleukin-6 antibody in Crohn's disease (ANDANTE I and II). *Gut* 68, 40–48. doi: 10.1136/gutjnl-2017-314562

Dulai, P. S., Singh, S., Jiang, X., Peerani, F., Narula, N., Chaudrey, K., et al. (2016). The real-world effectiveness and safety of vedolizumab for moderate-severe Crohn's disease: Results From the US VICTORY consortium. *Am. J. Gastroenterol.* 111, 1147–1155. doi: 10.1038/ajg.2016.236

Feagan, B. G., Rutgeerts, P., Sands, B. E., Hanauer, S., Colombel, J. F., Sandborn, W. J., et al. (2013). Vedolizumab as induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 369, 699–710. doi: 10.1056/NEJMoa1215734

Feagan, B. G., Sandborn, W. J., Gasink, C., Jacobstein, D., Lang, Y., Friedman, J. R., et al. (2016). Ustekinumab as induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* 375, 1946–1960. doi: 10.1056/NEJMoa1602773

Feagan, B. G., Sandborn, W. J., D'Haens, G., Panés, J., Kaser, A., Ferrante, M., et al. (2017). Induction therapy with the selective interleukin-23 inhibitor risankizumab in patients with moderate-to-severe Crohn's disease: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet* 389, 1699–1709. doi: 10.1016/S0140-6736(17)30570-6

Feagan, B. G., Sands, B. E., Rossiter, G., Li, X., Usiskin, K., Zhan, X., et al. (2018). Effects of Mongersen (GED-0301) on endoscopic and clinical outcomes in patients with active crohn's disease. *Gastroenterology* 154, 61–64 e6. doi: 10.1053/j.gastro.2017.08.035

Fischer, S., Rath, T., Geppert, C. I., Manger, B., Schett, G., Neurath, M. F., et al. (2017). Long-term combination therapy with anti-TNF plus vedolizumab induces and maintains remission in therapy-refractory ulcerative colitis. *Am. J. Gastroenterol.* 112, 1621–1623. doi: 10.1038/ajg.2017.242

Fleischmann, R., Kremer, J., Cush, J., Schulze-Koops, H., Connell, C. A., Bradley, J. D., et al. (2012). Placebo-controlled trial of tofacitinib monotherapy in rheumatoid arthritis. *N. Engl. J. Med.* 367, 495–507. doi: 10.1056/NEJMoa1109071

Gaujoux, R., Starosvetsky, E., Maimon, N., Vallania, F., Bar-Yoseph, H., Pressman, S., et al. (2018). Cell-centred meta-analysis reveals baseline predictors of anti-TNFalpha non-response in biopsy and blood of patients with IBD. *Gut*. 68 (4), 604–614. doi: 10.1136/gutjnl-2017-315494

Hazlewood, G. S., Rezaie, A., Borman, M., Panaccione, R., Ghosh, S., Seow, C. H., et al. (2015). Comparative effectiveness of immunosuppressants and biologics for inducing and maintaining remission in Crohn's disease: a network meta-analysis. *Gastroenterology* 148, 344–354 e5; quiz e14-5. doi: 10.1053/j.gastro.2014.10.011

Hirten, R., Longman, R. S., Bosworth, B. P., Steinlauf, A., and Scherl, E. (2015). Vedolizumab and infliximab combination therapy in the treatment of Crohn's disease. *Am. J. Gastroenterol.* 110, 1737–1738. doi: 10.1038/ajg.2015.355

Hueber, W., Sands, B. E., Lewitzky, S., Vandemeulebroecke, M., Reinisch, W., Higgins, P. D., et al. (2012). Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 61, 1693–1700. doi: 10.1136/gutjnl-2011-301668

Ito, H., Takazoe, M., Fukuda, Y., Hibi, T., Kusugami, K., Andoh, A., et al. (2004). A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 126, 989–996. discussion 947. doi: 10.1053/j.gastro.2004.01.012

Iwakura, Y., and Ishigame, H. (2006). The IL-23/IL-17 axis in inflammation. *J. Clin. Invest.* 116, 1218–1222. doi: 10.1172/JCI28508

Jiang, W., Su, J., Zhang, X., Cheng, X., Zhou, J., Shi, R., et al. (2014). Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel disease. *Inflamm. Res.* 63, 943–950. doi: 10.1007/s00011-014-0768-7

Kopylov, U., Ron, Y., Avni-Biron, I., Koslowsky, B., Waterman, M., Daher, S., et al. (2017). Efficacy and safety of vedolizumab for induction of remission in inflammatory bowel disease-the israeli real-world experience. *Inflamm. Bowel Dis.* 23, 404–408. doi: 10.1097/MIB.0000000000001039

Loftus, E. V., Colombel, J. F., Feagan, B., Vermeire, S., Sandborn, W., Sands, B., et al. (2017). P209 Long-term effectiveness and safety of vedolizumab in patients with ulcerative colitis: 5-year cumulative exposure of GEMINI 1 completers rolling into the GEMINI open-label extension study. *J. Crohns Colitis.* 11, S182–S183. doi: 10.1093/ecco-jcc/jjx002.334

Louis, E., Vermeire, S., Rutgeerts, P., De Vos, M., Van Gossum, A., Pescatore, P., et al. (2002). A positive response to infliximab in Crohn disease: association with a higher systemic inflammation before treatment but not with -308 TNF gene polymorphism. *Scand. J. Gastroenterol.* 37, 818–824. doi: 10.1080/gas.37.7.818.824

Mao, E. J., Hazlewood, G. S., Kaplan, G. G., Peyrin-Biroulet, L., and Ananthakrishnan, A. N. (2017). Systematic review with meta-analysis: comparative efficacy of immunosuppressants and biologics for reducing hospitalisation and surgery in Crohn's disease and ulcerative colitis. *Aliment. Pharmacol. Ther.* 45, 3–13. doi: 10.1111/apt.13847

Monteleone, G., Neurath, M. F., Ardizzone, S., Di Sabatino, A., Fantini, M. C., Castiglione, F., et al. (2015). Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *N. Engl. J. Med.* 372, 1104–1113. doi: 10.1056/NEJMoa1407250

Neurath, M. F., and Travis, S. P. (2012). Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut* 61, 1619–1635. doi: 10.1136/gutjnl-2012-302830

Nielsen, O. H., Li, Y., Johansson-Lindbom, B., and Coskun, M. (2017). Sphingosine-1-phosphate signaling in inflammatory bowel disease. *Trends. Mol. Med.* 23, 362–374. doi: 10.1016/j.molmed.2017.02.002

Ordás, I., Eckmann, L., Talamini, M., Baumgart, D. C., and Sandborn, W. J. (2012). Ulcerative colitis. *Lancet* 380, 1606–1619. doi: 10.1016/S0140-6736(12)60150-0

Pagnini, C., Saeed, R., Bamias, G., Arseneau, K. O., Pizarro, T. T., and Cominelli, F. (2010). Probiotics promote gut health through stimulation of epithelial innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* 107, 454–459. doi: 10.1073/pnas.0910307107

Pagnini, C., Arseneau, K. O., and Cominelli, F. (2015). Safety considerations when using anti-TNFalpha therapy to treat Crohn's disease. *Expert Opin. Drug Saf.* 14, 31–44. doi: 10.1517/14740338.2015.976610

Pagnini, C., Arseneau, K. O., and Cominelli, F. (2017). Natalizumab in the treatment of Crohn's disease patients. *Expert Opin. Biol. Ther.* 17, 1433–1438. doi: 10.1080/14712598.2017.1366444

Pagnini, C., Siakavellas, S. I., and Bamias, G. (2018). Systematic review with network meta-analysis: efficacy of induction therapy with a second biological agent in anti-TNF-experienced Crohn's disease patients. *Gastroenterol. Res. Pract.* 2018, 6317057. doi: 10.1155/2018/6317057

Panés, J., Sandborn, W. J., Schreiber, S., Sands, B. E., Vermeire, S., D'Haens, G., et al. (2017). Tofacitinib for induction and maintenance therapy of Crohn's disease: results of two phase IIb randomised placebo-controlled trials. *Gut* 66, 1049–1059. doi: 10.1136/gutjnl-2016-312735

Papp, K. A., Menter, A., Strober, B., Langley, R. G., Buonanno, M., Wolk, R., et al. (2012). Efficacy and safety of tofacitinib, an oral Janus kinase inhibitor, in the treatment of psoriasis: a Phase 2b randomized placebo-controlled dose-ranging study. *Br. J. Dermatol.* 167, 668–677. doi: 10.1111/j.1365-2133.2012.11168.x

Park, S. C., and Jeen, Y. T. (2018). Anti-integrin therapy for inflammatory bowel disease. *World J. Gastroenterol.* 24, 1868–1880. doi: 10.3748/wjg.v24.i17.1868

Parsi, M. A., Achkar, J. P., Richardson, S., Katz, J., Hammel, J. P., Lashner, B. A., et al. (2002). Predictors of response to infliximab in patients with Crohn's disease. *Gastroenterology* 123, 707–713. doi: 10.1053/gast.2002.35390

Pelletier, D., and Hafler, D. A. (2012). Fingolimod for multiple sclerosis. *N. Engl. J. Med.* 366, 339–347. doi: 10.1056/NEJMct1101691

Reinisch, W., Panés, J., Khurana, S., Toth, G., Hua, F., Comer, G. M., et al. (2015). Anrukinzumab, an anti-interleukin 13 monoclonal antibody, in active UC: efficacy and safety from a phase IIa randomised multicentre study. *Gut* 64, 894–900. doi: 10.1136/gutjnl-2014-308337

Sandborn, W. J., Colombel, J. F., Enns, R., Feagan, B. G., Hanauer, S. B., Lawrance, I. C., et al. (2005). Natalizumab induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* 353, 1912–1925. doi: 10.1056/NEJMoa043335

Sandborn, W. J., Feagan, B. G., Rutgeerts, P., Hanauer, S., Colombel, J. F., Sands, B. E., et al. (2013). Vedolizumab as induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* 369, 711–721. doi: 10.1056/NEJMoa1215739

Sandborn, W. J., Ghosh, S., Panes, J., Vranic, I., Wang, W., Niezychowski, W., et al. (2014). A phase 2 study of tofacitinib, an oral Janus kinase inhibitor, in patients with Crohn's disease. *Clin. Gastroenterol. Hepatol.* 12, 1485–1493 e2. doi: 10.1016/j.cgh.2014.01.029

Sandborn, W. J., Feagan, B. G., Wolf, D. C., D'Haens, G., Vermeire, S., Hanauer, S. B., et al. (2016). Ozanimod induction and maintenance treatment for ulcerative colitis. *N. Engl. J. Med.* 374, 1754–1762. doi: 10.1056/NEJMoa1513248

Sandborn, W. J., Su, C., Sands, B. E., D'Haens, G. R., Vermeire, S., Schreiber, S., et al. (2017). Tofacitinib as induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 376, 1723–1736. doi: 10.1056/NEJMoa1606910

Sands, B. E., Kozarek, R., Spainhour, J., Barish, C. F., Becker, S., Goldberg, L., et al. (2007). Safety and tolerability of concurrent natalizumab treatment for patients with Crohn's disease not in remission while receiving infliximab. *Inflamm. Bowel Dis.* 13, 2–11. doi: 10.1002/ibd.20014

Sands, B. E., Feagan, B. G., Rutgeerts, P., Colombel, J. F., Sandborn, W. J., Sy, R., et al. (2014). Effects of vedolizumab induction therapy for patients with Crohn's disease in whom tumor necrosis factor antagonist treatment failed. *Gastroenterology* 147, 618–627 e3. doi: 10.1053/j.gastro.2014.05.008

Sands, B. E., Chen, J., Feagan, B. G., Penney, M., Rees, W. A., Danese, S., et al. (2017). Efficacy and safety of MEDI2070, an antibody against interleukin 23, in patients with moderate to severe Crohn's disease: a Phase 2a study. *Gastroenterology* 153, 77–86 e6. doi: 10.1053/j.gastro.2017.03.049

Shaw, K. A., Bertha, M., Hofmekler, T., Chopra, P., Vatanen, T., Srivatsa, A., et al. (2016). Dysbiosis, inflammation, and response to treatment: a longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease. *Genome Med.* 8, 75. doi: 10.1186/s13073-016-0331-y

Siegel, C. A., and Melmed, G. Y. (2009). Predicting response to anti-TNF agents for the treatment of Crohn's disease. *Therap. Adv. Gastroenterol.* 2, 245–251. doi: 10.1177/1756283X09336364

Singh, S., Garg, S. K., Pardi, D. S., Wang, Z., Murad, M. H., and Loftus, E. V. Jr. (2014). Comparative efficacy of biologic therapy in biologic-naive patients with Crohn disease: a systematic review and network meta-analysis. *Mayo Clin. Proc.* 89, 1621–1635. doi: 10.1016/j.mayocp.2014.08.019

Singh, S., Fumery, M., Sandborn, W. J., and Murad, M. H. (2018). Systematic review with network meta-analysis: first- and second-line pharmacotherapy for moderate-severe ulcerative colitis. *Aliment. Pharmacol. Ther.* 47, 162–175. doi: 10.1111/apt.14422

Stidham, R. W., Lee, T. C., Higgins, P. D., Deshpande, A. R., Sussman, D. A., Singal, A. G., et al. (2014). Systematic review with network meta-analysis: the efficacy of anti-TNF agents for the treatment of Crohn's disease. *Aliment. Pharmacol. Ther.* 39, 1349–1362. doi: 10.1111/apt.12749

Targan, S. R., Feagan, B. G., Fedorak, R. N., Lashner, B. A., Panaccione, R., Present, D. H., et al. (2007). Natalizumab for the treatment of active Crohn's disease: results of the ENCORE Trial. *Gastroenterology* 132, 1672–1683. doi: 10.1053/j.gastro.2007.03.024

Targan, S. R., Feagan, B., Vermeire, S., Panaccione, R., Melmed, G. Y., Landers, C., et al. (2016). A randomized, double-blind, placebo-controlled Phase 2 study of brodalumab in patients with moderate-to-severe Crohn's disease. *Am. J. Gastroenterol.* 111, 1599–1607. doi: 10.1038/ajg.2016.298

Tew, G. W., Hackney, J. A., Gibbons, D., Lamb, C. A., Luca, D., Egen, J. G., et al. (2016). Association between response to etrolizumab and expression of integrin alphaE and granzyme A in colon biopsies of patients with ulcerative colitis. *Gastroenterology* 150, 477–487. doi: 10.1053/j.gastro.2015.10.041

Tozawa, K., Hanai, H., Sugimoto, K., Baba, S., Sugimura, H., Aoshi, T., et al. (2003). Evidence for the critical role of interleukin-12 but not interferon-gamma in the pathogenesis of experimental colitis in mice. *J. Gastroenterol. Hepatol.* 18, 578–587. doi: 10.1046/j.1440-1746.2003.03024.x

Trinchieri, G., Pflanz, S., and Kastelein, R. A. (2003). The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19, 641–644. doi: 10.1016/S1074-7613(03)00296-6

Van Assche, G., Vermeire, S., and Rutgeerts, P. (2010). The potential for disease modification in Crohn's disease. *Nat. Rev. Gastroenterol. Hepatol.* 7, 79–85. doi: 10.1038/nrgastro.2009.220

Van den Brande, J. M., Koehler, T. C., Zelinkova, Z., Bennink, R. J., te Velde, A. A., ten Cate, F. J., et al. (2007). Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease. *Gut* 56, 509–517. doi: 10.1136/gut.2006.105379

Varrin-Doyer, M., Zamvil, S. S., and Schulze-Topphoff, U. (2014). Laquinimod, an up-and-coming immunomodulatory agent for treatment of multiple sclerosis. *Exp. Neurol.* 262, Pt A:66–71. doi: 10.1016/j.expneurol.2014.04.002

Vermeire, S., O'Byrne, S., Keir, M., Williams, M., Lu, T. T., Mansfield, J. C., et al. (2014). Etrolizumab as induction therapy for ulcerative colitis: a randomised, controlled, phase 2 trial. *Lancet* 384, 309–318. doi: 10.1016/S0140-6736(14)60661-9

Vermeire, S., Schreiber, S., Petryka, R., Kuehbacher, T., Hebuterne, X., Roblin, X., et al. (2017). Clinical remission in patients with moderate-to-severe Crohn's disease treated with filgotinib (the FITZROY study): results from a phase 2, double-blind, randomised, placebo-controlled trial. *Lancet* 389, 266–275. doi: 10.1016/S0140-6736(16)32537-5

Vetter, M., and Neurath, M. F. (2017). Emerging oral targeted therapies in inflammatory bowel diseases: opportunities and challenges. *Therap. Adv. Gastroenterol.* 10, 773–790. doi: 10.1177/1756283X17727388

Vickers, A. D., Ainsworth, C., Mody, R., Bergman, A., Ling, C. S., Medjedovic, J., et al. (2016). Systematic review with network meta-analysis: comparative efficacy of biologics in the treatment of moderately to severely active ulcerative colitis. *PLoS One* 11, e0165435. doi: 10.1371/journal.pone.0165435

West, N. R., Hegazy, A. N., Owens, B. M. J., Bullers, S. J., Linggi, B., Buonocore, S., et al. (2017). Oncostatin M drives intestinal inflammation and predicts response to tumor necrosis factor-neutralizing therapy in patients with inflammatory bowel disease. *Nat. Med.* 23, 579–589. doi: 10.1038/nm.4307

Wong, U., and Cross, R. K. (2017). Primary and secondary nonresponse to infliximab: mechanisms and countermeasures. *Expert Opin. Drug. Metab. Toxicol.* 13, 1039–1046. doi: 10.1080/17425255.2017.1377180

Yamaoka, K., Saharinen, P., Pesu, M., Holt, V. E. 3rd, Silvennoinen, O., and O'Shea, J. J. (2004). The Janus kinases (Jaks). *Genome Biol.* 5, 253. doi: 10.1186/gb-2004-5-12-253

Yzet, C., Dupas, J. L., and Fumery, M. (2016). Ustekinumab and anti-TNF combination therapy in patients with inflammatory bowel disease. *Am. J. Gastroenterol.* 111, 748–749. doi: 10.1038/ajg.2016.66

Zundler, S., and Neurath, M. F. (2017). Novel insights into the mechanisms of gut homing and antiadhesion therapies in inflammatory bowel diseases. *Inflamm. Bowel Dis.* 23, 617–627. doi: 10.1097/MIB.0000000000001067

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership