Study of macrophage phenotype and function response to faecal supernatants of Inflammatory bowel disease patients treated with anti-TNF therapy.

Immune response to faecal supernatant of anti-TNF treated IBD patients

IRAS ID Number: 264405

Project Sponsor

West Hertfordshire Hospitals NHS Trust

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SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the Declaration of Helsinki, the Sponsor's SOPs, and other regulatory requirement.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature: Date: 29/12/2021

.....Fiona Smith.....

Name (please print): Fiona Smith

Position: Research and Development Director, West Hertfordshire Hospitals NHS Trust .....

Chief Investigator:

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Background

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that is without cure. It comprises two main disorders: Crohn's disease (CD) and ulcerative colitis (UC). These conditions can lead to debilitating symptoms of abdominal pain, weight loss diarrhoea and rectal bleeding. The incidence of IBD is increasing worldwide and the prevalence of IBD is currently the highest in North America and Western Europe (1)with cumulative prevalences of 0.3-0.8% (2, 3) and up to 600,000 people in the UK are thought to be affected (CCUK).

Although the range and efficacy of medical therapies is improving, up to 20% of UC patients and 50% of CD patients require surgery within 10 years from diagnosis (4). Current practice for medical therapy of IBD is changing, and standards are shifting from a gradual "step-up" approach, where only symptomatic patients are treated, to an approach where patients receive early intensive therapies in order to prevent severe disease (5, 6). Patients with severe disease or disease refractory to conventional immunomodulation frequently require anti-TNF therapy (7-9).

Anti-TNF therapy is the most effective medical therapy for IBD (5), but the use of these agents comes with extremely high costs and the risk of severe side effects. Infliximab and adalimumab are the most frequently used anti-TNF therapies at present. Several studies have supported their use for inducing and maintaining clinical remission in patients with CD and UC (7, 8, 10-12).

Primary non-response to anti-TNF induction therapy occurs between 20–40% of patients in clinical trials and in 10–20% in "real life" series. Secondary LOR is also a common clinical problem with incidence ranging between 23 and 46% at 12 months after anti-TNF initiation (13-15).

Treatment options for Inflammatory bowel disease are expanding with an increasing number of other biologic therapeutic agents now available (15-17). However, studies demonstrate a reduced efficacy of other treatment options in patients who have previously not responded to anti-TNF therapies and failure of medical therapy inevitably results in further bowel damage and the need for surgery.

Understanding the mechanisms involved in response to anti-TNF therapy and discovering predictors of efficacy are urgently needed in clinical practice in order to optimise treatments and to minimize side-effects and costs. This is particularly true given the increasing availability of biological therapies against other specific targets (i.e., anti-integrin and anti-p40 subunit of interleukin-12 and interleukin-23). There is an urgent need to personalise therapeutic choices to avoid unnecessary delays in treatment benefit, avoidance of adverse effects and to reduce costs.

There are a number of clinical and genetic factors associated with non- response to biologic agents, but none with accuracies high enough to be implemented in choosing an agent for a patient in the clinic prior to initiation of therapy. Biochemical markers including high levels of serum CRP (18) (19, 20) and faecal calprotectin (21) (22, 23) as well as serological markers including ASCA and pANCA seem to be strongly correlated with disease activity and response to infliximab. Demographic factors including young age at onset, colitis and concomitant immunosuppressive therapy and early trough drug levels have also been identified as variables predicting response to infliximab (14).

Genetic variants located in the genetic regions of *TNFRSF1A/B, FCGR2A/B, FAS, FASLG* and *CRP* and *MED15* are reported to decrease biological response to infliximab, but these findings are not unequivocally replicated in subsequent studies (24-26). The results of these studies suggest that

there might be a difference in single nucleotide polymorphisms (SNPs) associated with clinical response and biochemical response. Moreover, most of these reports have studied response immediately after induction while data regarding longer-term response is still lacking.

Responders and non-responders to anti-TNF threrapy were recently shown to express distinctly different patterns of mucosal antimicrobial peptides and microbiota (27-29). Recent data suggest that the microbiota may offer a non-invasive predictive tool to predict response to anti-TNF therapy as well as response to other biologic therapies in both inflammatory bowel disease and inflammatory arthritides (29, 30). Metabonomic analysis also suggests functional characteristics of patients' microbiota may distinguish anti-TNF responders and non responders (31-33).

A study of a small number of biologic naiive patients suggested ex-vivo and in vivo binding of topically administered, fluorescently labelled TNF antibodies using imunohistochemistry and confocal laser microendoscopy could predict anti-TNF response (34). In a small cohort of ulcerative colitis patients, serum cytokine analysis revealed a panel of cytokines that might predict response to anti-TNF with reasonable sensitivity and specificity. High Oncostatin M expression correlates with anti-TNF response and IL23 from CD14+ macrophages acitivates TNF receptor 2 expressing cells co-expressing IL23 receptors mediating resistance to TNF therapy in Crohn's disease (35).

TREM 1 expression on macrophages has been recently identified as a predictor of anti-TNF response in a small number of Crohn's disease patients (36), which acts upstream of the CCR2-CCL2 axis that regulates monocytes migration to the intestine prior to their differentiation into macrophages . These data suggest a link between monocyte-macrophage function in the intestine and physiological responses to anti-TNFα. Indeed, TREM-1 promotes inflammation by promoting inflammatory cytokine production such as TNFα following TLR activation. TREM-1 is upregulated by various TLR

stimuli indicating enhanced TLR responsiveness and/or altered microbiome composition may impact on responsiveness.

Trem1 regulates the CCR2-CCL7axis which controls monocyte recruitment into the intestine. Unlike other tissues of the body, macrophages in the intestine are predominantly seeded from blood monocytes (Bain et al Nat Immunol 2014; Bujko 2018 J exp med). These monocytes are responsive to TLR stimulation, producing high levels of TNFα, and differentiate through an intermediary "immature macrophage" phase before fully differentiating into mature macrophages that are characteristically hyporesponsive to TLR stimulation (Bain Mucosal Immunol 2012/2013). This is thought to be one of the major regulatory mechanisms by which harmful immune responses to the commensal microbiota such as those in IBD are avoided. This process is dysregulated in inflammation and IBD, with an accumulation of immature macrophages that have not fully differentiated expressing monocyte markers alongside inflammatory cytokines such as TNFα. These immature macrophages are responsive to TLR stimulation and accumulate in the inflamed mucosa in both mice and humans. Thus, the association of Trem 1 and anti-TNFα treatment non responsiveness is likely due to ability of Trem1 to regulate TLR responses to the local microbiota to govern monocyte function and differentiation into macrophages.

Given the importance in the local microbial environment in shaping monocyte function, the associations of both microbiota and Trem1 with anti-TNFα non responsiveness, and the potent capacity of bacterial derived metabolites in the intestine to shape monocyte and macrophage function (Garrett, Rooks Nat Rev Immunol), in this pilot study, we aim to assess differences of monocyte/macrophage phenotype and function when co-cultured with faecal supernatants (local metabolites) from anti-TNF responders and non-responders.

Hypothesis

Faecal supernatants from inflammatory bowel disease patients that are subsequently responsive to anti-TNF therapy will exert a distinct phenotype and function of monocytes/macrophages when co-cultured.

Primary objective

To characterise the phenotype of monocyte/macrophages co-cultured with faecal supernatants from anti-TNF responsive IBD patients prior to treatment

Secondary objectives

To assess changes in monocyte/macrophage response to faecal supernatant in primary anti-TNF responders that may predict secondary loss of response

Methods:

Patient Groups

Patients will be included if they are:

- >16 years with a clinical, radiological, endoscopic and histological diagnosis in keeping with Crohn's disease or Ulcerative Colitis.
- Crohn's disease patients will have ileal or colonic disease involvement.
- Ulcerative colitis patients must have at least left sided extent of disease.
- Under clinical consideration for initiation of anti-TNF therapy without contraindications to this therapy.

Exclusion criteria:

- Crohn's disease without ileal or colonic involvement
- Ulcerative proctitis
- Pregnancy

Definitions:

Primary non-response (PNR) is defined at week 14 by any of the following:

- Exit prior to Week 14 for treatment failure (including resectional IBD surgery)
- On-going corticosteroid use at week 12-14 (new prescriptions or failure to taper).
- Failure of CRP to fall to ≤5mg/L or by 50% from baseline (week 0)
- Failure of mHBI to fall to ≤4 or by 3 points (Crohn's patients) or
- a Mayo score that does not decrease by at least 3 points from baseline or at least 30% reduction in the Mayo score (Ulcerative colitis patients) with reduction in the rectal bleeding subscore of at least 1 point from baseline and without a reduction in faecal calprotectin >30% of baseline

Intermediate between PNR and response is defined at week 14 by any of the following:

Either but not both of the following criteria are met-

- CRP falls to ≤5mg/L or by 50% from baseline (Week 0) or faecal calprotectin falls >30% of baseline

or

- mHBI falls to ≤4 or by 3 points from baseline (Crohn's) or Mayo score that decreases >=3 points or a reduction in the Mayo score (Ulcerative colitis patients) of >=30% but post treatment score >=3

Response is defined at week 14 by both of the following:

- CRP falls to ≤5mg/L or by 50% from baseline (Week 0)

- faecal calprotectin falls >30% of baseline

- mHBI falls to ≤4 or by 3 points from baseline or Mayo score decreases to <3 or a reduction in the Mayo score (Ulcerative colitis patients) of >30% or >3 with a rectal bleeding subscore reduction of 1 from baseline

Remission will be assessed at each visit and defined by all of the following

- CRP of ≤5 mg/L and mHBI of ≤4 points or Mayo score of 0-2
- No ongoing steroid therapy
- No exit for treatment failure

Non-Remission will be assessed at week 52 and defined by any of the following:

- CRP of >5mg/L • mHBI of >4 points • Mayo score >2 Ongoing steroid therapy • Exit for treatment failure

Samples and schedule of research interventions

Blood and stool samples will be obtained from patients after consenting to participation and prior to initiation of anti-TNF therapy. For patients with primary anti-TNF therapy response continuing on therapy, further blood and stool samples will be collected at weeks 14, 30 and 52. Where endoscopy and biopsy is undertaken for clinical reasons prior to and following initiation of anti-TNF therapy, additional biopsies will be taken for research purposes.

Clinical evaluations, blood tests, faecal calprotectin and drug levels assessment will be conducted according to routine clinical practice.

Week 0	Week 14	Week 30	Point of LOR/Wk 52 if remission
Blood sample (50ml)	Blood sample (50ml)	Blood sample (50ml)	Blood sample (50ml)
Stool sample (frozen -80C)	Stool sample (frozen -80C)	Stool sample (frozen -80C)	Stool sample (frozen -80C)
+- Colonic biopsies			+- Colonic biopsies
Clinical evaluation	Clinical evaluation	Clinical evaluation	Clinical evaluation
Routine bloods including CRP, Albumin, Haemoglobin	Routine bloods including CRP, Albumin, Haemoglobin	Routine bloods including CRP, Albumin, Haemoglobin	Routine bloods including CRP, Albumin, Haemoglobin
Faecal calprotetin	Faecal calprotetin	Faecal calprotetin	Faecal calprotetin

Laboratory Experimental programme

Whole blood will be collected for flow cytometry analysis and mixed with Cytodelics Whole Blood Cell Stabilizer at a ratio of 1:1, incubated in room temperature for 10 minutes and transferred to a -80C freezer for long-term storage awaiting analysis. Whole blood samples preserved in Cytodelics Whole Blood Cell Stabilizer will be thawed at 20C. Cytodelics Fix/Lyse buffer was added at a blood:buffer concentration of 1:10 and samples were incubated at 20C for 5 minutes. Samples will then be diluted 1:4 with Cytodelics Wash buffer 1 and left to lyse for 15 minutes. Cells will then be washed twice with Cytodelics Wash buffer 2, filtered through a 35mm mesh and counted using a Bio-Rad TC20 cell counter prior to antibody labelling for flow cytometry (Olin, Henckel et al. 2018). Flow cytometry of blood monocytic cells will be used for phenotypic analysis of cell surface expression.

Monocytes will be assessed by flow cytometry for surface expression of Trem1, activation markers CD40, CD80, CD86, alternative activation markers CD206 and CD163, migration and adhesion/migration molecules including integrins β7, PSGL1, chemokine receptors CCR2, CCR4, CCR7, CXCR1, CXCR2 and CXCR6, adhesion molecules E- and P-Seelctin, Toll-like receptor TLR2 and TLR4.

Faecal samples will be collected and stored at -80C for less than 6 months prior to processing. Thawed samples will be weighed and then faecal supernatant will be obtained as previously described (37) and stored at -80C. Monocyte derived macrophages, differentiated *in vitro* from healthy human blood monocytes (already available) will be conditioned with faecal supernatants as previously described (38) and phenotypic analysis of cell surface expression will be undertaken as above (migration profiles). In addition, intracellular protein production will be assessed following cellular permeabilization and stimulation with TLR agonists. We will assess production of TNFα, IL-1β, IL-6, IL-12, cell cycle marker Ki67.

Intestinal biopsies taken at the time of endoscopy procedure will be stored in 4% paraformaldehyde for 24 hours prior to transfer into phosho-buffered saline (PBS). These samples will be used for immunofluorescent microscopy to characterise localisation of intestinal monocytes and macrophages with local extracellular matrix producing cells that have a profound impact on their function (e.g. stromal cells).

Statistics

For individual samples, a combination of flow cytometric techniques will be used allowing quantitation of the numbers of cells positive and level of labelling for different markers using 20-

colour flow cytometry to assess multiple parameters simultaneously. With the large amount of data collected by flow-cytometric analysis, it is possible to generate statistically useful information from limited numbers of samples. Normality testing will be carried out on all data; ANOVA (parametric) and Kruskal-Wallis (non parametric) tests will be used to compare data between multiple groups wih the appropriate corrections for multiple comparisons (Tukey test for ANOVA/parametric and Dunn's test for Kruskal Wallis/non parametric) . We will aim to recruit 10-20 patients with Crohn's disease and Ulcerative colitis initiating anti TNF therapy.

Ethical considerations and practicalities

Patients will be recruited from inflammatory bowel disease clinics or the departments IBD database. Clinical data will be recorded as per usual practice by the IBD clinician. The clinician or research nurse will be responsible for consent. Information will be given to the patient in the clinic. Patients will be given a patient information sheet (PIS) and consent form, explaining the study in general. Informed written consent will be obtained from all patients. Patients will be given time (a minimum of 30 minutes) to decide whether they want to take part in the research and will be able to ask the attending endoscopist or research nurse questions. The person taking research consent will be qualified to do so (appropriate research training) and will be aware of the study procedures. Patient data will be stored on hospital computers and password protected at all times. All data will be link anonymised and held securely. No individuals will be identified in published data. De-identified data will be analysed by the research investigators.

The samples stored will be stored in the Research and Development -80 freezers until transfer to the collaborators laboratories to perform the analysis as described. These samples will be labelled by the

participants' code number. The data will be seen by the research team and the data will be anonymised. We will ensure adherence to the General Data Protection Regulation 2018.

Risks: There are no additional invasive procedures to be used in this study, apart from that used in normal clinical care. Additional blood, stool and biopsy samples will be collected concurrently with samples taken for routine clinical purposes. Patients with inflammatory bowel disease are already very familiar with the need for these samples in their care.

All staff/clinicians involved in study recruitment and consent will have appropriate GCP training and be fully informed regarding the details of the study. If any participants are unhappy about any of the study processes they can contact the lead researcher in the first instance and use the NHS complaints service as per normal practice.

Outputs/benefits

The outputs/ benefits relate to a further understanding of the interaction of the microbiota and immune system in inflammatory bowel disease and enhanced means to identify patients that may or may not respond to anti-TNF therapy or are at increased risk of losing response. Understanding the immunological and microbiological mechanisms involved will ultimately contribute to our understanding of the pathogenesis of inflammatory bowel diseases as well as offer potential predictive markers for personalised therapeutics. There is no direct benefit to the individual patient.

Dissemination and publication policy

Anonymised findings will be disseminated to other health care workers and patient groups at meetings and conferences where the work will be presented. The data will be published in relevant peer-reviewed journals. Internal reports will update the progress of the work.

Timetable

The samples will be collected over 12 months. The key stages are as follows: Data will be collected from 2022 onwards. Interim reports will be performed as data is acquired and analysed. Data will be submitted to major annual gastrointestinal meetings (BSG, DDW, UEGW) to disseminate information.

Costs and funding issues

Of the principle investigators Drs Jonathan Landy requires no additional funding. Dr Mann is funded by The Wellcome Trust and The Royal Society.

Consumables - costs for the complete package will be

- £4000

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