Title:

CCR6- CCL20 Axis in IBD: What have we learnt in the last 20 years?

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Abstract

Inflammatory bowel disease (IBD) is a CC chemokine receptor 6 (CCR6) - associated immune mediated disorder which has attracted an extensive superfluity of experimental analyses. IBD has come to the fore of varied scrutinizing owing to its complexity by nature for comprising of two synergistic sub phenotypes; Crohn’s disease (CD) and Ulcerative colitis (UC). Both these disease entities cause potent immune dysregulation followed by intense tissue damage within the gut mucosal system, initiating symptoms which are severely debilitating. Multiple causative factors are said to be responsible for IBD but direct immune dysfunction is kindled by overplay of innate and adaptive immune responses produced against a pathogenic microbial attack through the weakened or leaky gut epithelial barrier. Once immune homeostasis is not achieved by tolerating agents, the self-assertive adaptive immunity mobilize its various T and B cell cohorts initializing their allied immune mechanisms by vigorously deploying them towards the site of infection. CCR6 and its unique solitary ligand CC-chemokine ligand 20 (CCL20) are small protein molecules which are abundantly expressed by T and B lymphocytes and act as chemotactic immune-modulatory envoys that help in the deployment of the effector lymphocyte arm of the immune system, producing two directly opposing outcomes in IBD. This dichotomous immunity consists of either immune tolerance or inflammation which then develops into a chronic state, remaining catatonic to inherent immunity or targeted clinical therapy. In this review, we have chronologically identified a plethora of experimental studies radiating into 14 different compartments highlighted in the visual depiction which have employed both mouse models and clinical subjects spanning a period of nearly two decades. In doing so, we expect the research community would further benefit by tracing through the history, thereby understanding the CCR6 –CCL20 axis in IBD and identifying the gaps in literature which can be fortuitously filled in the future.
1.0 Introduction

1.1 Inflammatory Bowel disease (IBD)

IBD consisting of two co-partnering disease models, CD and UC in the gastrointestinal tract is fast reaching lethal proportions with poor prognosis and treatment combinations. CD is a transmural, segmental disease which can affect any part of the alimentary canal whereas UC is restricted to the colon and rectum. IBD has a global presence spreading into all continents, with majority of cases being recorded from Europe, America and Australia and the average cost of managing the disease per annum is in the range of $250,000 in Australia alone. People from all age groups coming from all walks of life are at risk of contracting IBD although genetic predisposition plays a critical role with over 200 susceptibility gene loci being already identified along with a host of environmental determinants, dietary preferences and the gut microbiome being listed as causative factors. Its excruciating symptoms are abdominal pain, bloody diarrhoea, vomiting, nausea, urgency to evacuate, severe weakness and necrotic fibrosis which could recur intermittently between bouts of remission. If clinical therapy fails to produce responsiveness, surgical intervention becomes
compulsory. The downside of IBD is that it could well develop into colorectal carcinoma or other cancers in the gut [1-8].

### 1.2 Chemokines – Chemokine Receptor 6 (CCR6) and CC - Chemokine ligand 20 (CCL20)

Chemokines are an exclusive group of small molecular cytokines which are chemoattractant by nature and direct leukocyte migration to inflamed tissue microenvironments within the gut mucosa during IBD. Chemokines comprise of receptors and their bonding partners called ligands and the complimentary receptor-ligand pairs together actively mobilize the leukocytes during maintaining immune homeostasis or defending inflammation. There are four major divisions of chemokines named as CC, CXC, XC and C, the common feature of these being separated by cysteine motifs having disulphide bonds. One chemokine receptor may bind with several chemokine ligands or multiple receptors could partner one chemokine ligand. Biochemically, chemokines are small proteins consisting of lower molecular weights within 8-14 kilo Daltons. Biological activities of chemokines are numerous, which include, embryogenesis, wound healing, development of vasculature, T lymphocyte development and differentiation, leukocyte homeostasis, B cell development and maturation [9-12].

CC motif chemokine receptor 6 abbreviated by CCR6 is a chemokine receptor which is abundantly expressed by T and B memory lymphocytes, antigen-presenting cohorts (APC) including the dendritic cells, macrophages, the CD4⁺ T helper lymphocytes and epithelial cells. It propels these cells towards its chemokine ligand 20 (CCL20) which is released in copious quantities by the gut epithelium in response to microbial stimulation. CCR6 is identified in the tissues of thymus, appendix, foetal liver, small intestine, colon, spleen and lymph nodes whilst CCL20 known by several other names {liver and activation regulated chemokine (LARC), macrophage inflammatory protein – 3 alpha (MIP-3α) and Exodus-1} is secreted by endothelium, neurons and the gut epithelium. A vital aspect of the CCR6-CCL20 axis in IBD is that it maintains the T helper lymphocyte 17 (TH17)/regulatory Treg balance during initiating immune tolerance although inflammation arises as a direct result of the dysregulation of TH17-Treg paradigm. Naïve T helper lymphocytes upon antigen priming by APC develop into effectors consisting of T helper lymphocyte 1 (TH1), T helper lymphocyte 2 (TH2), TH17 and regulatory Treg cell subsets in the lymphoid organs and migrate towards the inflammatory locations in the gut mucosal compartment. Contrary to the earlier belief of TH1/TH2 dual immune activation pathways in understanding IBD, the recent hypothesis which attracted more attention has been to deeply evaluate the TH17-Treg dysfunction, of which the immune-driven cues and inherent mechanisms still remain obscure. TH17 cells are associated with an inflammatory phenotype whereas the regulatory Treg cells are linked to an anti-inflammatory suppressive role [9-12].

CCR6 expression on TH17 is induced by the pro-inflammatory cytokines interleukin (IL), IL-17A-F, IL-21, IL-22, IL-23, IL26, IL-1β, IFN-γ, IL-6 and tumour necrosis factor- alpha, (TNF-α) with the transcription factors retinoic-acid-receptor-related orphan nuclear receptor gamma t (RORγt), signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-kB). CCR6 expression on regulatory Treg cells are induced by TGF-β and IL-10 together with the nuclear receptor forkhead box P3 (FoxP3). Interestingly, there is evidence obtained from the experimental models...
where both TH17 and Treg cells being able to oscillate between both inflammatory and suppressive roles [9-12]. Therefore, it is immensely important to be able to identify the immune ingredients which tilt this precarious immune imbalance towards a cure pathway. This review examines the various research studies (shown in figure 2) in the past two decades in the broad context of inflammatory and immune suppressive associated analyses with a view to link the distinctly dichotomous nature of the TH17/Treg axis in IBD as seen in figure 1.

Figure 1: The dichotomous nature of the CCR6-CCL20-mediated TH17/Treg axis.

Figure 2: The list of pro-inflammatory and anti-inflammatory research studies in IBD which have been undertaken so far.

2.0 Pro-inflammatory Analysis

2.1 Effect of Chemokines (CCR6 and CCL20) on T cells

The earliest recorded experimental work on the analyses on CCR6 and colitis is in 2001 by Scheerens and co-workers where they had demonstrated that CCR6 expression was negatively induced by IL-
10, the anti-inflammatory cytokine, using mice deficient in IL-10 and quantifying messenger ribonucleic acid (mRNA) expressions of a number of chemokines. The chemokines examined included Monokine induced by gamma interferon (MIG), regulated upon activation normal T cell expressed and secreted (RANTES), Macrophage inflammatory protein -3 alpha (MIP-3α), T-cell-activation protein -3 (TCA-3), Thymus and activation regulated chemokine (TARC), Lipopolysaccharide-induced CXC chemokine (LIX), Monocyte chemoattractant protein-1 (MCP-1), Macrophage inflammatory protein – 1 beta (MIP-1β) and lymphotactin, in which CCR6 and CCL20 were found to be up regulated in IL-10 −/− mice having chronic colitis compared with the wild type (WT) healthy controls. In a second model, they used Recombination activating gene 2 (Rag2) −/− mice into which CD4+CD45RB high T cells were adoptively transferred during the preliminary stages of colitis and discovered that CCR6 was clearly linked with the acute phase of disease progression. Their findings revealed that chemokines were expressed in both types of mice and were associated with an acute TH1 type immune response [13].

One of the primary functions of CCR6 was targeted by Varona and researchers in 2003 which is maintaining leukocyte homeostasis via influencing the cytokine environment. They used dextran sodium sulphate (DSS) induced colitis in a murine model deficient in CCR6. Absence of CCR6 had remarkably reduced intestinal pathology (shortening of the long axis of intestine, thickening of intestinal wall) in DSS mice emphasizing that CCR6 is a compulsory chemokine receptor in determining gut mucosal aberrations because they showed recovery in between DSS free periods. WT mice otherwise resistant to tri nitro benzene sulfonic acid (TNBS) -induced colitis became more susceptible to it when CCR6 was deleted. They also showed accumulation of TCRαβcells in the gut mucosa with a significant elevation of mRNA by many folds in CD8+ T cells compared to CD4+ at the beginning of DSS treatment. Interestingly in CCR6−/− mice, there was no major difference in these T cell populations during the acute phase of colitis although in the WT, the numbers decreased drastically which emphasizes that CCR6 is needed for trafficking and limiting T cell populations into the intestinal mucosa during acute IBD [14].

The effect of adhesion molecules and CCL20 expression in the inflamed gut in colitis afflicted mice was investigated by Teramoto and teammates in 2005. Inflammation in the gut mucosa is linked to lymphocyte trafficking and associated adhesion molecules, such as the mucosal addressin cell adhesion molecule -1 (MAdCAM-1), which is up regulated in the Inflamed colonic mucosa in patients suffering from CD and UC. They had employed a murine model of DSS –induced experimental colitis into which fluorescent-labelled T and B lymphocytes taken from the spleen of healthy controls were introduced and lymphocyte adhesion inside colonic micro vessels was observed using an intravital microscope. T and B cell adhesion to the colonic vessels was significantly increased in DSS-induced colitis. The use of anti- MAdCAM-1 monoclonal antibodies and anti-CCL20 antibodies or desensitization of CCR6 receptors with excess CCL20 resulted in a remarkable reduction in the accumulation of T and B lymphocytes in DSS-induced colitis [15].

A TNBS -induced model of Balb/c murine colitis was utilized by Katchar and group in 2007 to investigate the effect of CCL20 secreted by the colonic epithelial cells to determine its impact on T cell activation. They used an anti-CCL20 monoclonal antibody to neutralize CCL20 concentration in the gut of these mice to elucidate the effect of CCR6 mediated leukocyte mobilization and recruitment to inflammatory sites in the gut. They observed elevated CCL20 protein levels in diseased mice and
that the neutralizing antibody therapy yielded favourable responses (i) in the recovery of ulcers in the mucosa, (ii) lessened histological injury, (iii) reduced myeloperoxidase activity and (iv) decreased colonic weight to length ratio which are otherwise characteristic of acute disease. In the acute phase of TNBS-induced disease, a significant increase of CCR6 expressing lamina propria (LP) T cell populations (CD4+ and CD8+) was noted. The monoclonal antibody treatment had remarkably reduced these numbers thereby assigning an inflammatory role to CCR6/CCL20 in IBD pathophysiology [16].

More evidence to support the effects of CCR6 in chronic colitis was demonstrated by Eijkelkamp and teammates in 2007 using a DSS–induced colitic model of G protein coupled receptor kinase 6 (GRK6) KO mice. They had investigated the activity of GRK6 which regulates the functions of G protein-coupled receptors such as CCR6. GRK6+/− and GRK6−/− mice groups developed severe colitis measured by histological scores, clinical parameters and colon length while keratinocyte-derived chemokine levels, granulocyte infiltration and interleukin – 1 beta (IL-1β) levels were increased with a decrease in the expression of FoxP3 transcription factor proteins in colonic tissue. WT mice had displayed complete recovery whilst the GRK6 deficient mice continued into chronic colitis suggesting the importance of the intracellular kinase GRK6 in determining the disease progression in colitis [17].

CCR6−/− mice challenged with oral ovalbumin to develop allergic diarrhoea was examined by Blazquez and group in 2010 in which they had observed protection against disease with a significant decrease in TH2 associated cytokines in the intestine. Further using a T cell transfer model, they demonstrated that CCR6 was required even on the transferred T cells within the mouse recipients to develop allergic gut disease [18].

Researching into the activities of chemokines in IBD, Atreya and team in 2010 pointed out that unregulated effector cell responses act as a causative mechanism in the development of mucosal instability during IBD. These effector cell over activation is said to initiate and sustain inflammation in the gut mucosa and are amply aided by chemokines which help leukocyte adhesion and migration to sites of injury. Among the numerous functions of chemokines, they had listed out the secretion of lipid mediators and reactive oxygen species from leukocytes, matrix release of metalloproteinase, stimulation of tissue fibrosis and tumorigenesis [19].

The chemokine influence of CCR6 and CCL20 on the progression of UC was researched by Uchida and co-workers in 2015 in two groups of patients divided into paediatric onset and adult onset of disease. According to their investigations, CCR6 and CCL20 caused significant tissue damage and disease-related pathology more in paediatric patients indicating a difference in chemokine functionality and disease onset changing with the age of human subjects [20].

The requisites for T cell migration within mucosal tissues was studied by Pezoldt and Huehn in 2016 and recorded that in addition to CC chemokine receptor 9 (CCR9) and integrin α4β7, CCR6 and Neuropilin-1 (Nrp-1), two gut homing related molecules are needed for the early differentiation of T effectors within peripheral lymph nodes (pLNss) but not in mesenteric lymph nodes (mLNss). They also revealed that inflammation in the intestine had promoted negative induction of CCR6 and Nrp-1 in pLNss suggesting that localized inflammation in the gut could systematically impact upon T cell differentiation [21].
Yang and researchers in 2017 reported that the severity of experimental colitis depends upon the congenital traits of the mouse models in driving T cell proliferation. BALB/c mice fed with DSS developed a propensity towards a TH2/TH17/ Treg polarized immune mechanisms to seek protection while C57BL/6 mice adopted a TH1-polarized inflammatory pathway becoming more sensitive to DSS-triggered colitis [22].

### 2.2 The Influence of TH17 Cells on IBD

CCR6 is described as predominantly a non-lymphoid tissue (intestine) receptor and a homeostatic chemokine receptor, borne by TH17 cell subsets by Lim and teammates in 2008. CCR6 was shown to direct T cell migration into the gut mucosa and within secondary lymphoid organs to modulate immune homeostasis during an inflammatory episode in the human body. They used human peripheral blood, adult tonsils (secondary lymphoid tissue) and neonatal cord blood mononuclear cells to determine that CCR6 is expressed by both TH17 cells as well as regulatory Treg cells bearing the nuclear transcription factor, FoxP3. Furthermore, TH17 cell subsets were also found to express TH1 and TH2 associated T cell trafficking chemokine receptors described as CXCR6, CCR5, CCR2, CXCR3 and CCR4 respectively [23].

Remarkable observations on TH17 cells were made by Wang and co-workers in 2009 emphasizing that CCR6 is a signatory receptor of this cell population and it is an important intestine homing receptor which regulates T cell migration within the gut tissue microenvironments. They had noted reduced T cell migration into Peyer’s patches in CCR6 deficient mice compared to the WT although it was normal in the large intestine. Reconstitution of severe combined immunodeficiency (SCID) mice with CCR6−/− TH17 cells had caused severe aberrant lesions in the gut because in the absence of CCR6, these cells differentiate into a pro-inflammatory TH1 type. Further, CCR6 expression on TH17 cells was down regulated by IL-2 in both *in vitro* and *in vivo* experiments as much as it was up regulated by transforming growth factor—beta sulphate (TGF-β) [24].

More observations are added to the list of characteristics of TH17 cells by Kleinschek and teammates in 2009 describing the expression of CD161, a C –type, lectin like receptor, along with CCR6 and integrin beta 7 to be responsible for the disease severity in CD patients. They had demonstrated that TH17 cells noted for their gut homing capacity are prone to inducing colitis, confirmed by marked expression of the cytokines IL-17, IL-22 and IL-23 receptor. CD 161 expressing CD4 T cells are known to readily release IL-17 and gamma interferon (IFN-γ) when stimulated by IL-23 in CD patients whereas IL-1β was additionally needed to stimulate IL-23 induced cytokine production in healthy controls [25].

Ahern et al in 2010 studying the multiple responses of the cytokine IL-23 had observed its effects using an IL23R−/− T cell model had revealed that IL-23 (i) initiated intestinal accumulation of TH17 cells, (ii) suppressed the differentiation of FoxP3+, IL-10–producing Treg cells and (iii) stimulated the emergence of a group of IFN-γ- IL-17A+ T cells. The deficiency of IL-23R on T cells however had negligible impact on TH1 cell differentiation. When mutated, *IL23R* gene is known to enhance susceptibility to IBD [26].

El-Bassat et al in 2016 highlighted the importance of IL-23 in UC where increased expression of IL-23p19 gene was positively correlated to increased IL-23 serum levels [27].
TH17 cells are further evaluated by Esplugues and the team in 2011 using an anti-CD3 antibody induced tolerance model, a sepsis model and a viral infective model to study their characteristic behaviour in driving antigen-specific intestinal pathology in the small intestine. They revealed that TH17 cells deliver two different mechanisms; firstly they become eliminated across the gut lumen and secondly, they are transformed into an immune suppressive phenotype described as rT (H) 17 both in vitro and in vivo. They suggested that both these mechanisms function to delimit the TH17 cell populations in the gut thereby regulating pathogenicity in the gut mucosa [28].

The activity of nicotinic acetylcholine receptors (nAChRs) on T cells in enhancing gut inflammation was examined by Galitovskiy and team members in 2011 using two mouse models of colitis. Nicotine was shown to abrogate oxazolone –colitis via the up regulation of colonic Treg cells and decreased TH17 although TNBS-colitis was exacerbated with an elevation of TH17 cells in the colon. They suggested that nicotinic receptors expressed bispecific action by stimulating IL-10 release and attenuating IL-17 secretion or stimulating IL-17 production which confirmed that smoking could induce opposing effects in CD and UC affected individuals [29].

A small subset of cells within the TH17 population which expressed IL-17 type cytokines, namely, IL-17, TNF, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-22 and fostered autoimmune pathogenesis was identified by Bengsch and group in 2012. These were CD4+ T cells expressing CD26 also known as dipeptidylpeptidase IV, an ectoenzyme which plays a role in T cell activation. CD26+ T cells were shown to co-express type 17 molecules, among which was CCR6 including IL-23R, RORγt, CD161 [30].

Genomic loci within CD4+ memory T cells and particularly in the TH17 compartment which are linked to immune-mediated diseases were examined by Zhang and teammates in 2012, using RNA sequencing and promoter methylation studies. IL17A and CCL20 were genes solely expressed by TH17 cells and increased promoter methylation was correlated to negligible RNA sequence levels in them. They concluded that gene expression in T cell subsets combined with disease-related signals give an insight into the pathways promoting immune-compromised pathogenicity [31].

Duhen and Campbell in 2014 identified IL-1β as a critical cytokine that availgs CCR6+CXCR3+TH17 cells sensitive to IL-12. Both IL-1β and IL-12 together induced the differentiation of TH17 into a population which secretes IFN-γ, IL-17 and GM-CSF during a pathogenic episode. Those TH17 cells were of TH1/17 type and expressed the gut homing receptor, integrin β7 and receptors capable of recognizing extracellular bacteria. They concluded that this particular cell subset was specific for microbial induced pathogenesis [32].

A novel TH17 cell subset named the suppressor TH17 which produces immune suppressor activity in patients with CD was reported by Huang in 2015. The interesting feature from their results is that they are generated from induced Treg cells (iTreg) when they are exposed to conditions which favour TH17 activation. Suppressor TH17 have been detected to express a membrane bound catalytic protein, CD 39, which generates nucleosides such as adenosine from pro-inflammatory extracellular nucleotides. Suppressor TH17 cell type is present in healthy peripheral blood and the LP but appear to rigorously decrease in CD patients [33].

In studying the pathways adopted by lymphocytes in performing their effector functions, Hildner and group in 2016 recorded that the B cell activating transcription factor (Batf) expressed on T cells
acts as an important regulator of TH17 cells during their developmental stages and is strongly expressed in intestinal tissue during IBD. Batf positive T cells are thought to actively contribute to the T cell mediated component of colitis. Further, unlike STAT3, the absence of Batf did not influence the intestinal homeostasis \textit{ex vivo} [34].

The effect of unconjugated bilirubin (UCB), which is formed as a result of haem oxidation on experimental colitis was investigated by Longhi and co-workers in 2017 because it has known protective immune suppressive properties. Immune suppressive effects of UCB is mediated by the aryl hydrocarbon receptor (AHR) ligated to TH17 cells which also upregulate CD39 and FoxP3. In IBD they reported negligible immune suppressive effects of UCB upon TH17 cells owing to reduced AHR levels \textit{in vitro}. However the conclusion was that boosting of CD39 immune innervation or the UCB induced AHR pathway could provide substantive therapeutic outcomes to overcome TH17 dysfunction in IBD [35].

2.3 The influence of γδ T cells

An interesting phenomenon involving gamma delta (γδ) T cells is described by Haas and researchers in 2009 which differentiate into two different lineages upon acquiring the ability to produce cytokines that occur within the thymus. This cell population expresses two mutually exclusive receptors, namely, CCR6 and natural killer 1.1 (NK1.1) and the CCR6 expressing gamma delta T cells acquired an IL-17A releasing phenotype while the NK1.1 bearing cohort was prone to produce IFN-γ with or without T cell receptor (TCR) dependent stimuli [36].

The inflammatory potential of a new subset of CD4 T cells, namely the IL-17 expressing gamma delta T cells was discovered by Do and team in 2011 which are responsible for driving TH17 cell differentiation and therefore, T cell innervated colitis. They found that mice deficient in the receptor TCRβγ displayed resistance to disease whilst TCRβ deficient mice became susceptible. However simultaneous reconstruction with IL-17+ gamma delta T cells and CD4 T cells had made TCRβγ deficient mice to produce severe colitis, thereby suggesting that IL-17+γδ T cells have the capacity to induce intense intestinal pathology by supporting TH17 subset differentiation, \textit{in vivo} [37].

2.4 The influence of Innate Lymphoid Cells (ILC)

Geremia with her group in 2011 introduced the concept involving a novel cell lineage identified as innate lymphoid cells (ILC) which release the cytokines, IFN-γ, IL-17 and IL-22 when stimulated by IL-23 which were shown to mediate intestinal pathology in colitis mice. They had observed a significant increase in CD127+ CD56+ ILC in the intestine of CD subjects but not in those having UC. CD56+ILC had released IL-22 and IL-26 when stimulated by IL-23 and CD56 negative ILC expressed IL-17A-F indicating the functional axis of IL-23/IL-17 pathway in IBD. ILC are involved in triggering inflammation in the gut via cytokine release and recruitment of lymphocytes in mice enduring colitis [38].

The fate of the T cells specific for commensal bacteria in IBD was disclosed by Hepworth and teammates in 2015. They demonstrated the involvement of CCR6+ major histocompatibilitycomplex class II (MHCII+) ILC3 directly mediating the cell death of activated T cells which induce tolerance towards commensal microbial flora in the gut. They further recorded that in paediatric patients, these
colonic ILC3 cells expressed reduced MHCII. They further had suggested that this process of apoptosis in commensal-specific T cells was deregulated in human IBD subjects [39].

2.5 The effect on intestinal epithelial cells

In 2002, Kwon et al reported their investigation on CCL20 also known as MIP-3α describing its expression at sites other than predominantly lymphoid locations such as the intestine. Using Caco-2 and HT-29 human colonic cell lines the researchers demonstrated elevated mRNA levels after analysing colonic tissue with real time –polymerase chain reaction (RT- PCR) and enzyme-linked immunosorbent assay (ELISA) and found that IL-1β and TNF-α stimulated CCL20 in a dose dependent manner. CCL20 expression in patients afflicted with Crohn’s disease (CD) was significantly increased in the primary epithelium compared to healthy controls or those having ulcerative colitis (UC). They deduced, CCL20 was essentially raised in the human intestine paving way for mobilization and recruitment of lymphocytes during IBD [40].

Lee and teammates in 2005 revealed that peripheral blood mononuclear cells (PBMC) obtained from UC patients had exhibited up regulated CCL20. They had measured mRNA of CCL20 in patients with UC compared with healthy controls where increasing CCL20 levels were correlated with disease progression. A follow up study after 3 months revealed that CCL20 mRNA was severely reduced in PBMC followed by disease amelioration in those who were treated with glucocorticoid and 5-aminosalicylic acid containing drugs indicating that CCL20 levels are modulated by anti-inflammatory medication. Further, HT-29 cells obtained from the human intestinal epithelium showed markedly reduced CCL20 secretion when treated with anti-inflammatory drugs which is otherwise induced by the pro-inflammatory cytokines, IL-1β and TNF-α. They concluded that CCR6 expressing PBMC are greatly diminished in acute UC with no such apparent difference in mild UC and the finding of nucleotide-binding oligomerization domain 2 (NOD2) gene susceptibility as a risk factor in IBD underpins the importance of PBMC in its disease pathogenesis [41].

The connectivity between chemokines and leukocyte recruitment into inflamed gut mucosa in IBD was described by Puleston and group in 2005. They used a microarray of copy deoxyribonucleic acid (cDNA) to represent the members of the chemokine superfamily along with their receptors and found certain sub groups of chemokines were specifically abundant in the colon of active IBD patients compared with healthy uninflamed tissue controls. CCL20 as well as CXCL 1-3 and 8 along with the receptors, CCR6, CXCR1 and 2 were found to be distinctive in the acute phase of colonic IBD. An examination of Caco-2 and HT-29 cell lines had shown a similar expression of these chemokines when stimulated by IL-1β. Caco-2 cells were unresponsive to TNF-α although both IL-1 β and TNF-α synergistically activated HT-29 and primary keratinocytes indicating that these two cytokines prominently influence the epithelial chemokine expression in inflamed tissue of IBD patients [42].

Macho-Fernandez et al in 2015 had reported that lymphotoxin beta receptor (LTBR) signal transduction in the gut epithelium promotes intestinal homeostasis by wound healing and self-repair after an epithelial injury. The cue for this process is provided by IL-23 produced by the epithelial cells. IL-23 induces IL-22 mediated, cell proliferation and increased mucous secretion by a group of lymphoid tissue inducer cells expressing CCR6 and RORγt [43].
The importance of the innate immune apparatus in activating the gut epithelium during an episode of UC was described by Skovdhal and group in 2015 stating toll-like receptor 3 (TLR3) stimulation had increased CCL20 expression while it was decreased when TLR3 was silenced. Further, the expression of CCR6 and its ligand was found to be more during active disease compared to the inactive stages of UC and CD afflicted individuals [44].

The CCL20 production by the intestinal epithelial cell (IEC) in CD subjects which was supposed to be very high due to increased TNF-α, was examined by Marafini and co-workers in 2016 and recorded it was decreased upon TGF-β1 induction but this effect was reversed by a similar to mothers against decapentaplegic 7 (Smad7) antisense oligonucleotide which is normally elevated in CD patients. During an episode of CD, Smad7 has been shown to downregulate TGF-β which causes negative regulation of TNF-α signalling, responsible for inflammatory induction. Via the negative control of TGF-β, Smad7 brings about lowered production of CCL20 in serum during CD [45].

The CCR6 dependent processes of intestinal homeostasis was explored by Lin and group in 2017 pinpointing that CCR6 deficiency affects B cell production and its related activities in Peyer’s patches (PP) germinal centres such as lowered generation of immunoglobulin A (IgA) memory B cells, impaired IgA class switching ability and decreased affinity to IgA. Further they reported decreased numbers of ILC within isolated lymphoid follicles (ILFs) similar to mothers against decapentaplegic bringing forth a marked reduction in IL-22 secretion which negatively impacts upon anti-microbial peptide production by the IEC. Absence of CCR6 functionality also leads to higher levels of Alcaligenes in PP and filamentous bacteria thriving within the epithelium delineating a role for CCR6 in regulating intestinal symbiotic relationships [46].

2.6 The impact on expression patterns in histology

The histological development of the intestinal mucosa along with quantifying the proportions of T lymphocyte numbers in the gut correlated with CCL20 activity was examined by Lugering and team in 2005. They used an experimental CCR6 enhanced green fluorescent knock-in mouse model. Their study revealed that CCL20 was abundantly produced by the follicle associated epithelium (FAE) in PP and the PP were significantly reduced in size in mice deficient in CCR6. They also noted a proportional decrease in T and B cells. Another observation was the specific reduction in regulatory CD4+ CD45RB low T cells with a reduced CD4/CD8 ratio. Importantly, they recorded a decrease in microfold cell numbers within the FAE in CCR6−/− mice and additionally, IL-12 secretion by CCR6 expressing dendritic cells (DCs) was also noted [47].

Williams, IR who is well known for introducing CCR6 mediation in intestinal lymphoid structure organogenesis, in 2006 published his study using CCR6 KO mice in which was reported that CCR6+ B cells are recruited into cryptopathches (CPs) in the intestinal mucosa and their expansion allows the CPs to differentiate into ILF. Also it was stated that the developmental defects in intestinal morphology obvious in CCR6 KO mice become responsible for reduced IgA secretion against oral antigens. Not only CCR6, but other chemokines, CCR7 and CXCR5 are also said to participate in moulding lymphoid tissues into organized structures associated with the gut mucosal immune system [48].
Researching into the immunogenic role of CCR6 utilizing a pathogenic bacterium, *Yersinia enterocolitica*, Westphal and co-workers in 2008 showed this infection in CCR6 deficient mice produced limited symptoms whereas in control mice lethal severe lesions were produced. They demonstrated the use of M cells present in PP as conduits for the penetration of the mucosal barrier by these microbes underscoring the importance of CCR6 deficiency and its relationship with microbial uptake by M cells. Through analysis by immunohistochemistry, they had discovered the invasion of PP by *Yersinia* in control mice which was also augmented by pro-inflammatory cytokines whilst bacteria were completely absent in the intestinal tissues of CCR6\(^{-/-}\) mice. It is to be noted that both groups of mice showed equal susceptibility when infected intraperitoneally [49].

An evaluation of CCL20 expression in individuals suffering from prolonged UC using immunohistochemistry (IHC), by Hashimoto and team in 2013 recorded a significant elevation in CCL20 biomarker in the rectal mucosa correlating the IHC scores with the development of UC-related neoplasia. This study provided evidence of chemokines released by the colonic epithelium to be important for maintaining and repairing the epithelial barrier because prolonged inflammation is a proven risk for developing malignant diseases in the gut [50].

### 2.7 The association with cancer

CCR6 is specifically expressed by IEC as well as colorectal cancer (CRC) cells and CCR6 is also up regulated during IEC differentiation, stated Brand and researchers in 2006 using the techniques RT-PCR and immunohistochemistry. They used western blotting to analyse signalling, MTS assays to investigate cell proliferation and wounding assays to study chemotactic cell movement. When IEC are stimulated with CCL20 in a pro-inflammatory cytokine setting of TNF-\(\alpha\), IL-\(\beta\) and lipopolysaccharide (LPS), its mRNA expression was markedly up regulated synonymous with the observation that CCL20 mRNA is remarkably high in colonic tissue of CD patients and it was also correlated to increased IL-8 mRNA expression in their lesions. They suggested that CCR6 modulates cell proliferation and cell migration via CCL20 activated migratory pathway of extracellular signal-regulated kinases 1/2 (ERK 1/2), stress-activated protein kinase / jun kinase (SAPK/JNK) and protein kinase B / (Akt) signal transduction in IEC and CRC cells pointing out that CR6 as an important marker for maintaining intestinal homeostasis and CRC metastasis [51].

CCR6/CCL20 chemokines are described as a critical link between non-malignant UC and malignant colorectal diseases, by Frick and members in 2013 through quantifying mRNA and protein profiles using qRT-PCR, ELISA and IHC. It is well established that cancer cells use CCR6 for its metastatic spread, and in this study resection tissue specimens were observed from patients having UC, colorectal adenoma (CRA), colorectal adenocarcinoma (CRC) and colorectal liver metastases (CRLM) compared with healthy tissue. CRA, CRC and CRLM exhibited specific up regulation of CCR6/CCL20 proteins suggesting a definite association between these chemokines and colorectal carcinoma [52].

The effect of CCR6 and its ligand in colonic carcinoma was investigated by Nandi and teammates in 2016 using a CCR6 deficient mouse model into which colon cancers were adoptively transplanted. Tumorigenesis is associated with increased macrophage infiltration and the depletion of these macrophages weakened tumour growth in the transplantable tumour model. CCL20 is known to promote monocyte migration into tissues in *vitro* and the gathering of macrophages within the tumours *in vivo*. This group thus recorded that CCR6 deficiency in the host exhibits reduced tumorigenesis in syngeneic CCR6-expressing colonic cancers [53].
2.8 The impact on Human Immunodeficiency Virus (HIV)

The capacity of CCR6+ T cells to induce permeability to HIV was recorded by Monteiro and group in 2011 via a newly recognized HIV-gp120 binding receptor which happens to be α(4)β(7) integrin, vital for gut homing in the gut associated lymphoid tissue (GALT). They found T cells expressing combined CCR6 and integrin β(7) have the ability to propagate HIV and the mechanisms of differentiation of the memory T cells positive for CCR6 are crucial when targeting therapeutic interventions to treat HIV [54].

2.9 The influence of genetic studies

The expression of mRNA in the colon and ileum was evaluated by Bogaert and group in 2010 using biopsies taken from healthy controls, CD and UC afflicted patients utilizing RT-PCR. They observed high expression of genes associated with the TH17 pathway (IL-17A-F, IL-21, IL-22, IL-26) and the genes related to cell recruitment such as CCR6 and CCL20, all of which were elevated in the diseased colonic samples. They also analysed the genes involved in cell differentiation (STAT3, TGFβ1, IL1B, IL6, IL23A) and revealed CCR6, STAT3, TGFβ1 and TNF were high in the inflamed ileum but was less in the inflamed colon. IL8 and TNF levels had been measured as indicators of inflammation [55].

A hospital-based study of the Chinese Han population displaying susceptibility to CD when associated with three polymorphisms in the signal transducer and activator of transcription 3 (STAT3) gene was reported by Wang and researchers in 2014. Significant differences had been noted between the CD subjects and healthy controls in the genotyping using PCR. They had suggested that single nucleotide polymorphism (SNP) in STAT3 could be used as a predictive index of contacting CD in a given population [56].

38 genomic loci associated with susceptibility to IBD was recorded by Liu and members in 2015 using Immunochip data from GWAS and examining individuals of European, Asian, Indian and Iranian descent. They suggested such metadata are useful for mapping the genomic architecture of IBD among diverse ethnic populations worldwide [57].

Intestinal T cell transcriptomes were analysed by Raine and teammates in 2015 to identify risky gene loci which predispose individuals to gut mucosa-associated disorders such as CD, UC and coeliac disease. A distinct signalling pathway had been encountered for each different T cell subset and identified candidate genes and cell types responsible for gut inflammatory pathologies [58].

2.10 The effect of obesity

A link between obesity and colitis-associated CRC was demonstrated by Wunderlich and group in 2018. CCR6 expressing B cells and γδ T cells are avidly recruited by CCL20 polarized lymphocyte migration to the gut. Obesity has been shown to induce IL-6 secretion which enhances the proliferation of tumour-promoting macrophages that release CCL20. They showed that dietary-induced obesity could leave colitis patients at a higher risk of developing cancer via increased recruitment of inflammatory immune cells to the gut which promote active inflammation [59].
3.0 Anti-inflammatory Analysis

3.1 The effect of Regulatory Treg cells

The gene profile of the chemokine superfamily was examined by Kristensen and co-workers in 2006, isolated from the rectum of mice having colitis and mice revamped with CD25+ regulatory T cells which protect them against colitis. Analysis of mRNA revealed the up regulation of inflammatory chemokines (CCR1, CXCR3 and their ligands) in colitic mice whilst down regulation of mRNA in the chemokines CCR9, CCL17, CCL25 and CXCL1 in protected mice. What is more relevant in the context of CCR6 is marked increase in CCL20 transcripts in protected mice which were re-introduced with CD4 CD25 regulatory T cells [60].

The fact that Treg cells require the expression of CCR6 to induce its suppressive effects during an inflammatory event is re-confirmed by Kitamura and teammates in 2010. Revamping of a Rag2-/- immune deficient mouse model with CCR6 deficient CD4 T memory cells had developed exacerbation of colitis with elevated production of IFN-γ. Thus CCR6 is explicitly needed for Treg-modulated immune suppression of colitis to direct IL-10-releasing, antigen-specific iTreg cells to the colon of diseased mice [61].

Rivino and team in 2010 further contributes towards enhancing the immune functions of Treg cells by demonstrating that CCR6 expressing CD4+ memory T cells differentiate into two cohorts of IL-17 and IL-10 releasing TH17 and Treg cells respectively. Both types were induced by TGF-β whilst the IL-10 stimulated suppression of the tolerogenic Treg cells was abrogated by IL-2 in a colony of autologous immature myeloid dendritic cells ex-vivo [62].

Kryczek et al in 2011 brings forth a new term, namely the “inflammatory” T reg cells, by investigating the attributes of a small subset of FoxP3+ IL-17 expressing CD4 T cells in human peripheral blood because they had become pathogenic in UC afflicted patients by stimulating the release of inflammatory cytokines and halting T cell –mediated immune responses. The researchers described this particular cohort of cells as having similar characteristics as that of TH17 and Treg cells in a co-existing fashion and arise from the differentiation of CCR6+ CD4 T memory cells which require priming by IL-2, TGF-β and APCs of myeloid origin [63].

The effect of microbial infection on the migration of a CD4+ CD25[high] Treg population in the gut was examined by Cook and team members in 2014 using gastric biopsies taken from Helicobacter pylori infected patients. IL-8, CXCL1-3 and CCL20 levels were markedly raised in those subjects and CCR6 and CXCR1-2 were significantly elevated in Tregs obtained in peripheral blood of patients. They concluded that H. pylori infection had induced more FoxP3 Tregs to migrate towards the gastric mucosa, attracted by CCL20 produced by the gut epithelial cells [64].

Another subset of regulatory T cells identified as TGF-β - induced IL-8 FoxP3 T cells, was described by Kryczek and group in 2016 which consist of mostly naïve T cells expressing CD127 which are capable of both immune suppressive and inflammatory roles. T cell proliferation and the production of cytokines which initiate effector functions were inhibited by this subset whilst IL-8 induced inflammatory cytokine release and neutrophil infiltration was also recorded. The researchers labelled the cells as an inflammatory Treg subgroup which could initiate tumour development in an inflammatory setting such as the development of CRC from chronic UC [65].
The non chemotaxis functions of the CCR6 –CCL20 axis was investigated by Kulkarni and group in 2018 and recorded (i) negative correlation with FoxP3+ CD4+ T cells in UC subjects compared to healthy controls (ii) Tregs positive for CCR6 had enhanced RORγt expression compared with CCR6 negative Tregs (iii) TGF-β1- induced iTreg differentiation was abrogated by CCL20 and channeled towards transforming into pro-inflammatory TH17 type (iv) Impaired suppression of the surface molecules CD39, CD73, FasL expressed on iTreg cells in the presence of CCL20 and (v) CCR6 induced phosphorylation of the molecules Akt, STAT3 and mamalian target of rapamycin (mTOR) in T cells [66].

Godefroy and co-workers in 2018 report of a newly identified T cell subset named CD4+/CD8α+ or double positive 8 alpha cells (DP8α cells) which reportedly express CCR6 and proliferate when stimulated by a gut inhabiting faecal microbe, Faecalibacterium prausnitzii, a firmicute of the Clostridium IV group. These microbes are said to be low in numbers in IBD patients but in healthy individuals, they promote the release of the cytokine, IL-10 and inhibit T cell proliferation in a CD39-dependent pathway. The researchers suggested that more investigations are needed of this cell subtype in IBD disease models [67].

3.2 The influence of TH17/Treg balance

A novel breakthrough in CCR6 biology is introduced by Yamazaki and team members in 2008 by revealing the CCR6 expressing TH17/Treg axis and their importance in inflammatory diseases which was validated by a murine model of experimental autoimmune encephalitis. However it is highly relevant also to colitis as both these cell types form an exclusive signalling pathway which is active in IBD. CCR6 expression on TH17 is induced by the cytokine TGF-β and transcription factors RORα and RORγ. Along with CCR6, TH17 synergistically secretes its ligand CCL20 thus contributing towards chemotaxis of TH17 cells towards inflammatory sites. CCL20 expression is induced by TGF-β, IL-6, IL-21 and requires RORγ and STAT3 transcription factors. In addition, induced regulatory T cells (iTreg) and natural regulatory T cells (nTreg) too have been shown to up regulate CCR6 expression, by which disease amelioration is brought about via their immune suppressive functions. The highlight of this research was reiterating the importance of CCR6-driven immune cell migration to inflamed sites thereby modulating the disease outcome in both autoimmune and inflammatory disorders [68].

The stepping stones into introducing the TH17/ Treg equilibrium paradigm was laid down by Lochner and group in 2008 which most of the researchers in recent years believe is a factor which determines the pathological outcome in IBD. They describe the co-existence of IL-17+ Tαβ cells expressing RORγt induced by the cytokines IL-6 and IL-23 with FoxP3+ IL-10 producing regulatory Treg cells. Moreover they had established that the ratio of IL-17+ to FoxP3+ RORγt+ CCL20+ Tαβ cells remain constant in RORγt- green fluorescent protein (GFP) transgenic mice afflicted with inflammation and infection [69].

Chaudry et al in 2009 adds more validation to the TH17/Treg imbalance hypothesis by demonstrating that Treg cells restrain TH17 immune responses in colitogenic mice in a STAT-3 dependent process. STAT-3 is a TH17-specific nuclear receptor which is crucial for the differentiation of TH17 cells and Treg-specific ablation of this receptor resulted in the loss of the immune suppressive role of Treg cells which are thought to adapt to its environment by producing lethal intestinal lesions [70].
3.3 The effect of Dendritic Cells (DC)

In a study involving umbilical cord blood derived CD34+ mononuclear cells, which are a subset of human Langerhans cells (LC), Dieu-Nosjean et al in 2001 explored the immunogenic mechanisms of CCR6 and CCL20 in which they demonstrated the involvement of cytokine mediated immunity. LC are described as a unique sub cohort of dendritic cells (DC) accumulating on epithelial surfaces and eliciting immune responses. IL-10, the key anti-inflammatory cytokine which is notable for the induction of regulatory T<sub>reg</sub> cells was found to up regulate CCR6 expression during LC development. When IL-10 influence was withdrawn, CCR6 failed to maintain its expression during the maturation of DC while IL-4 and IFN-γ directly acted to block CCR6 expression as well as the ability to respond to CCL20 during different stages of LC development. They suggested the recruitment of LC to the epithelial surface was quelled by TH1/TH2 inflammatory pathways while it was consistently promoted by IL-10 and TGF-β induced tolerogenic immunity [71].

CCR6 is described as a receptor involved in recruiting DC and memory T lymphocytes into lymphoid follicles in the intestinal mucosa by Kaser and teammates in 2004 whereas CCL20 was identified in follicle associated epithelium. Large numbers of immature DCs positive for the marker Langerin were found to accumulate in sub epithelial space. These results were observed from quantifying mRNA expression using Taqman-PCR in colonic explant cultures obtained from individuals afflicted with IBD, non IBD colitis and irritable bowel syndrome compared with healthy controls. Another fact identified by them was that TNF-α had served to increase CCL20 secretion in the healthy controls whilst anti-TNF-α acted to decrease it. This study had illustrated the contribution of CCL20 in regulating the mobilization of T lymphocytes and antigen sampling cell populations into the gut mucosa during IBD [72].

An interesting mechanistic insight into the routine functions of leukocytes was introduced by Brenner and co-workers in 2004 by utilizing runt-related transcription factor 3 (RUNX3) knockout (KO) mice, where RUNX is a lineage-specific transcription factor belonging to the runt domain family. It regulates the development of T cells, sensory neurons and TGF-β signalling effects of DCs. The importance of this transcription factor is that mice devoid of RUNX are prone to developing spontaneous colitis and hyperplasia in the gut mucosa. At an early age as 4 weeks, Runx3 KO mice tend to develop characteristic clinical and histological signs associated with IBD such as infiltration of leukocytes into the gut, erosion of the gut mucosa, increased release of IgA and lymphoid cluster development. Absence of RUNX in KO mice has revealed TH1/TH2 type immune response associated cytokine profile by using RNA in situ hybridization and immunohistochemistry. Loss of RUNX is related to loss of autonomous functions in leukocytes and mutations of this gene is thought to become a risk factor for developing IBD in humans [73].

The importance of CCR6 expressing DCs in mobilizing and activating T lymphocytes was demonstrated by Salazar-Gonzalez and group in 2006, specifically in the sub epithelial domes in PP in response to pathogen activation by *Salmonella typhimurium* in a C57BL/6 murine model. In contrast, CCR6 deficient DCs failed to mount such a response to activate T cells resulting in decreased immunity against an oral infection of invasive enteric bacteria. DCs are important for antigen sampling, pathogen recognition and priming of naïve T lymphocytes into effectors and thereby initiating T cell activation during adaptive immune responses. The study used mouse strains having
CCR6 enhanced by green fluorescent protein or a deletion of CCR6 to be induced by CCL20 and bacterial flagellin-specific TCR transgenic mice to identify DC subsets which stimulate T cells in PP and an inducible diphtheria toxin system for the depletion of DCs. They demonstrated that CCR6+ DCs mediate localized T cell activation in the PP and CCR6 deficiency impairs activation of S. typhimurium antigen specific CD4+ T cells by those DCs [74].

More information supporting the DC infiltration to inflammatory sites in the gut is described by Watanabe and team in 2007 in UC afflicted patients demonstrating a positive correlation between immature CCR6+ DCs and the level of inflammation in the crypts of colonic mucosa. They found a validated positive relationship between crypt atrophy and mononuclear cell infiltration during active inflammation. Their observations included the clustering of S-100 protein + CCR6+ MIP-3α+ DCs becoming localized within crypts when they were inflamed using histological scores, immunohistochemistry and mRNA analyses suggesting that CCR6 expressing DCs play a significant role in inducing crypt inflammation [75].

The behaviour of an E-cadherin expressing population of monocyte-derived DCs was examined by Siddiqui and researchers in 2010 using an experimental murine model. E-cadherin is the receptor for CD103, and is known to promote inflammation in the intestine. E-cadherin expressing DCs gather inside mesenteric lymph nodes, express toll-like receptors and produce pro-inflammatory cytokines IL-6 and IL-23 during an inflammatory incident in the intestine. It is already known that CD103+DCs in the gut promote regulatory immune responses of Treg cells but the transfer of E-cadherin + DCs into immune deficient mice reconstituted with T cells, had produced TH17 immune responses leading to worsening of intestinal colitis. They concluded that these monocyte-derived DCs positive for E-cadherin are a potential therapeutic target in IBD [76].

The villous epithelial microenvironment was investigated by McDonald and group in 2014 which carries out immune surveillance of the luminal contents in the gut using a mouse model deficient in CCR6. Small intestine resident lamina propria DCs associated with villous epithelium co-express receptors lymphotixin beta (LTBR) and CCR6 but they were notably reduced in CCR6 deficient mice because the DCs expand in a LTBR- dependent as well as CCR6- dependent manner [77].

### 3.4 The influence of Natural Killer T Cells (NKT Cells)

Hornung et al in 2011 demonstrates the ability of DX5+NKT cells to attenuate colitis in Balb/c mice when transferred from healthy mice to SCID mice where they were later detectable in MLN after adoptive transfer. Fluorescence Activated Cell Sorter (FACS) had identified the expression of a repertoire of chemokine receptors on this cell sub population involved in migration in vivo, among which CCR6 was also present [78].

### 4.0 Future Directions

In tracing the experimental research which involved the contributions of CCR6 - CCL20 dual chemokines into the immune mechanisms of IBD, we have come across 64 different studies spanning nearly 18 years which on average records 4 analyses per year. The studies have been sporadic, running into various different themes, trying to build up headways into elucidating immune networks without focussing on the highlight of the CCR6-CCL20 axis which happens to be the TH17/Treg paradigm dysregulation. Goal oriented specifically targeted innovative research models
are needed to construct the bigger picture behind the CCR6–CCL20 immune modulatory scenario which will open avenues to test more effective therapeutic interventions to treat IBD in the future.

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