Role of Chemokine Ligand CCL20 and its Receptor CCR6 in Intestinal Inflammation

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Abstract Chemokines are important players in the immune system with individual chemokine axes demonstrating significant associations with numerous inflammatory disorders. The chemokine receptor CCR6 and its ligand CCL20 are reported to be involved in the pathogenesis of inflammatory bowel disease, however the exact mechanism remains elusive. The *Ccr6* gene has been identified as a susceptibility gene in Crohn's disease, while the expression of its ligand, CCL20 is up regulated following inflammatory stimulus in the intestine. The identification of the role of CCR6-CCL20 axis during the inflammation will shed valuable light into the pathogenesis of IBD as well as providing a potential therapeutic target for treatment for IBD.

Keywords CCR6, CCL20, Inflammatory Bowel Disease

pattern in continents such as America, Australia, and also in some European countries[10]. However an increase in the incidence of IBD has been observed over the last decade in some countries in the Asian continent[10]. Such a change in IBD incidence may be attributed to factors such as westernization of lifestyle, diet, increased consumption of foods high in sugar, improved hygiene and increased use of antibiotics [10].

Figure 1. Multiple factors contributing to the initiation of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is an immune mediated disorder characterized by intestinal inflammation[1,2]. Crohn's Disease (CD) and Ulcerative Colitis (UC) are the two most common forms of IBD affecting the gastrointestinal tract[3]. Overlapping symptoms of CD and UC are chronic relapsing flares associated with rectal bleeding, abdominal pain and / or diarrhea[4]. CD is a transmural, granulomatous condition commonly involving ileum and colon, while UC specifically involves the colon and manifests as superficial inflammation confined to the mucosal and submucosal layers of the intestinal wall[3,4]. Colorectal cancer and toxic-mega colon are life threatening gastrointestinal complications of UC and CD patients[3,5,6]. Extra-intestinal manifestations are observed in 25-40% of IBD patients [7].

Globally, IBD is highly prevalent among young adults and has a huge impact on their quality of life[8]. The onset of IBD occurs at a peak age of 15-29 in both men and women[9]. Epidemiological studies reveal the high incidence and prevalence of IBD is now in a stabilized

Although the pathogenesis leading to the disease process is widely researched, detailed mechanisms remain elusive[11]. While the etiology of IBD remains unclear, evidence suggests that impaired innate and adaptive immune responses play a significant role in the initiation of IBD[8,12-14]. An altered immune response evoked against luminal bacteria in genetically predisposed individual is reported as a primary risk factor for IBD[11]. Mucosal breaches by luminal microflora initiate an abnormal immune response, altering the balance between commensal and pathogenic bacteria is also considered as another risk factor.

Additionally, environmental factors are also indicated to have significant involvement in the development of IBD.

Genetic predispositions of individuals play a major role in the initiation of IBD. Environmental factors and luminal microflora may act as triggers, in the presence of a dysregulated immune response which exacerbates IBD. Environmental factors such as the use of antibiotics, smoking, westernized diet, psychological stress, appendectomy, oral contraceptives, antibiotics, atypical mycobacterial infection, episodes of childhood infections, and increased intestinal permeability can possibly act as triggers for the initiation of IBD [8]. Mawdsley et al [15] reported psychological stress augments disease activity in IBD. In IBD patients, episodes of acute and chronic stress influence the immune mechanisms and may be relevant to the initiation of IBD pathogenesis.

Genetic susceptibility is one of the major contributing factors to the onset of IBD [16,17]. Numerous studies have investigated the genetic factors associated with IBD and have found it to be polygenic [18]. Genome wide association studies (GWAS) have specifically shown new insights into the complex pathway of IBD pathogenesis [19]. GWAS have subsequently identified up to 71 new associations to the existing loci adding up to around 163 risk conferring loci associated with IBD [20].

The first IBD susceptible gene, identified using candidate gene studies, was mapped to chromosome 16, and identified as nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) also known as caspase recruitment domain-containing protein 15 (*CARD15*) [4,18]. The identification of the gene *NOD2/CARD15* was pivotal in providing a better understanding of IBD pathogenesis and emphasizing a link between development and innate immunity [18]. The association between *NOD2* mutations and CD is much stronger than with UC [4]. Of the genes identified some are specific to CD or UC, whereas some are shared between CD and UC [4,18,20]. Interestingly many of the genes associated with IBD such as *NOD2*, *TLR*, *JAK2* and *STAT3* play a role in the innate immune response [4,18].

Dysregulation of chemokines and chemokine receptors are suggested to contribute in mucosal immune responses in IBD. Recent research reports suggest chemokine and chemokine receptors could be targeted for therapy in human diseases [21,22]. The C-C chemokine receptor type 6 (*Ccr6*) gene has recently been identified to be associated with Crohn's disease [23].

2. The Intestinal Immune System

The intestinal epithelium consists of a single layer of epithelial cells and is interconnected by close knit junctions covered by mucus providing a mucosal barrier preventing the entry of luminal toxins, microorganisms and foreign antigens [24]. The intestine normally encounters enormous numbers of luminal microbes of (up to 10^{14}) and incoming pathogens [25,26], comprising over 500 different species

[27]. It has selective permeability and allows passage of dietary nutrients, water and electrolytes to enter into the circulation from the intestinal lumen [24,28,29]. The prevention of bacterial penetration, invasion and systemic spread is essential to maintain immune homeostasis [24].

The intestinal mucosa is rich in tissue macrophages and lymphocytes [30]. The balance between the tolerance towards commensal microbes and the effective responses mounted to an external pathogen needs to be maintained for the prevention of disease as well as to maintain immune homeostasis [31]. Mucin, a protein produced by intestinal goblet cells, forms an integral part of the mucus layer covering the intestinal epithelium along with antibodies, defensins and lysozymes [32-34]. The outer loose mucus layer serves as the first physical and chemical barrier to the luminal microbes, while the inner mucus layer is sterile and is highly concentrated with antimicrobial molecules [30].

A range of antimicrobial peptides are produced by Paneth cells, which are granulated cells, located at the base of the small intestinal crypts. Paneth cells prevent microbial invasion in the small intestine by secreting α -defensins, lysozymes and secretory phospholipase A2 [35]. The epithelial cells involved in the host defense mechanism internalize the incoming gram negative bacteria through phagocytosis. Goblet cells are also involved in delivering the luminal antigens to the immature dendritic cells for antigen sampling [34]. Microbial cell wall components such as lipopolysaccharide (LPS) can stimulate the enterocyte, provoking an innate immune response with the increased production of interleukin 8 (IL-8) via an activation of the transcription factor nuclear factor kappa B (NF κ B) [36].

The microfold cells (M-cells) are a unique cell type present in the follicle associated epithelium (FAE) covering the Peyer's patches and these along with the intestinal dendritic cells are involved in luminal antigen sampling [37]. Neutra et al [38] suggested that the uptake of immune complexes by M cells may regulate mucosal immune responses, but the exact significance of the interaction between the M cells and the intestinal antibodies remain unknown.

2.1. Th17 and T-regulatory Cells

The dendritic cells mature as they migrate from intestinal peripheral /inflamed tissue and pass on the antigens to the mesenteric lymph node where naïve T cells undergo differentiation after an antigen encounter [39]. The differentiation of T cells is often dependent on the cytokine environment, and an intricate association between cells of innate and adaptive immune responses [40,41]. CD4+ T cells differentiate into T regulatory cells, Th1, Th2, and Th17. Both Th1 and Th2 produce some cytokines such as interferon- γ (IFN γ), lymphotoxin- α (LT α), interleukin-4 (IL-4), IL-5 and IL-13, in response to stimuli by pathogens [42]. Until recently Th1 was shown to be involved in mediating a number of autoimmune conditions [42,43]. T helper lymphocytes requiring IL-23 for differentiation and

producing IL-17, IL-17F, IL-21 and IL-22, were recently identified as a new subset known as Th17 effector cells [42]. Th17 effector sub-populations have been observed to be more plastic in phenotype [40]. Th17 cells mediate intestinal inflammations leading to the progression of the disease but the mechanisms are highly debated and remains unresolved [44]. T regulatory cells are stimulated by IL-10 secretion of the dendritic cells [45-48].

Antigenic peptides are presented by MHC molecules to T cells which initiate a cascade of events to destroy the pathogen [49]. The intestinal tissue microenvironment and the local cytokine environment enhances the T cell receptor (TCR) activated CD4⁺ T cell differentiation into IL-10 and TGF- β producing cells [50]. Interestingly both T-regulatory cells and Th17 cells have opposite roles yet require cytokine TGF β . IL-6 is necessary for Th17 development and this suppresses transcription factor forkhead box P3 (FoxP3) and modulates the T cell differentiation process toward Th17 cells [51]. Weaver et al [44] reported expression of IL-17 and IL-23 was up regulated in Crohn's disease lesions, Th17 cells exist in a sub-population of CD4 T cells that are CCR6⁺, IL-23R⁺, and CD161⁺. The effective communication between the intestinal epithelial barrier, innate and adaptive immune cells are vital in maintaining intestinal homeostasis and also to prevent any disease process [52]. Impaired innate and adaptive immune response involving TH17/TH1 inflammatory response and increased inflammatory cytokine production is thought to initiate IBD [53].

3. Chemokines

Chemokines are cytokines with chemotactic and chemo attractant ability and are part of a large family of small globular proteins secreted by a wide range of cells such as macrophages, T lymphocytes, neutrophils and monocytes [54]. Chemokines are involved in both innate and adaptive immunity [55-57] and around 40 to 50 human chemokines have been identified [58-62]. The chemokine activities are mediated by chemokine receptors which belong to a superfamily of seven-transmembrane domain G-protein coupled receptors. Around 20 chemokine receptors have been identified, and these receptors are located on the surface of a range of immune cells [62-65]. Chemokines are becoming attractive potential targets for the development of new therapeutic agents [66].

Chemokines function as signaling proteins of the immune system [57,67] and are of two fundamental types - homeostatic and inflammatory, based on their gene expression regulation [14,59]. Homeostatic chemokines are involved in recruiting lymphocytes, neutrophils and macrophages during an immune response while inflammatory chemokines are strongly implicated in many acute and chronic inflammatory diseases such as respiratory diseases, arthritis and atherosclerosis [68].

Chemokines are essential in the transmigration of the

immune cells by binding to their specific G-protein coupled receptors present on the surface of the immune cells whilst mediating their own biological functions. This emphasizes the need for the tight regulation of potent pro-inflammatory chemokines [64]. During inflammation chemokines are also involved in directing the lymphocytes to the site of inflammation thereby exhibiting their inflammatory properties [14,62]. The ongoing release of chemokines at the site of inflammation facilitates the migration of effector cells in a chronic inflammatory environment [69]. Chemokines are instrumental in recruiting immature and activated cells to the site of inflammation, whereas the inhibition of chemokine activities results in an anti-inflammatory environment and leads into wound healing process.

3.1. Chemokine Structure

Chemokines are proteins with a molecular weight of 8-14 kDa [59,61,64,70] and have three β -pleated sheets and a carbon terminal α -helix with disulphide bonds connecting cysteine residues [71]. Chemokines consist of structurally related secreted proteins of 67-127 amino acids in length [62] and they exist as a monomer, dimer or tetramer, with the functional form being a monomer. Dimers can form at the NH₂-terminal which is the activation and primary binding site, the secondary binding site is present in the flexible loop region that follows the second cysteine [69]. Chemokines are divided into subclasses CXC, CC, CX3C and XC [65,67,72] based on the presence of an amino-acid between (CXC) or adjacent (CC) to the first two N-terminal cysteine residues [59]. Chemokines have very similar monomeric structures and all have a flexible NH₂-terminal region connected to a loop and have three antiparallel β strands and a single COOH-terminal α -helix [69]. Chemokines have a preference to bind with a superfamily of G-protein coupled receptors; these chemokine receptors are found on T cells and B cells and also on other cells like neurons and endothelial cells. Amongst the chemokines, IL-8 was one of the first to be discovered and characterized [65].

3.2. Chemokines and Chemokine Receptors

Chemokines are named based on their receptors they bind (CXC, CC, CX3C, XC) with an addition of R for receptor, followed by a number indicating the order of discovery [62]. CC chemokines bind with CCR chemokine receptors, when bound they tend to attract monocytes, lymphocytes, eosinophils and basophils. CXC chemokines bind with CXCRs and thereby attract neutrophils [71]. Many chemokines bind to multiple receptors, whereas some chemokines bind specifically to a single receptor [73]. Chemokines are important factors in mast cell and eosinophil degranulation and is involved in differentiation of T helper cells and their phenotypes [41].

3.3. Chemokine receptor CCR6

The *Ccr6* gene in humans is located on chromosome 6q27

[74]. The chemokine receptor CCR6 is expressed in the intestine by dendritic cells (DC), subsets of T cells ($CD4^+$, $CD8^+$), and in most B cells [59,75]. It was initially found to be expressed by memory T and B cells [76,77]. Furthermore CCR6 is also expressed by the central and effector memory T cells which are characterized by the expression of CCR7 [78,79]. Apart from the expression of CCR6 on Th17 cells, recently CCR6 was reportedly expressed by IL-22 producing NK cells [80], IL-17 producing γ/δ T cells [81] and a sub population of $CD4^+$ FoxP3⁺ regulatory T cells (Treg)[82-85]. All cells expressing CCR6 are found in the intestine [80,86,87].

The $CD4^+$ T cells and dendritic cells both migrate into the gut mucosal tissue when IL-17 is and their migration is said to be mediated by CCR6 [73]. Recent studies have identified CCR6 as a specific marker for Treg cells and Th17 cells [82,88]. In the intestine Th17 cells are generated depending on the intestinal flora [86,89]. CCR6 is expressed by both Th17 and T regulatory cells; recent studies suggesting that they have opposite roles in autoimmune diseases [90]. *Ccr6* is a susceptibility gene, strongly involved in Crohn's disease [91,92]. An in-vitro study of differentiated T effector and T helper subsets by Yamazaki et al [88] showed mRNA and protein expression of CCR6 being very high in Th17 cells when compared with the small amounts of expression seen in Th1 and Th2 cells [90]. Evidence of CCR6 expression in Treg cells both in vivo and in vitro were reported by Kleinewietfeld et al [82], and showed consistency with the findings of Yamazaki et al [90].

CCR6 deficient mouse models are used to understand the immune responses and the role of chemokine axis CCR6/CCL20 in the intestine [93]. CCR6 is found to be expressed selectively by the T cells, B cells and also in sub-populations of dendritic cells of mice and human [94]. The chemokine ligand CCL20 has its specific chemokine receptor CCR6 [95], only found to interact in mice and humans [93].

The *Ccr6* knock-out mice possess under-developed Peyer's patches, as a result of a reduction in CCL20 expression by follicle associated epithelium (FAE). Compromised development of isolated lymphoid follicles is also noted in CCR6 knock-out mice [14]. Evident decrease of $CD11b^+$, $CD11c^+$ myeloid dendritic cells in the sub epithelial dome of Peyer's patches was noted in *Ccr6* knock-out mice, and is displaced into inter-follicular region and may not exist in Peyer's patches of CCR6 deficient mice [96,97]. CCR6 deficient mice have an increased T cell sub-population within the mucosal layer of the intestine [96,97] with CCR6 being expressed on the cell surface of Th17 cells [98]. Interestingly not all mouse Th17 cells express CCR6 unlike in humans where all Th17 cells are shown to express CCR6 [90]. It was observed that the alterations of the gut leukocyte homeostasis and the cytokine environment might confer disrupting effects on the functionality of the immune system [93,97].

The *Ccr6* knock-out mice have an impaired humoral response to orally administered antigen and they

also fail to respond to rotavirus, an enteric pathogen as demonstrated by Cook et al [97]. The developmental defect of mucosal inductive places in CCR6 deficient mice is the reason behind decreased IgA production to orally administered antigens [14]. *Ccr6* knock-out mice are useful in studying the function of M cells, as the interaction of the chemokine axis CCR6/CCL20 is said to play an important role in the M cell differentiation [99]. Studies using *Ccr6* knock-out mice also show an increase in TCR α/β T cell subpopulations which indicates a possible role of this chemokine receptor in immune regulation [100]. In contrast, the *Ccr6* knock-out mice confer normal systemic immune responses to subcutaneous antigens [97]. Research suggested CCR6 to be a regulator of both humoral and lymphocyte homeostasis in the intestine [97].

The *Ccr6* knock-out mice when treated with DSS developed less severe intestinal inflammations compared to wild type mice [93]. Interestingly, *Ccr6* knock-out mice treated with 2, 4, 6 trinitrobenzenesulphonic acid (TNBS) showed increased susceptibility to intestinal inflammation in otherwise genetically resistant strains [93,101,102]. *Ccr6* knock-out mice were used to demonstrate the role of CCR6 and its importance in many lung and gut disorders [75]. Studies using CCR6 deficient mice suggest that CCR6/CCL20 axis has an important role in the immune process leading to inflammatory bowel disease [93].

Wild Type Th17 cells or CCR6^{-/-} T cells when transferred into a Rag1^{-/-} severe combined immune-deficient mouse (SCID) resulted in severe intestinal inflammation [85,103]. Reduction in the population of Th17 and T regulatory cells was noted in this process [85,103]. According to these results Lee et al [103] suggests the function of CCR6 is more important to Treg cells than Th17 cells. However the relationship of this chemokine duo and the balance between Treg and Th17 largely relies on the selective skewing of CCR6-CCL20 ascertaining the prominence of either Treg or Th17 may be an essential factor in the process of intestinal inflammation.

3.4. Chemokine ligand CCL20

CCR6 uniquely interacts with the chemokine CCL20 also known as Exodus-1, macrophage inflammatory protein (MIP-3 α), liver and activation-regulated chemokine (LARC) [75,93,95,104]. Hieshima et al [105] first identified CCL20, a novel chemokine ligand expressed in the liver. The *CCL20* gene is located on chromosome 2q33-37 [105,106]. The chemokine ligand CCL20 is expressed in thymus, appendix, fetal liver and by peripheral blood lymphocytes [104]. Furthermore expression of CCL20 is also found in various types of epithelial cells such as pulmonary epithelial cells, keratinocytes and also in intestinal epithelial cells [75]. Follicle – associated epithelial cells (FAE) covering the Peyer's patches of the intestine and the isolated lymphoid follicles (ILFs) are involved in the making of CCL20 the only ligand for CCR6 [14]. CCL20 was reported to be expressed only in Th17 cells and it was not expressed by

either Treg cells or T helper subsets [90]. Up-regulation of CCL20 expression in intestinal epithelial cells can be seen in response to induction by invasive or non-invasive flagellated bacteria [107]. Furthermore, pro-inflammatory cytokines such as IL-1 α or tumor necrosis factor- α (TNF- α) produced during acute intestinal inflammation also stimulate and upregulate CCL20 expression [108].

3.5. The CCR6-CCL20 axis

The CCR6-CCL20 axis is an important factor in the intestinal immunity. Ito et al [75] describes the involvement of CCR6-CCL20 axis during the normal development of innate immunity and immune homeostasis. In intestinal immune responses, especially in the innate immune response where CCR6 mediated signals are important, the CCR6-CCL20 axis might have a very significant impact during tissue damage and injury [75]. CCR6 is suggested to be associated with regulation of leucocyte migration followed by effector function of T cells, are all considered as important factors in gut immunity [75,91]. Induction of CCL20 by bacterial lipopolysaccharide (LPS) endotoxin in response to infection occurring in intestinal epithelial cells has been demonstrated [109]. The CCR6-CCL20 axis also mediates the chemotaxis of dendritic cells and macrophages and recruits them to the site of inflammation [75].

Figure 2. CCR6 is the pivotal link between the anti-inflammatory and pro inflammatory cells.

Le Borgne et al [110] demonstrated a significant involvement of CCR6-CCL20 in the migration of dendritic cells from the blood to the lamina propria of the intestine during the inflammation process [110]. Th17 cells and Treg cells are present in large numbers in the lamina propria during normal and in inflammatory conditions suggesting that the axis is critical for the outcome of intestinal pathology [102]. The immune system importantly has to select to either suppress or initiate inflammation while maintaining immune self-tolerance. Maintaining the balance between inflammatory cells and T regulatory cells is vital for the outcome of intestinal pathologies [102]. The CCR6-CCL20 axis has been demonstrated to play an important role in many lung and gut disorders [75]. Furthermore

CCR6-CCL20 axis is of notable importance in cancer and autoimmune diseases [82,111]. The CCR6-CCL20 axis appears to hold promise as the new target for therapeutic interventions in many diseases including IBD [102].

4. Conclusion & Future Directions

Current conventional therapies involve the use of anti-inflammatory drugs; aminosalicylates and steroids. Immunomodulators such as azathioprine, 6-mercaptopurine, methotrexate or cyclosporine are also being used in patients who are resistant to or dependent on steroids. Biological therapies have gained importance in IBD therapy, and targeted therapies with anti-inflammatory cytokine-therapy like anti-TNF-alpha antibody, anti-IL-6R, and anti-IL-12 or toxin-conjugated anti IL-7R, recombinant cytokines (IL-10 or IL-11) are the recent advancements in the field of IBD therapy [28].

Research involving CCR6-CCL20 and their role in various autoimmune disorders are of particular interest to global scientific community. The CCR6-CCL20 axis needs to be further dissected as there is need to provide us with a clear understanding of its role with regards to inflammatory bowel disease. CCR6 appears to play a pivotal link between the anti-inflammatory and pro-inflammatory cells with the CCR6-CCL20 chemokine axis mediating the inflammatory pathway, and the factors leading up to triggering this duo to exacerbate inflammation or suppress the inflammatory environment and facilitate homeostasis need to be clearly identified. Future studies need to investigate the exact mechanisms of this axis, and if identified it would be an innovative breakthrough to solving the IBD puzzle.

Conflict of Interest

None to disclose.

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