Chemokines orchestrate leukocyte trafficking in inflammatory bowel disease

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1. ABSTRACT

Inflammatory Bowel Disease (IBD) is a chronic disorder characterized by recurrent and serious inflammation of the gastrointestinal tract. Genetic, immunologic and environmental factors all contribute to the pathogenesis of IBD. Crohn's disease and ulcerative colitis represent 2 common forms of IBD. Recent discovery of Crohn's disease-associated gene mutations suggests that compensation of disrupted innate immunity in IBD patients leads to abnormal T lymphocyte response to antigenic stimulation and subsequent inflammation by producing pro-inflammatory mediators including chemokines. Chemokines are a group of chemoattractant cytokines that exert double-edged effects on both host defense and inflammation. Chemokines have been shown to play an essential role in the recruitment of inflammatory cells. Leukocyte infiltration and increased production of certain chemokines are all observed in IBD. In this review, we discuss the current literature and present our recent studies on the role of different chemokines in the pathogenesis of IBD. Controlling the expression and neutralizing the function of chemokines are an approach that would allow the development of a novel treatment strategy with effective anti-inflammatory effect.

2. INFLAMMATORY BOWEL DISEASE

Crohn's disease and ulcerative colitis, collectively referred to as inflammatory bowel disease (IBD), are gastrointestinal disorders characterized by chronic and relapsing inflammation (1, 2). However, Crohn's disease and ulcerative colitis represent 2 classic types of IBD, and there are many overlapping subgroups within IBD (3). It is generally believed that Crohn's disease has a potential to affect any part of the gastrointestinal tract, whereas ulcerative colitis is largely confined to the large intestine. The common histological changes associated with IBD include inflammation and ulceration of the intestinal mucosa with leukocyte infiltration (4, 5). However, transmural inflammation and granuloma formation are the pathological hallmarks of Crohn's disease, whereas the intestinal mucosa layer is the target of ulcerative colitis. In addition to the distinctive histological features, the immunopathogenesis of these two diseases appears to be distinct as well. Crohn's disease involves a predominant Th1 response, while ulcerative colitis is thought to be mediated by the Th2 T-cell subpopulation. However, recent discovery of Th17 cells may alter the notion of this Th1 and Th2 dichotomy in IBD (6, 7). Th17 cells are a subset of T lymphocytes that

uniquely produce interleukin (IL)-17. They are highly pathogenic and implicated in a number of autoimmune diseases. Although the etiology of IBD is complex and multifactoral, cumulative evidence suggests that a defect of innate immune response to microbial agents is involved in IBD pathogenesis (1, 2). Recent genetic studies find that mutations in the NOD2 gene are associated with Crohn's disease (8, 9). The NOD2 gene encodes an intracellular receptor for a unique bacterial wall component called muramyl dipeptide, and plays a pivotal role in innate immunity. Thus, it is feasible to postulate that compensation of disrupted innate immunity in IBD patients leads to abnormal T lymphocyte response to antigenic stimulation and subsequent inflammation by producing pro-inflammatory mediators including chemokines.

Although glucocorticoid and T cell suppression are current standard treatment for IBD, severe side effects of these therapies have promoted active research of more specific immunomodulation. Recent development of infliximab, a chimeric monoclonal antibody against tumor necrosis factor-alpha (TNF-α), in Crohn's disease treatment is a successful example demonstrating the great promise of this cytokine-based immunomodulatory approach. In addition, inhibition of leukocyte trafficking has received a great deal of attention as a novel anti-inflammatory strategy for the treatment of IBD.

3. CHEMOKINES

A large number of immune cells traffic through the gastrointestinal tract daily at a steady but controllable level to screen, detect and interact with numerous gut microorganisms and antigens. This process is sometimes referred to as "physiological inflammation". However *in situ*ations such as IBD, the physiological balance is disrupted, and excessive inflammatory cells invade the gastrointestinal system, resulting in pathological inflammation and tissue destruction by releasing harmful cytokines and proteases (10). During both physiological and real inflammation, chemokines are essential for recruiting immune cells from the circulation system to local tissue. Furthermore, some chemokines can activate leukocytes, and cause degranulation as well as reactive oxygen species (11, 12), thereby playing a pivotal role in the inflammatory phase of IBD.

Chemokines are a family of small secreted glycoprotein with molecular weight of 7-10Kda (13), and demonstrate their important chemotactic activity during the inflammatory process. Chemokines are mainly produced by phagocytes, endothelial and epithelial cells. Currently, over 50 chemokines have been discovered. According to their structure and conservative amino acid sequence in the N terminal, chemokines are classified in 4 groups, namely C CC CXC and CX3C. CXC chemokines are further divided into ELR+ and ELR-CXC, depending on whether they possess Glu-Leu-Arg residue (14, 15). ELR residue is crucial for the chemotactic activity of CXC chemokines through interaction with CXC receptor (CXCR2) in the neutrophils. Thus ELR+CXC are neutrophil chemoattractant, while ELR-CXC primarily acts on monocytes, lymphocytes, and NK cells. C chemokines consist of XCL1 and XCL2 and they have a structural characteristic of composing only one conservative cysteine in their N terminal. CX3CL1, also called fractalkine, is the only CX3C chemokines known to date. It has 3 amino acids between 2 conservative N-terminal cysteines. In addition, CX3C is distinct from other chemokines because it can serve as a membrane-bound adhesion molecule (16, 17).

The production pattern of chemokines varies among different groups. Some are constitutively expressed. Others require induction. The constitutively secreted chemokines are implicated in directing baseline leukocyte trafficking and organizing lymphoid tissue formation. Nevertheless, inducible chemokines mainly attract immune cells to local sites and set a stage for inflammation (18). CC chemokines only bind CC receptors; whereas CXC chemokines interact with CXC receptors. However, most chemokine receptors bind more than one chemokine. Chemokine receptors have 7 transmembrane domains and couple with G protein eliciting downstream signal transduction. Recent studies show that certain chemokine receptors play a critical role in facilitating tissue specific homing of lymphocytes (19).

4. CHEMOKINES IN INFLAMMATORY BOWEL DISEASE

IBD is characterized by intestinal infiltration of dysregulated immune cells (20-22). This inflammatory cell infiltration is mainly caused by the elevation of various chemokines in the serum and intestinal mucosa of IBD patients (23-26). Cumulative study demonstrates that all 4 types of chemokines are involved in the development of IBD (Table 1).

4.1. CC chemokines

It has been shown that CCL2 expression is increased in the intestinal epithelial cells of IBD patients. Furthermore, the CCL2 level appears to be correlated with the severity of gut inflammation. Macrophages and endothelial cells in the mucosa and submucosa are main CCL2 producing sources (26-29). The levels of CCL3 and CCL4 are hardly detectable in the normal gastrointestinal tract. However, they are significantly elevated in active IBD (26, 30). CCL5, also called RANTES, is minimally expressed in an acute-inflamed site. Conversely, its production is markedly augmented in chronic inflammation (31). Recently, 2 groups investigated the expression of CCL5 and its receptor CCR5 in the intestinal biopsy specimens from patients with IBD (26, 27). Using immunohistochemistry, they found strong staining of these proteins in the granuloma of Crohn's disease. However, CCL5 expression is even higher in ulcerative colitis than in Crohn's disease (26). CCL5 is responsible for the recruitment of CD4+CD45RO+ memory T cells into the gut, leading to further activation of monocytes, macrophages, mast cells, eosinophils, and basophils. Therefore, CCL5 is indispensable for promoting and maintaining chronic inflammation (32-34). CCL8, produced by intestinal macrophages and epithelial cells, is

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Table 1. Summary of Chemokines Implicated in Inflammatory Bowel Disease

Chemokines	Alternative Names	Corresponding Receptors	Involvement in Inflammatory Bowel Disease	
			Crohn's disease	Ulcerative colitis
CC chemokine family				
CCL2	MCP-1	CCR2	Yes	Yes
CCL3	MIP-1a	CCR1, CCR5	Yes	Yes
CCL4	MIP-1b	CCR5	Yes	Yes
CCL5	RANTES	CCR1, CCR3, CCR5	Yes	Yes
CCL7	MCP-3	CCR1, CCR2, CCR3	Yes	Yes
CCL8	MCP-2	CCR3	Yes	Yes
CCL11	Eotaxin	CCR3	Yes	Yes
CCL17	TARC	CCR4	Yes	Yes
CCL18	DC-CK1	Unknown	Yes	Yes
CCL19	ELC	CCR7	Yes	Yes
CCL20	MIP-3a	CCR6	Yes	Yes
CCL21	SLC	CCR7	Yes	
CCL22	MDC	CCR4	Yes	Yes
CCL25	TECK	CCR9	Yes	
CCL28	MEC	CCR3, CCR10		
CXC chemokine family				
CXCL1	GROa	CXCR2	Yes	Yes
CXCL2	GROb	CXCR2	Yes	Yes
CXCL3	GROc	CXCR2	Yes	Yes
CXCL4	PF4	Unknown	Yes	
CXCL5	ENA-78	CXCR2	Yes	Yes
CXCL6	GCP-2	CXCR1, CXCR2	Yes	Yes
CXCL8	IL-8	CXCR1, CXCR2	Yes	Yes
CXCL9	MIG	CXCR3	Yes	Yes
CXCL10	IP-10	CXCR3	Yes	Yes
CXCL11	I-TAC	CXCR3	Yes	Yes
CX3C chemokine family				
CX3CL1	Fractalkine	CX3CR1	Yes	Yes
C chemokine family				
XCL1	Lymphotactin	XCR1	Yes	Unchanged

a major chemoattractant of neutrophils. Its level is also associated with the disease activity of IBD. Moreover, CCL8 level is significantly reduced in IBD patients after anti-inflammatory treatment (26, 35-37).

Recent studies discovered 3 intestinally specific CC chemokines, namely CCL20, CCL25, and CCL28 (39). CCL20 is produced in the epithelium of the appendix and colon, and its receptor CCR6 is predominantly distributed in the T cells including newly discovered CCR6 and monocytes in the colonic mucosa (38), especially near the crypts of the small intestine where immature T cell precursors reside (39). In addition, CCR6 plays an important role in the development of lymphoid tissues (40). The absence of CCR6 disrupts the formation of normal lymphoid structures. Thus, it is conceivable that inhibition of CCR6 may abrogate chronic intestinal inflammation through suppression of gut-associated lymphoid tissue. CCL20 is responsible for the chemotaxis of immature dendritic cells expressing CCR6 receptor in the intestinal epithelium and Peyer's patch (40). Interaction between CCL20 and CCR6 mediates the chemoattraction of effector/memory T cells and B cells in several disease states including cancer and rheumatoid arthritis (38).

Microarray and real-time PCR analysis show an increased level of CCL20 transcription in colonic specimens of IBD patients (41, 42). Choi et al. also demonstrated that the CCL20 is upregulated in ulcerative colitis (43). Furthermore, CCL20 level is proportional to the disease activity. Both 5-aminosalicylic acid and glucocorticoid dramatically inhibit CCL20 transcription in these patients (19, 44, 45). In contrast to CCL20, CCL25 is mainly expressed in the crypt and vascular endothelium of the small intestine. CCL25 is selectively expressed in the thymus and small intestine but not in the colon (42, 43). The corresponding receptor of CCL25 is CCR9. Ninety percent of the small intestinal lymphocytes express surface CCR9. CCR9 knockout mice display abnormal T and B cell development. Thymocytes from CCR9 knockout mice are unable to respond to CCL25, and these mice have fewer B cell precursors. In addition, they have less intraepithelial γδ+ T cells in the small intestine (46-48). Expression of CCR9 and integrin α4β7 on the cell surface provides the small intestinal with a homing signal for circulating gut specific T cells (46-48). It is evident that CCR9+ T lymphocytes are markedly elevated in Crohn's disease patients with small bowel involvement but not in patients with colonic Crohn's disease (49). Stimulation of CCR9+ T cells from Crohn's disease patients leads to greater production of inflammatory cytokines such as IL-17, indicating that CCR9+ T cells in Crohn's disease are pathogenic (48). Similar to CCL20, CCL28 is mostly present in the colonic epithelial cells but not in the small intestine (50, 51), and is found to interact with CCR10 on memory T cells and CCR3 on eosinophils (50). Hence, it is feasible to postulate that CCL25 contributes to the small intestine inflammation of Crohn's disease, and that CCL20 and CCL28 are implicated in IBD colitis.

Other 2 CC chemokines related to IBD are CCL19 and CCL21. These 2 chemokines bind CCR7 and play a role in the development of Peyers patch and lymph nodes. Recent studies report that CCL19 and CCL21 are elevated in a number of chronic inflammatory diseases. In
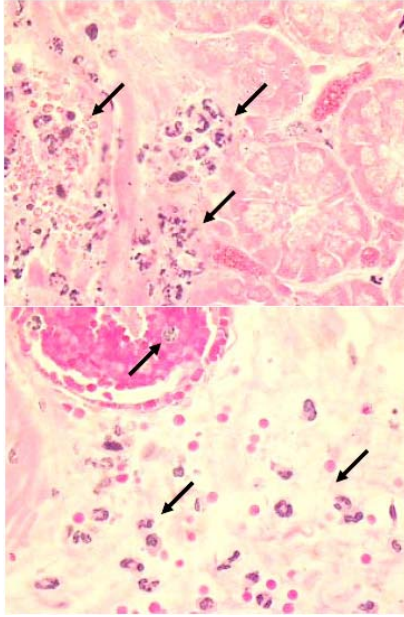
Figure 1. Representative histology of inflamed mouse colon at 24 hours after TNBS challenge. Notice marked neutrophil-predominant leukocyte migration and infiltration.

the mesenteric lymph nodes of patients with Crohn's disease, the levels of these chemokines are significantly higher, suggesting their pathogenic role in IBD and possibility as a potential therapeutic target (16, 52, 53).

4.2. CXC chemokines

Many ELR+CXC chemokines are implicated in IBD. For instance, CXCL1 CXCL2 CXCL5 CXCL6 and CXCL8 are markedly induced in the diseased areas of IBD compared to in normal tissues (26, 54-59), indicating that these chemokines actively participate in the inflammatory process. During the disease state, CXCL8 positive cells congregate in the lamina propria. However, the distribution of these cells is different between Crohn's disease and ulcerative colitis. CXCL8 is widely distributed in ulcerative colitis, whereas its expression is patchy and located in isolated areas in Crohn's disease (60, 61). Furthermore, the increase of CXCL8 level in the colonic mucosa is significantly associated with the exacerbation of the disease (59). This immunopathological finding is consistent with clinical and endoscopic observations that Crohn's disease often presents with skipped lesions, and that ulcerative colitis has more diffuse tissue inflammation.

Immunohistochemistry studies reveal that epithelial cells in the crypt and immune cells in the lamina propria are primary sources of CXCL5 production. Although both CXCL5 and CXCL8 are neutrophil chemoattractants, these two chemokines have different kinetic expression profiles. Upon the stimulation of inflammatory cytokines such as IL-1β and TNF-α, CXCL8 production peaks approximately 10 hours earlier than CXCL5 (60). This suggests that CXCL5 and CXCL8 compensate each other in the recruitment of neutrophils

throughout the entire inflammatory reaction. Recently, the presence of CXCL6 is also observed in the base of ulcers in IBD (55). Taken together, these results support the role of ELR+CXC chemokines in IBD.

CXCL9, CXCL10, and CXCL11 are ELR-chemokines. Similar to ELR+ chemokines, they are increased in the patients with IBD. CXCL10 is secreted by several cell types including monocytes and endothelial cells (54, 62, 63). It facilitates the adhesion of T cells and monocytes to the vasculature. Furthermore, anti-CXCL10 antibody blocks the ulceration in the mouse IBD model, and recombinant CXCL10 protein inhibits epithelial proliferation (64). Therefore, CXCL10 and other ELR-CXC chemokines have become an active research focus in understanding their detrimental roles in gastrointestinal inflammation.

4.3. CX3C and C chemokines

Epithelium-derived chemokines are essential for the chemotaxis of immune cells in the gastrointestinal mucosa. CX3CL1 is produced by epithelial cells. In addition to attract CX3CR+ T cells and monocytes, CX3CL1 uniquely recruits dendritic cells into the lamina propria (65, 66). CX3CL1 further mediates the interaction between intestinal epithelial cells and dendritic cells in the process of antigen presentation and immune cross-talking (66, 68). Especially, intestinal CX3CR1+ dendritic cells in the ileum show the ability to sample and uptake luminal bacteria. CX3CR1 deficiency impairs the dendritic cells to process invasive pathogens (68). Results show production of CX3CL1 along with CCL8 is significantly enhanced in the lamina propria of Crohn's disease patient, and CX3CR1+ cells are increased in active IBD compared to inactive IBD or healthy controls (69). The expression of CX3CL1 is enhanced by inflammatory cytokines in Crohn's disease. A CX3CR1 T280M polymorphism is correlated with fibrostenosing phenotype of CD (70). Similarly, C chemokine XCL1 expression is augmented in activated CD8+ T cells in Crohn's disease. However, it is unclear whether XCL1 is involved in ulcerative colitis.

4.4. Chemokines in animal models of colitis

Consistent with human studies, similar profiles of chemokine alteration are observed in rodents developing inflammatory colitis (71, 72). These animal models allow us to further define the role of chemokines in IBD and develop potential therapeutic strategies. Using a trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse model, we intended to mimic the situation of colonic inflammation seen in IBD patients (73). Six week-old C57BL/6J mice were intrarectally administered with TNBS. For comparison purposes, control mice received vehicle. After exposure to TNBS, the mice manifested bloody diarrhea, then the colons were processed for histology, tissue myeloperoxidase (MPO) assay, and chemokine analysis.

As illustrated in Figure 1, severe colitis was observed in TNBS-treated mice, characterized by marked infiltration of inflammatory cells mainly neutrophils (arrows).
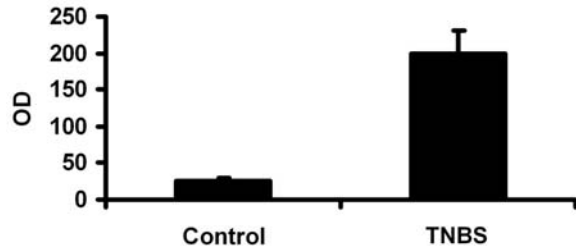
Figure 2. Significant increase of neutrophil invasion in the colonic tissues of TNBS-treated mice. The mice were intrarectally challenged with TNBS. Twenty-four hours later, the colons from both control and treated mice were collected and the tissue homogenates were prepared for myeloperoxidase (MPO) assay. Results represent the mean $\pm$ SEM of 6 mice.
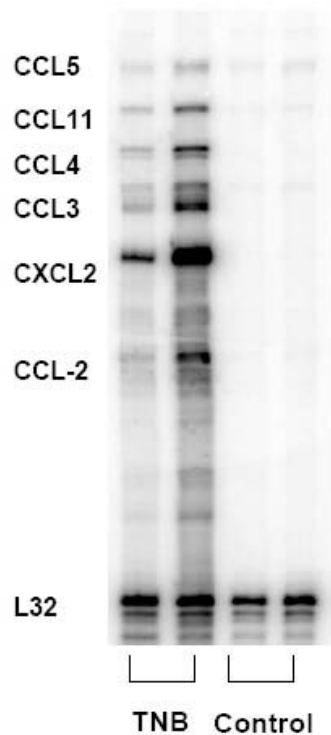

Figure 3. Marked induction of chemokines in the colonic tissues of TNBS-treated mice. The mice were treated with TNBS intrarectally. Twenty-four hours later, the colons from both control and treated mice were collected and mRNA for RNase Protection assay. Note: Housekeeping gene (Ribosomal protein L32).

Since MPO is restricted to neutrophils and some monocytic cells, we assessed the level of MPO activity in the colonic tissue to further reflect the degree of neutrophil/monocyte influx during the inflammatory process. As demonstrated in Figure 2, compared to the control group, a greater than 8-fold increase in MPO activity was observed in the TNBS-treated mice. Thus, these histological and biochemical changes indicate that leukocyte infiltration is a predominant feature of IBD and experimental colitis.

In order to investigate the role of chemokines in inflammatory colitis, we examined whether TNBS treatment caused an increase in chemokine expression. The colon was dissected and the mucosa was harvested by scraping the surface of colonic tissue. Total RNA was isolated from the mucosal tissue using Trizol reagent, and the transcription of several chemokines was examined by RNase Protection Assay (RPA). Compared to control mice receiving vehicle alone (Figure 3), TNBS treatment significantly increased the expression of both CC and CXC chemokines. These chemokines exert inflammatory activity through their transmembrane-spanning receptors (74, 75). Recently, targeted deletion of chemokine receptors has provided pivotal information on the role of specific ligands and receptors in many disease models. Deletion of the CXCL8 receptor, leads to reduced neutrophilic infiltration (76), whereas deletion of CCR2 or CCR5 protects mice from dextran sulfate sodium-induced colitis (77). Intestinal inflammatory diseases have been reproduced in several gene-knockout models greatly enhancing our understanding of immuno-pathophysiology (78, 79).

5. CHEMOKINE-TARGETING THERAPY

5.1. Chemokine antagonists

Given the importance of chemokines in inflammation, basic research and clinical trial are underway to modulate chemokine production and activity in order to attenuate inflammatory responses. Neutralizing chemokines and antagonizing chemokine receptors potentially prevent leukocyte infiltration and attenuate inflammatory responses.

Recently, numerous studies have been conducted to prove the concept of chemokine and chemokine receptor-targeting therapy. Since CCR1 is expressed on neutrophils and monocytes during inflammatory response, nonpeptide CCR1 receptor antagonists BX471 and J-113863 were tested in various animal disease models, and found to mitigate sepsis, pancreatitis-associated lung injury, renal graft injury, and arthritis (80-82). Likewise, CXCR2 and its corresponding chemokines play a key role in acute inflammation. Thus, the effect of Antileukinate, a CXCR2 antagonist, was assessed in a mouse pancreatitis model. This peptide protected mice against acute pancreatitis (83). As discussed previously, CCR9 uniquely mediates lymphocyte homing to the small intestine. Therefore, CCR9 has been considered as a potential therapeutic target for the treatment of Crohn's disease. Neutralization of CCR9 and its ligand CCL25 are shown to ameliorate rodent ileitis (84, 85). These preclinical studies have laid the foundation for clinical trials in evaluating the efficacy of anti-chemokine/chemokine receptor therapy.

A randomized anti-CCR1 trial was first carried out in 16 patients with active rheumatoid arthritis. The patients received CCR1 antagonist (MRA) or placebo for 14 days. The clinical improvement with the CCR1 antagonist was higher than placebo. Overall cell counts including CD4+ and CD8+ lymphocytes were significantly decreased in the synovium. Severe adverse events were not
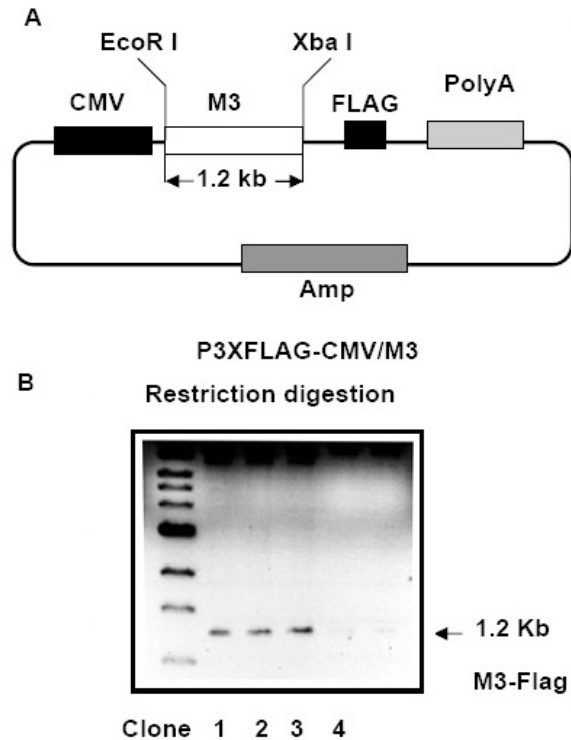
A

Figure 4. Generation of Plasmid Vector Encoding cDNA of M3-FLAG fusion protein. (A) Schematic of M3- FLAG fusion construct. Its expression is driven by cytomegalovirus (CMV) promoter. (B) Representative gel imaging showing restriction digestion-proven colonies positive for M3-FLAG construct.

reported in the trial group; thus MRA treatment was generally well tolerated (85). Although this study demonstrates the potential of anti-chemokine therapy for inflammatory diseases in humans, it remains an interest to further investigate their efficacy and safety in IBD.

5.2. Inhibition of other adhesion molecules involved in inflammatory cell trafficking

Similar to chemokines and chemokine receptors, many integrins and adhesion molecules play an important role in inflammatory cell trafficking in IBD (86, 87).

Lymphocytes expressing α4β7 integrin specifically recognize mucosal addressing cell adhesion molecule 1 (MAdCAM-1). The interaction between α4β7-integrin and MAdCAM-1 is important in mediating lymphocyte homing to the intestinal mucosa (88). Natalizumab is a humanized IgG4 anti-α4-integrin monoclonal antibody, inhibits α4β7-integrin/MAdCAM-1 binding. In 2 initial placebo-controlled randomized trials, 40% patients with moderate to severe Crohn's disease appeared to respond to the treatment and went to remission, while only 8% control patients were in remission by the end of the study (89, 90). However, a recent large phase 3 trial shows that Natalizumab is more efficacious to maintain disease remission or control active Crohn's disease in conjunction with other immunosuppressants (91,

92). Since Natalizumab inhibits the adherence of inflammatory lymphocytes to gut mucosa, it can prevent the reoccurrence of Crohn's disease by blocking pathogenic leukocyte infiltration. Nevertheless, it does not affect the cells that have already homed in the local tissue. Thus, this antibody alone may not be able to restrain the ongoing inflammation unless used in conjunction with other immunosuppressants. Natalizumab is also investigated to treat multiple sclerosis (93). However, it is very alarming that 3 patients receiving Natalizumab developed a rare yet devastating condition called multifocal leukoencephalopathy (94-96). This raises the concern of anti-α4 integrin therapy-related risks, and highlights the need of developing new immune modulators with more effective therapeutic effect and better safety profile. However, the therapeutic effect of anti-integrin therapy is encouraging. It further supports the feasibility of chemokine-targeting treatment because both approaches share the similar therapeutic mechanism of regulating inflammatory cell trafficking.

5.3. New generation of potential chemokine inhibitors

Inflammation is a bustling and intricate process, requiring coordination of multiple mediators including chemokines. As discussed previously, many chemokines have overlapping activity. However, each one emerges at a different stage during inflammation to exert its unique function. Thus, a broad-spectrum chemokine inhibitor would be ideal to achieve broader anti-inflammatory effects. Have that said, Herpesviruses have naturally evolved strategies to counteract host chemokine systems by encoding chemokine-binding proteins. A secreted protein called M3 was identified from murine gammaherpesvirus 68 (97). This protein is encoded by the gammaherpesvirus 68 M3 open reading frame (ORF). M3 ORF encodes a secreted protein that interacts with host cytokines. Although M3 protein lacks homology to currently known chemokine receptors, it exhibits inhibitory activity for a broad spectrum of chemokines, including CXCL10 and CCL5 (97, 99). M3 protein binds CC and CXC chemokines with high affinity, and blocks chemokine effects by abolishing calcium signaling. Therefore, this protein is of great interest for its potential therapeutic applications.

Recently, we have generated a M3 expression construct. Gamma herpesvirus 68 (GHS 68) and baby hamster kidney (BHK)-21 cells were obtained from ATCC (Mannassas, VA). GHS 68 virus was grown in BHK-21 cells. Then the viral genomic DNA was extracted from cell supernatants and served as a template for amplification of M3 by polymerase chain reaction (PCR). M3 gene, with an EcoR I site at the 5' end and a Xba I site at the 3' end, was amplified with Taq polymerase (Perkin-Elmer, Foster City, CA) using two primers (5'-AAG CTT GAA TTC ACC ACT ATG GCC TTC TA TCC ACA TCT GTG-3' and 5'-TGA CTA TCT AGA ATG ATC CCC AAA ATA CTC CAG CCT-3'). The PCR fragment was cloned into pCR2.1 (Invitrogen, Carisbad, CA) for propagation. After EcoR I/Xba I digestion, the 1.2–kbp product was ligated into p3XFLAG-CMV-14 expression vector (Sigma-Aldrich, St. Louis, MO) (Figure 4).
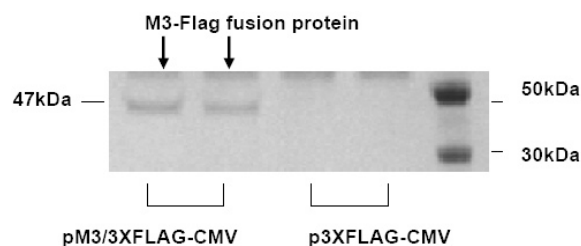
Figure 5. Expression of M3-FLAG fusion protein. The recombinant protein was produced in NIH 3T3 cells after transient transfection of the CMV promoter-driven plasmid carrying M3-FLAG construct. The M3-FLAG protein was then immunoprecipitated via exploiting FLAG epitope from the supernatants of transfected cells, and further identified by Western blot analysis using an anti-FLAG antibody.
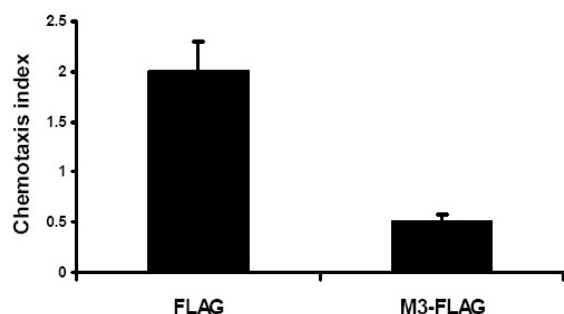

Figure 6. Inhibition of Chemotaxis by M3-FLAG Protein. Mouse splenocyte migration was measured *in vitro* in the presence or absence of recombinant M3-FLAG fusion protein. M3 significantly inhibited CXCL10-induced chemotaxis.

The recombinant protein was produced in NIH 3T3 cells transiently transfected with the CMV promoter-driven M3-FLAG construct using a lipofectamine based on manufacturer's protocol (Gibco BRL, Gaithersburg, MD). The 47-kDa M3 protein was then immunoprecipitated via exploiting FLAG epitope from the supernatants of transfected cells, and further identified by Western blot analysis using an anti-FLAG antibody. Briefly, media from transfected cells were collected. Thirty μg of total protein from each sample were electrophoresized through a 4-12% gradient Tris-glycine SDS gel and then transferred to nitrocellulose membrane using Xcell SureLock mini cell (Novex, San Diego, CA). After milk blocking, the nitrocellulose membrane was incubated with primary antibody against FLAG (Sigma-Aldrich, St. Louis, MO), followed by HRP-conjugated anti-primary antibody immunoglobulins. The signals of FLAG fusion protein were detected by enhanced chemiluminescence reagent. As illustrated in Figure 5, the cells transfected with M3-FLAG construct produced a FLAG positive band, showing the typical profile of M3 protein at 47 kDa. This result indicates that we have successful generated M3-FLAG fusion protein.

We further tested the biological activity of M3-FLAG fusion protein using a 96-well microchemotaxis assay. Briefly, mouse splenocytes were harvested and resuspended in RPMI 1640 media with 10% FBS. These splenocytes were incubated with 400 U/ml of recombinant IL-2 for 3 days. After IL-2 stimulation, the splenocytes were stained with Calcein AM. Approximately 5,000 labeled splenocytes in 50 μl were applied on the top of chemotaxis filter (6.4-mm diameter; 5-μm ore size) (Neuroprobe Inc, Gaithersburg, MD). Recombinant CXCL10 (100 ng/ml) was placed underneath the chemotaxis filter after being reconstituted in the conditioned medium of the cells transfected with M3-FLAG construct or p3XFLAG-CMV-14 vector as a control. Following a 2-hour incubation at 37^0C and 5% CO_2, cell migration was quantified using a fluorometer (Excitation: 488 nm / Emission: 530 nm). Numbers of migrating cells extrapolated from standard curves. A chemotaxis index was used to reflect the degree of cell migration. It is calculated by dividing the number of cells that migrated in response to CXCL10 by the number of cells that migrated in response to chemotaxis buffer alone.

As shown in Figure 6, the M3-FLAG fusion protein significantly inhibited CXCL10 (a potent CXC chemokine)-induced chemotaxis of mouse splenocytes. Thus, M3-FLAG fusion protein appears to be a potential therapeutic agent to treat inflammatory diseases associated with chemokines, including IBD.

6. SUMMARY

Chemokines, a group of pro-inflammatory small peptides, are involved in recruitment of leukocytes to local tissue. Chemokines comprise over 50 ligands that interact with approximately 2 dozens of receptors. They act in a coordinated manner to recruit and activate leukocytes to sites of infection and inflammation. Chemokines have been subdivided into 4 families (CXC, CC, C and CX_3C) based on the position and number of conserved cysteine as well as the presence of intervening amino acid(s) between the first two conserved cysteine residues. Both CXC and CC chemokines have been extensively characterized. CXC chemokines are mainly chemotactic for neutrophils, whereas CC chemokines activate and recruit NK cells, monocytes, and lymphocytes. Therefore, chemokines play an important role in determining the pathogenic sequence of specific leukocyte infiltration in various inflammatory conditions including IBD. Recently, the role of chemokines has been investigated in the pathogenesis of IBD, and chemokines are elevated in serum and intestinal tissue of IBD patients with acute exacerbation. In IBD, chemokine expression is localized in the areas of intestinal inflammation. Mucosal chemokine levels correlate with the severity of intestinal inflammation. By understanding the role of chemokine in IBD pathogenesis, it opens an avenue for developing novel therapeutic approaches for treating IBD (e.g. prevention and amelioration of intestinal inflammation may be achieved by chemokine-neutralizing therapies). Thus, this endeavor will hopefully lead to the transition from basic scientific findings to discovery of practical and clinical applications. In addition, it will help advance immunotherapy of IBD.

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