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ARTICLE IN PRESS
JPT-06509; No of Pages 15
Pharmacology & Therapeutics xxx (2012) xxx–xxx
Contents lists available at SciVerse ScienceDirect
Pharmacology & Therapeutics
journal homepage: www.elsevier.com/locate/pharmthera

Associate Editor: Ikuko Kimura

Immune aspects of the pathogenesis of inflammatory bowel disease

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ARTICLE INFO

Keywords:
Susceptibility gene
Autophagy
ER stress
Micobiota
Cytokines
Innate and acquired immunity
Molecular targeting therapy

ABSTRACT

Although the precise etiologies of inflammatory bowel disease (IBD) (ulcerative colitis and Crohn's disease) remain obscure, several reports have indicated that dysfunction of the mucosal immune system plays an important role in its pathogenesis. Recent progress with genome-wide association studies has identified many IBD susceptibility genes. In individuals with genetic risk, abnormal interactions between the host immune system and gut flora, and dysregulation of cellular responses such as autophagy and ER stress, induce an abnormal host immune response in the gut resulting in intestinal inflammation. Research progress animal models in IBD, and in human IBD, has identified several key molecules in IBD pathogenesis such as TNFα and adhesion molecules, and molecular targeting therapies based on these molecules have been developed. Here, we review immunological aspects in IBD pathogenesis and the development of immunoregulatory therapy.

Contents

1. Introduction

Inflammatory bowel disease (IBD) is classified according to two typical phenotypes, namely ulcerative colitis and Crohn's disease. Although the precise etiology of IBD remains obscure, several reports have indicated that dysfunction of the mucosal immune system plays an important role in its pathogenesis (Xavier & Podolsky, 2007). The gastrointestinal tract is continuously exposed to a variety of antigens including enteric bacteria and foods. However, homeostasis of the gut is maintained in the normal state, i.e. without the development of intestinal inflammation, by suppressing excessive immune responses to foreign antigens. In both innate and acquired immunity, the disruption of regulatory mechanisms may lead to abnormal immune responses to enteric antigens and cause chronic intestinal inflammation. Here, we review the immunological aspects of the pathogenesis of IBD.

2. Discovery of susceptibility genes indicates immunological dysregulation in IBD

It has been well established that host genetic susceptibility plays a key role in the risk of development of IBD. For example, the risk of development of IBD in homozygous twins is higher than the risk in the general population. In 2001, nucleotide oligomerization domain receptor (NOD) 2, also known as CARD15, an intracellular pathogen recognition molecule, was identified as a susceptibility gene for Crohn's disease by linkage analysis (Hugot et al., 2001; Ogura et al., 2001). Since the discovery of NOD2, innate immune response has been highlighted in the research of IBD pathogenesis. More recently, genome-wide association studies (GWAS) have enabled the identification of approximately 99 single nucleotide polymorphisms that confer risk for either ulcerative colitis or Crohn's disease. The observation that several risk loci are shared between ulcerative

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http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

colitis and Crohn's disease (Franke et al., 2010; Anderson et al., 2011) suggests that there is some overlap of disease pathogenesis. Importantly, many of these recently identified IBD susceptibility genes are associated with host immune function including epithelial barrier function, host defense mechanisms in response to pathogens, and host immune responses (Fig. 1). For example, *NOD2* (Hugot et al., 2001; Ogura et al., 2001), *CARD9* (Zhernakova et al., 2008)and *ITLN1* (Barrett et al., 2008) in the innate immune response; *IL-23R* (Barrett et al., 2008), *STAT3* (Barrett et al., 2008), and *TNFSF15* (Yamazaki et al., 2005) in the IL-23-Th17 pathway; *ATG16L1* (Hampe et al., 2007; Prescott et al., 2007) and *IRGM* (Parkes et al., 2007) in autophagy; *XBP-1* (Kaser et al., 2008) and *ORMDL3* (McGovern et al., 2010) in ER stress; and protein–tyrosine phosphatase, non-receptor type*(PTPN)2* (Wellcome Trust Case Control Consortium, 2007; Wiede et al., 2011) in T cell response, have been identified. Research into IBD susceptibility genes has also shown differences in genetic risk between several human races. In Japanese IBD patients, many of the IBD susceptibility genes identified in other populations (including *NOD2*, *IL-23R*, and *ATG16L1*) did not show any association (Inoue et al., 2002; Yamazaki et al., 2007). *TNFSF15*, originally identified as a Crohn's disease susceptibility gene in a Japanese population, showed a lesser association in one European population (Picornell et al., 2007; Tremelling et al., 2008), but it showed a definite association in another European cohort (Thiebaut et al., 2009).

Thus, many susceptibility genes and loci have been identified by GWAS; however, the functional roles of these genetic loci are not fully understood. Glocker et al. identified children with mutations in the interleukin-10 receptor (*IL-10R*) (Glocker et al., 2009) and *IL-10*genes (Glocker et al., 2010). Kotlarz et al. (2012) identified loss of function mutations in *IL-10* and *IL-10R* in patients with very early onset IBD. These findings indicate that infant IBD patients with perianal disease should be screened forIL-10 and IL-10R deficiency.

3. Cellular responses — autophagy and ER stress

Recent advances in GWAS have identified several IBD susceptibility genes which are associated with cellular responses such as autophagy and endoplasmic reticulum (ER) stress. These cellular responses play a homeostatic role in epithelial barrier function and host immune responses.

3.1. Autophagy

Autophagy-related gene 16-like1 (*ATG16L1*) and *IRGM*are two genes recently identified by GWAS as Crohn's disease susceptibility genes, both of which are associated with "autophagy" (Hampe et al., 2007; Parkes et al., 2007; Prescott et al., 2007; Rioux et al., 2007). Autophagy is one mechanism for maintaining cellular homeostasis, and means "to eat oneself" or "self-cannibalization." While apoptosis is a cell death pathway, autophagy is involved in recycling cellular organelles for cell survival. Autophagy is now also considered to be important for host defense against intracellular microorganisms. The *ATG16L1* gene is located in chromosome 2q37.1 and encodes a protein that is known to mediate resistance against intracellular microorganisms, such as bacteria, and viral particles. Interestingly, *ATG16L1* did not show a positive association with Crohn's disease in a Japanese population, similar to the case for *NOD2/CARD15* studies (Inoue et al., 2002; Yamazaki et al., 2007). The association of these autophagy genes with Crohn's disease strongly supports the hypothesis that abnormal function in elimination and innate immune responses to intracellular pathogens contributes to the pathogenesis of Crohn's disease. In contrast to a report by Rioux et al. (Hampe et al., 2007; Parkes et al., 2007; Prescott et al., 2007; Rioux et al., 2007), Fujita et al. reported that in *Atg16L1*-deficient mouse embryonic fibroblasts with a stably expressed WD repeat domain mutant of *Atg16L1*, an *Atg16L1* WD repeat domain deletion and the T300A mutant have little impact on *Salmonella* infection (Fujita et al., 2009).

Another possible pathogenic contribution of a mutation of *ATG16L1* is associated with Paneth cell function and differentiation. Cadwell K et al. demonstrated that Paneth cells of *Atg16L1*-deficient (*Atg16L1^{HM}*) mice exhibit notable abnormalities in the granule exocytosis pathway. Importantly, Crohn's disease patients homozygous for the *ATG16L1* Crohn's disease risk allele displayed Paneth cell granule abnormalities similar to those seen in this mouse model (Cadwell et al., 2008). An

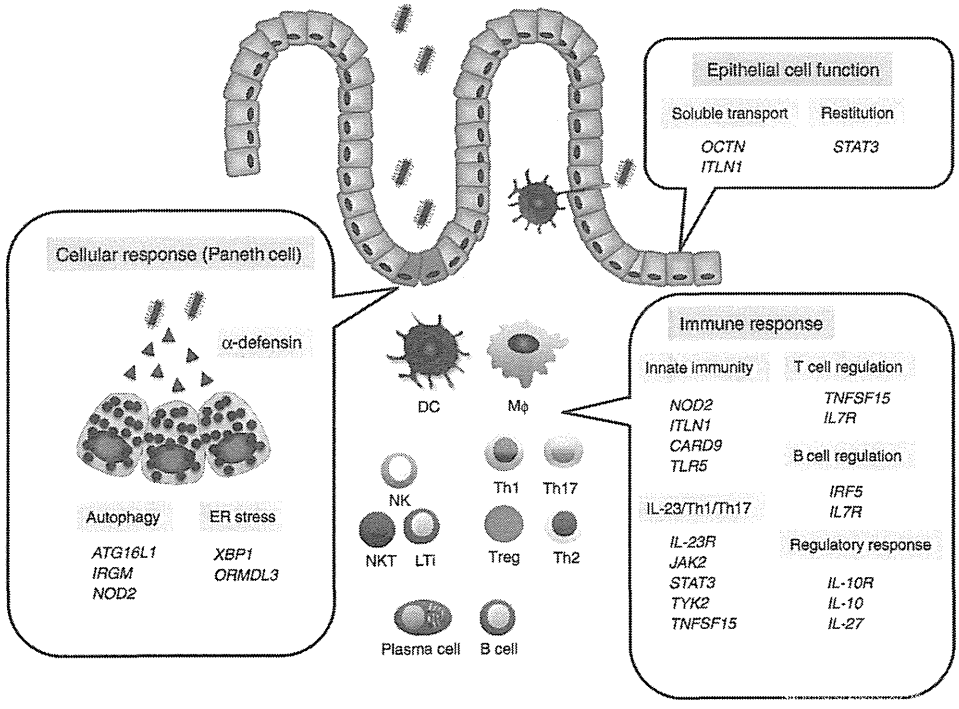

Fig. 1. Susceptibility genes in the immunopathogenesis of IBD. Recently identified IBD susceptibility genes suggest disruption of immunological regulation in IBD pathogenesis including epithelial barrier function, immune response (e.g. innate immunity, IL-23/Th17 pathway), and cellular responses (e.g. autophagy, ER stress).

Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

interaction between a specific strain of an enteric virus, murine norovirus (MNV) and *ATG16L1* mutation has been reported (Cadwell et al., 2010). MNV infection caused morphological and granule packaging abnormalities in Paneth cells of *Atg16L1*^{HM} mice, but not in those of wild type mice. Interestingly, in *Atg16L1*^{HM} mice infected with MNV, DSS treatment exhibited multiple histopathological characteristics of human Crohn's disease including muscular layer inflammation, mesenteric fat and blood vessels, lymphoid aggregates, and subserosal fibrosis.

Analysis using intestinal biopsies from patients with pediatric Crohn's disease showed that autophagy is specifically activated in Paneth cells, that this activation is associated with a significant decrease in the number of secretory granules and with features of crinophagy, and that it occurs independently of *ATG16L1* or *IRGM* variants, which are associated with Crohn's disease (Thachil et al., 2012). Plantinga et al. analyzed the response to NOD2 ligands in peripheral blood mononuclear cells (PBMCs) from healthy individuals and patients with Crohn's disease with different *ATG16L1* genotypes. PBMCs from individuals with the *ATG16L1* Thr300Ala risk variant displayed increased production of the pro-inflammatory cytokines IL-1β and IL-6, after stimulation with NOD2 ligands compared with other genotypes (Plantinga et al., 2011). Collectively, ATG16L1 plays several important roles in autophagy, innate host defense by Paneth cells, and regulation of proinflammatory response (Fig. 2). Mutation of *ATG16L1* may lead to abnormal cellular responses in Crohn's disease.

3.2. ER stress

The unfolded protein response (UPR) has been identified as a critical pathway in the maintenance of cellular homeostasis. *De novo* protein synthesis occurs in the ER, and the folded proteins are then transported to Golgi apparatus. However, in the case of unfolded or misfolded proteins, these are stored in the ER. Excess accumulation of these structurally abnormal proteins will cause ER stress, and finally induces apoptotic cell death and may cause several inflammatory and degenerative diseases. The UPR is important in a variety of cell phenotypes, especially in secretory cells such as goblet cells and Paneth cells in the intestine. The UPR is initiated by binding of unfolded proteins with glucose-regulated protein 78 (GRP78) in the ER. Subsequent recognition of these unfolded proteins leads to homeostatic cellular responses such as ER-associated degradation and translational responses through the pancreatic endoplasmic reticulum kinase (PERK)-elongation initiation factor 2α (eIF2α) pathway and the inositol-requiring enzyme 1(IRE-1)-X-box binding protein 1 (XBP1) pathway (Walter & Ron, 2011) (Fig. 3).GWAS have identified the association of several ER stress associated genes with IBD susceptibility (Barrett et al., 2008; Kaser et al., 2008; McGovern et al., 2010). An intestinal epithelial cell (IEC)-specific isoform of *Ire1* deficient (*Ire1β*$^{-/-}$) mice showed increased susceptibility to dextran sodium sulfate (DSS)-induced colitis (Bertolotti et al., 2001), suggesting dysregulation of UPR in IECs may become a trigger for chronic intestinal inflammation. Subsequent to the report of *Ire1β*$^{-/-}$ mice, Kaser et al. (2008) demonstrated that mice with an IEC-specific deletion of *Xbp1* (*Xbp1*$^{Vil\text{-}fl/fl}$ mice) developed spontaneous small intestinal inflammation. Furthermore, in *Xbp1*$^{Vil\text{-}fl/fl}$ mice, Paneth cells and goblet cells (which are typical secretory epithelial cell phenotypes) showed numerically, structurally and functionally abnormal development. *Xbp1*$^{Vil\text{-}fl/fl}$ mice exhibited increased susceptibility to *Listeria monocytogenes* infection, as did *Nod2*$^{-/-}$ mice (Kobayashi et al., 2005).

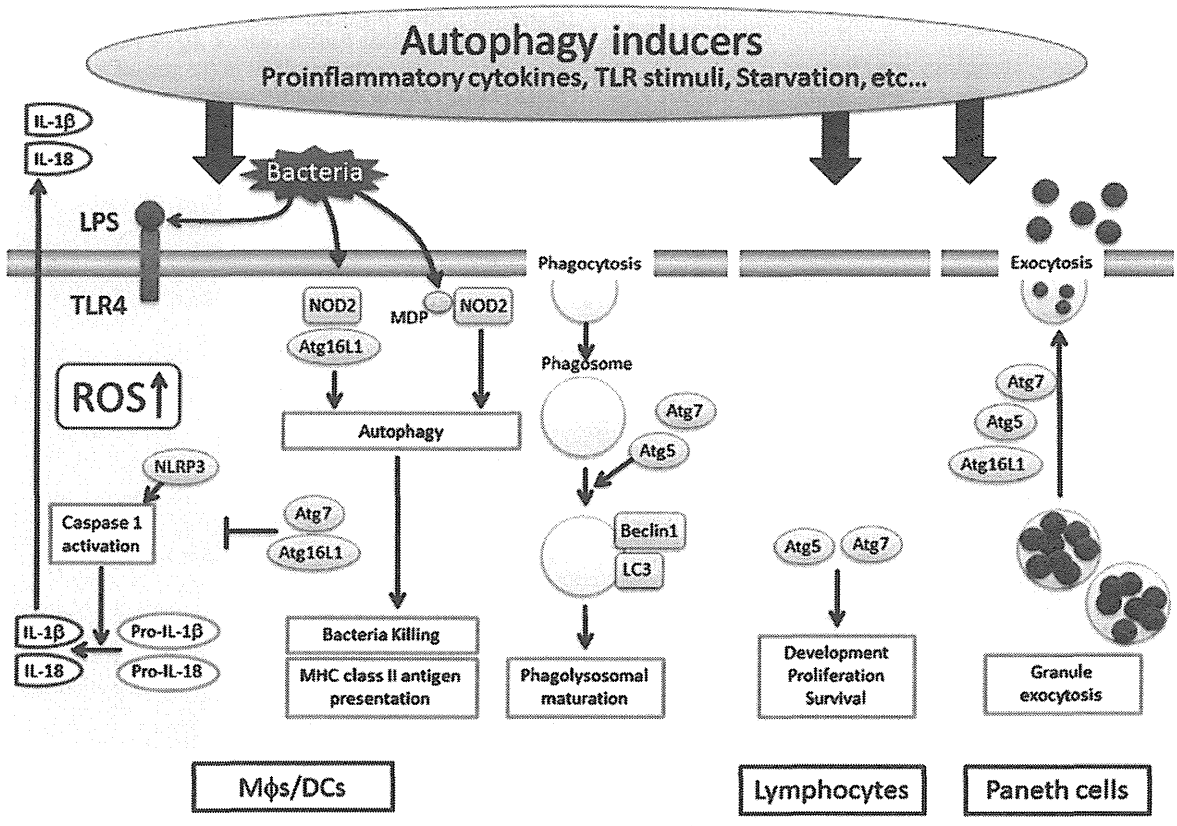

Fig. 2. Contribution of ATG16L1 in cellular responses. Autophagy inducers (e.g. proinflammatory cytokines, TLR stimuli, starvation) induce not only autophagy, but also several cellular responses. LPS/TLR4 signal promotes cleavage of pro-IL-1β and pro IL-18 and induce the production of IL-1β and IL-18 in the macrophages and DCs. In another pathway, NOD2 recognizes bacterial muramyl-dipeptide (MDP) and recruits ATG16L1. Subsequently, autophagy is induced resulting in bactericidal effects and presentation of endogenous antigens to MHC class II. These processes are impaired in patients with mutation in NOD2 or Atg16L1. Dysregulated autophagy caused by the mutation in also affects the excessive production of proinflammatory cytokines, such as IL-1β and IL-18. Autophagy genes, Atg5 and Atg7, promote autophagosomal formation and maturation. Atg5 and Atg7 contribute to the development, proliferation and survival in lymphocytes, and promote granuleexocytosis together in Paneth cells.ATG16L1 is also involved in this process in the Paneth cells.

Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

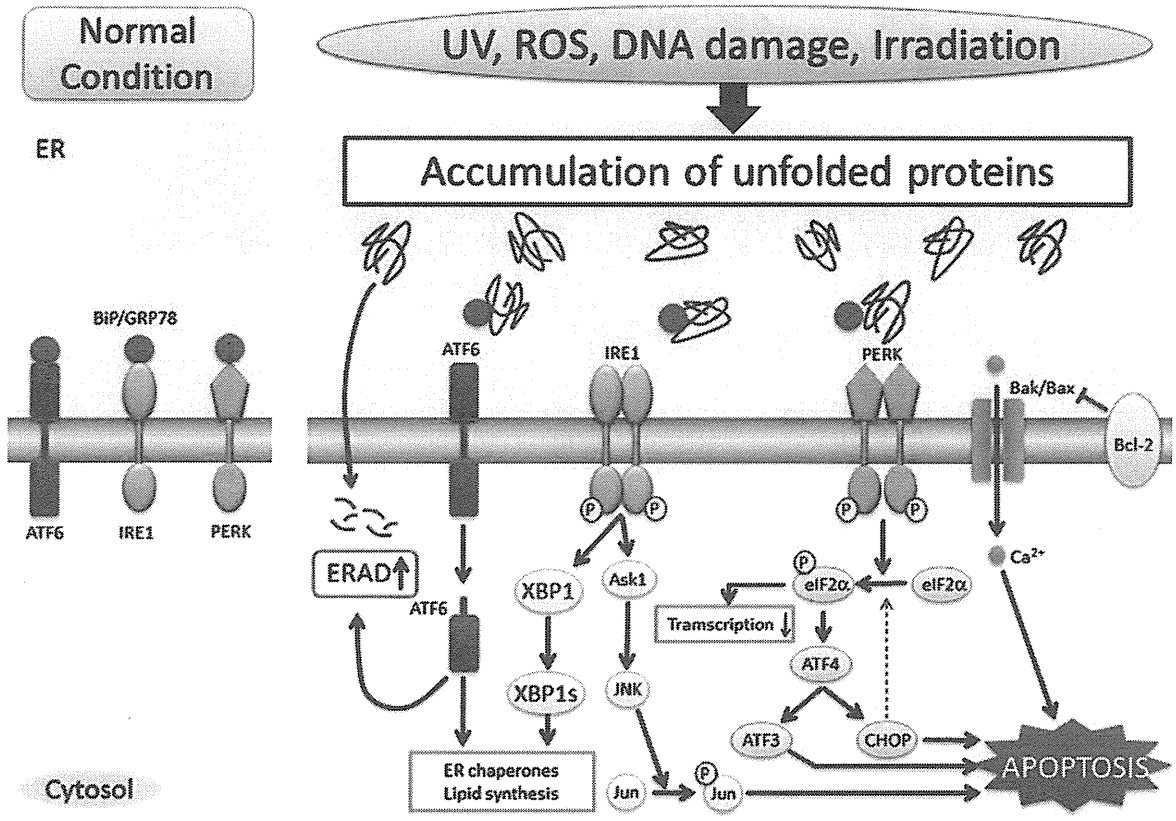

Fig. 3. ER stress induces cellular responses. Under the normal condition, three main ER stress transducers activating transcription factor (ATF) 6, IRE1, and PRKR-like endoplasmic reticulum kinase (PERK) are inactive due to binding to BiP (immunoglobulin heavy-chain binding protein, also referred to as glucose-regulated protein (GRP) 78). When unfolded proteins accumulate in the ER lumen, BiP dissociates from the ER stress transducers. Dissociation of BiP activates them. BiP-free ATF6 translocates to the Golgi apparatus and is cleaved from the membrane. This active form of ATF6 induces transcription of ER chaperones, such as BiP, and XBP1 and promotes export of misfolded proteins and ER-associated protein degradation (ERAD). Active IRE1 has an endoribonuclease activity and causes frame switch splicing of XBP1 mRNA. The spliced form of XBP1 (XBP1s) protein has potential transcription activity and regulates genes involved in ER chaperones. These mechanisms clear misfolded proteins and restore ER homeostasis. On the other hand, IRE1 also activates JNK and stimulate the phosphorylation of Jun. Active PERK phosphorylates eIF2α, which results in successive downregulation of protein translation. eIF2α also induces preferential translation of transcription factor ATF4. Thereby, ATF4 induces expression of C/EBP-homologous protein (CHOP) and ATF3. These proteins trigger an apoptotic program.

Importantly, several key players in IBD pathogenesis (NOD2, ATG16L1, XBP1, and α-defensin) can be found together in Paneth cells, strongly suggesting that Paneth cells play a key role in IBD pathogenesis.

4. Microbiota and IBD

4.1. Host–microbe interactions for gut immunological homeostasis

The mammalian intestine is home to ~100 trillion bacteria that perform important metabolic and protective functions for their hosts. Interactions between the host immune system and the resident microbes have been highlighted as particularly important in this context. In mouse studies, it has become evident that enteric flora regulate intestinal immune cell development. In differentiation of IL-17 producing memory T cells (T helper 17 (Th17) and T regulatory (Treg) cells), a delicate balance of soluble factors, such as transforming growth factor (TGF)-β and IL-6, plays an essential role (Weaver et al., 2006; Stockinger & Veldhoen, 2007). The recent findings that commensal bacteria can regulate the development of both Th17 and Treg cells suggests the importance of the local environment induced by commensal microorganisms in immunological homeostasis of gut-associated lymphoid tissues (GALT) (Fig. 4). Ivanov II. et al. demonstrated that a single commensal small intestinal microbiota, segmented filamentous bacterium (SFB), is sufficient to induce the appearance of Th17 cells in the lamina propria. Interestingly, this exciting discovery that a single commensal microbiota can induce a specific T cell subset is derived from the comparative analysis of the intestinal microbiota in B6 mice bred in Taconic Farms compared with those bred in the Jackson Laboratory. SFB were present in conventionally raised B6 mice from Taconic Farms but were undetectable in the same strain of mice obtained from the Jackson Laboratory. Colonization of germ free mice by SFB induced intestinal Th17 cells. In addition, the introduction of fecal material from germ free mice colonized with SFB into Jackson B6 mice by oral gavage induced robust Th17 cell differentiation. SFB colonization resulted in reduced growth of an intestinal pathogen, suggesting that intestinal commensal microbes can contribute to Th17 cell-mediated mucosal protection (Ivanov et al., 2009). In addition, Atarashiet al. reported that commensal bacteria-derived adenosine 5′-triphosphate (ATP) activates a unique subset of colonic lamina propria cells, $CD70^{high}CD11c^{low}$ dendritic cells (DCs) cells, leading to the differentiation of Th17 cells. A $CD70^{high}CD11c^{low}$DC subset expresses Th17-prone molecules, such as IL-6 and IL-23p19, in response to ATP stimulation, and preferentially induces Th17 differentiation of co-cultured naive CD4+ T cells (Atarashi et al., 2008). These observations highlight the importance of commensal bacteria for Th17 differentiation in health and disease. Not only the development of Th17 cells, but also that of Foxp3transcription factor-positive Treg cells, are regulated by commensal bacteria. Atarashi et al. reported indigenous *Clostridium* species promotecolonic Treg cell accumulation. Colonization of mice by a defined mix of *Clostridium* strains provided an environment rich in TGF-β, and this affected Foxp3+ Treg cell number and function in the colon (Atarashi et al., 2011). *Bacteroides fragilis* may also regulate the promotion of Treg development. Mazmanianet al. reported that the prominent human symbiont *B. fragilis* protects animals from experimental colitis induced by

Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

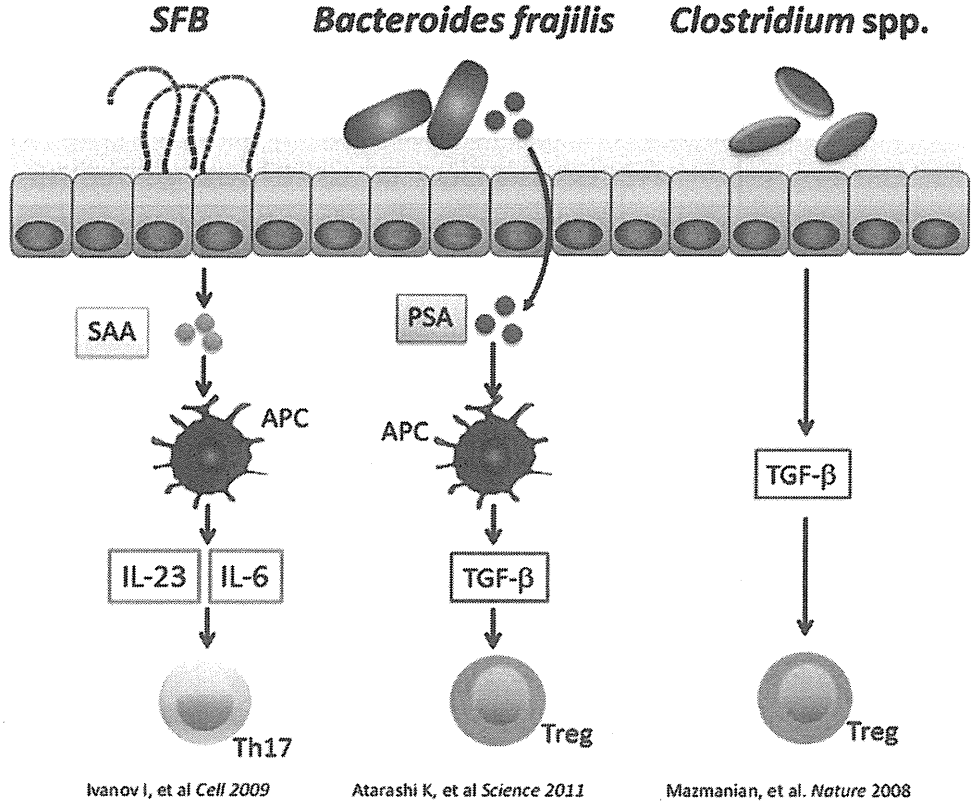

Fig. 4. Intestinal microbiota regulates intestinal mucosal immunology. Enteric flora regulate intestinal immune cell development. SFB induces Th17 cells. *Clostridium* species promoteTreg cell accumulation. PSA derived from *Bacteroides fragilis* also promotes Treg development.PSA; polysaccharide A.

Helicobacter hepaticus. This beneficial activity requires a single microbial molecule, polysaccharide A (PSA). In animals harboring *B. fragilis* not expressing PSA, *H. hepaticus* colonization leads to disease and pro-inflammatory cytokine production in colonic tissues. PSA is required to suppress pro-inflammatory interleukin-17 production by intestinal immune cells and protects from inflammatory disease through an IL-10-producing CD4+Treg cell type (Mazmanian et al., 2008). Further investigations by Roundet al. revealed that PSA derived from *B. fragilis* directs the development of Foxp3^{+}T cells. Monocolonization of germ-free animals with *B. fragilis* increases the suppressive capacity of Tregs and induces anti-inflammatory cytokine production exclusively from Foxp3^{+} T cells in the gut (Round & Mazmanian, 2010). The homeostatic functional property of PSA from *B. fragilis*is through Toll-like receptor (TLR)2-dependent signal transduction on Foxp3^{+}Tregs (Round et al., 2011). For the development of colonic Tregs, commensal bacteria antigen-specific post-thymic education is important. Colonic Tregs use T-cell antigen receptors (TCRs) different from those used by Tregs in other locations. Many of the local antigens seemed to be derived from commensal bacteria. These TCRs did not facilitate thymic Treg cell development, implying that many colonic Treg cells arise by antigen-specific peripheral education (Barrett et al., 2008).

It also seems likely that host immunological properties might affect the composition and function of the enteric flora. Mice with T-bet deficiency in theinnate immune system (T-bet−/− x RAG2−/− mice) developed spontaneouscolitis. This colitis was observed to be communicable to genetically intact mice, suggesting loss of T-bet influences bacterialpopulations to become colitogenic (Garrett et al., 2007). Deficiency of the NOD-like receptor family pyrin domain containing (NLRP) 6 in mouse colonicepithelial cells resulted in reduced IL-18 levels and altered fecal microbiota, characterized by expanded representation of the bacterial phyla *Bacteroidetes*(*Prevotellaceae*). NLRP6 inflammasome-deficient mice were characterized by spontaneous intestinal hyperplasia, inflammatory cell recruitment, and exacerbation of DSS-induced colitis. Cross-fostering and co-housing experiments revealed that the colitogenic activity of this microbiota is transferable to neonatal or adult wild-type mice. Wild type (WT) mice cohoused with antibiotic treated NLRP6 deficient mice developed significantly less-severe DSS colitis compared to WT mice cohoused with untreated NLRP6 deficient mice. This reduction in severity correlated with decreased abundance of Prevotellaceae. These findings indicated the role of *Prevotellaceae* as a key representative of this microbiota-associated phenotype (Elinav et al., 2011). Anti-microbial peptides produced by intestinal epithelial cells or Paneth cells play an important role in host innate immunity. RegIIIγ, a secreted antibacterial lectin, is essential for maintaining host–bacterial segregation at the small intestinal epithelial surface. In*RegIIIγ*$^{-/-}$ mice, increased bacterial colonization of the intestinal epithelial surface and activation of intestinal immune responses by the microbiota were observed. Thus, RegIIIγ regulates the spatial relationships between microbiota and host (Vaishnava et al., 2011). Paneth cell α-defensins are antimicrobial peptides that contribute to host defense against enteric pathogens, as well as governing intestinal microbial ecology. In analysis of the intestinal microbiota of mice expressing a human α-defensin gene, and in mice lacking an enzyme required for the processing of mouse α-defensins, significant α-defensin-dependent changes in microbiota compositionwere observed. Furthermore, human α-defensin gene-expressing mice had striking losses of segmented filamentous bacteria and fewer Th17 cells (Salzman et al., 2010). Thus, symbiosis of commensal microorganisms contributes to intestinal immunological homeostasis and protection from pathogens, while dysbiosis of commensal bacteria induces abnormal immune responses and causes intestinal inflammation.

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However, in contrast to data from mouse studies, evidence suggesting that a single commensal bacteria strain can regulate the host immune system has not yet been reported in humans. In human microbiota studies, there are some methodological difficulties such as the bacterial culturing systems, and differences in the results from culture-independent and culture-based approaches. Recent progress in anaerobic culturing techniques using gnotobiotic animals, and in metagenomic techniques, make it possible to retrieve components of microbiota that have coexisted in single donors who have physiologic or disease phenotypes of interest (Goodman et al., 2011). Systematic analysis of the human gut microbiome has also progressed rapidly. Intensive global research combining fecal metagenomes of individuals from four countries identified three robust clusters or "enterotypes" that are not specific to a single nation or continent. The enterotypes are mostly driven by species composition, butabundant molecular functions are not necessarily provided by abundant species and are often provided by low-abundance species (Arumugam et al., 2011). The National Institutes of Health-funded Human Microbiome Project Consortium has established a population-scale framework to develop metagenomic protocols, resulting in abroad range of quality-controlled resources and data including standardized methods for creating, processing and interpreting distinct types of high-throughput metagenomic data,a nd these have been made available to the scientific community. Results derived from human microbiome analysis might be useful for diagnosis and prediction or prognosis of several human diseases such as IBD.

The incidence of IBD has increased considerably over the past half century, matching developments in cultural Westernization. The number of patients with IBD is also increasing in Asian countries. The rapid increase in the incidence of IBD cannot be explained by genetic drift. It may be caused by exposure to non-genetic factors, such as diet and lifestyle, in genetically susceptible individuals, leading to the abnormal host immune responses that are characteristic of IBD. So far, the hypothesis that an interaction between genetic background, diet, and flora causes chronic intestinal inflammation has not been proved in mice models of colitis, although it is considered a promising hypothesis to explain IBD pathogenesis. Recent findings reported by Devkota et al. have clearly demonstrated that the interaction between flora and diet is important for the development of chronic intestinal inflammation in genetically susceptible mice. Consumption of a diet high in saturated (milk-derived)fat, but not polyunsaturated (safflower oil) fat, changed the conditions for microbial assemblage and promoted the expansion of a sulfite-reducing pathobiont, *Bilophila wadsworthia*, which is associated with a pro-inflammatory Th1 immune response and increased incidence of colitis in IL-10 deficient mice. The milk-derived-fat promoted taurine conjugation of hepaticbile acids, which increased the number of sulfite-reducing microorganisms such as *B. wadsworthia*. Thus, dietary fats, by promoting changes in host bile acid composition, can alter the composition of gut flora, resulting in dysbiosis that can induce chronic intestinal inflammation (Devkota et al., 2012).

Exposure to microbes during early childhood is associated with protection from immune-mediated diseases such as IBD (von Mutius, 2007). Age-sensitive exposure to commensal microbes is critical for establishing mucosal immune tolerance mechanisms. Olszak et al. showed that colonization of neonatal GF mice with a conventional microbiota protected the animals from mucosal invariant natural killer T (NKT) cell accumulation and related intestinal inflammation (Olszak et al., 2012).

4.2. Pathogenic contribution of gut microbiotain human IBD

Adherent *Escherichia coli* have been assigned a putative role in Crohn's disease (Darfeuille-Michaud et al., 1998; Swidsinski et al., 2002; Martin et al., 2004). Boudeauet al. isolated pathogenic adherent-invasive *E. coli* (AIEC) from the ileal mucosa of a patient with Crohn's disease (Boudeau et al., 1999), and several reports support the contribution of AIEC to the pathogenesis of ileal Crohn's disease (Darfeuille-Michaud et al., 2004; Martinez-Medina et al., 2009).

AIEC may target ileal Peyer's patches (PPs). This strain can interact with mouse and human PPs via long polarfimbriae (LPF). The prevalence of AIEC strains harboring the LPF operon was markedly higher in CD patients compared with controls (Chassaing et al., 2011).

Gelatinase (GelE), a metalloprotease (MMP) from *Enterococcus faecalis*, may play a role in the development intestinal inflammation. Stimulation with *E. faecalis* or purified GelE from patients with Crohn's disease and ulcerative colitis revealed proteolytic activity on epithelial barrier function (Steck et al., 2011).

Mammalian gut contains not only bacterial flora, but also a rich fungal community that interacts with the immune system through the innate immune receptor Dectin-1. Mice lacking Dectin-1 exhibited increased susceptibility to chemically-induced colitis, which was the result of altered responses to indigenous fungi. In addition, a polymorphism in the gene for Dectin-1 (CLEC7A) in humans is strongly linked to a severe form of ulcerative colitis (Iliev et al., 2012).

Recent advances in technology for analysis of human microbiota, such as 16S rRNA sequencing, has made it possible to analyze the microbiota in human IBD (Conte et al., 2006; Sokol et al., 2006; Martinez et al., 2008; Takaishi et al., 2008; Andoh et al., 2011; Frank et al., 2011; Kellermayer et al., 2012; Li et al., 2012). Frank et al. reported the results of a culture-independent rRNA sequence analysis of GI tissue samples obtained from CD and UC patients. Comparison of clone libraries of GI tissue samples reveals statistically significant differences between the microbiota of CD and UC patients and those of non-IBD controls (Frank et al., 2007). Papa et al. have attempted to diagnose pediatric IBD non-invasively by assessing differences in the microbiota using 16S rRNA sequencing analysis of fecal samples (Papa et al., 2012). Furthermore, transplantation of fecal microbiota from healthy donors to patients with IBD has also been reported. Systematic review by Anderson et al. evaluated 17 articles of case series/case reports of patients receiving fecal microbiota transplantation for management of their IBD. Whilst the available evidence is limited and weak, they concluded that fecal microbiota transplantation has the potential to be an effective and safe treatment for IBD (Anderson et al., 2012).

5. Abnormal immune regulation in animal IBD models and human IBD

As the intestinal mucosa is continuously exposed to numerous commensal bacteria and food antigens, it is thought that the gut possesses both innate and acquired regulatory immune systems to prevent excessive inflammatory responses against antigen stimulation. As susceptibility gene profiles reveal, disruption of the innate and acquired immune systems of the gut may cause the development of chronic intestinal inflammation. Gut innate and acquired immunity is composed of several key players, such as intestinal epithelial cells (IECs), macrophages (Mϕs), DCs, NKT cells, innate lymphoid cells (ILCs), T cells, and B cells.

5.1. Intestinal epithelial cells

Intestinal epithelial cells play a role as a barrier to prevent invasion by pathogens, and the influx of antigens. IECs produce mucins and trefoil factors which are important protective components of the mucus layer covering the surface of the intestinal lumen. IECs also produce several types of anti-microbial peptide such as defensins, which are a classical innate immune mechanism. Therefore, disruption of this barrier system may allow invasion by not only pathogenic micro-organisms, but also commensal bacteria and food antigens. IECs also act as sensors for pathogens through several innate immune receptors such as toll-like receptors (TLRs) and NODs, and produce cytokines and chemokines to recruit immune cells. TLR signaling pathways induce the production of pro-inflammatory cytokines and chemokines in

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IECs, and also affect epithelial integrity. The activation of TLR2 signaling preserves IEC integrity by claudin 2 up-regulation through PI3 kinase and MyD88 pathways (Cario et al., 2007). Claudins 2, 5 and 8, tight junction proteins in IECs, exhibit altered expression levels and distribution in Crohn's disease. Destruction of tight junction structures may cause increased permeability and bacterial translocation (Zeissig et al., 2007). Restitution of IECs is important for rapid recovery of the mucosal barrier. Recently, IL-22, which belongs to the IL-10 family of cytokines, has been reported to enhance STAT3-dependent expression of mucus-associated molecules and restitution of goblet cells (Sugimoto et al., 2008). Recent studies have demonstrated that a major cell type producing IL-22 is ILCs, suggesting that ILCs modulate the barrier function of IECs (Spits & Di Santo, 2011).

IECs produce constitutive and inducible anti-microbial peptides. In particular, Paneth cells located at the bottom of the crypt have intracellular granules that produce α-defensin. Paneth cells expressing TLRs and NOD2 may play a role as pathogen sensors, and thus contribute to maintenance of the crypt environment. Nudinget al. demonstrated reduced mucosal antimicrobial activity in Crohn's disease of the colon (Nuding et al., 2007). Wehkamp et al. reported that antimicrobial activity was decreased in Crohn's disease patients with ileal lesions, with a specific decrease of α-defensin production by Paneth cells being observed (Wehkamp et al., 2005). Recent studies by the same group revealed a link between α-defensin deficiency in ileal Crohn's disease and Wnt/Tcf-4, which is known as a regulator of Paneth cell differentiation (Wehkamp et al., 2005). Tanabe et al. have reported that s–s binding of α-defensin stored in Paneth cells is important for escape from the degradation induced by protease activity. Interestingly, they also reported that some Crohn's disease patients have an abnormally denatured form of α-defensin that lacks s–s binding (Tanabe et al., 2007). As a novel function of defensins, Shi et al. found that MMP-7-deficient mice, which do not produce the mature form of α-defensin, are susceptible to DSS-induced colitis (Shi et al., 2007). Thus, abnormal function of antimicrobial peptides, especially α-defensins produced by Paneth cells, alters intestinal microbial ecology (Salzman et al., 2010)and may play at least a partial role in the pathogenesis of Crohn's disease.

Recently, very important findings regarding the role of cross-talk between IECs and immune cells were reported. NF-κB signaling in IECs plays an important role in gut immune homeostasis (Nenci et al., 2007; Zaph et al., 2007). Nenchiet al. reported that mice lacking NEMO (also named IKKγ) in their IECs conditionally developed spontaneous colitis. In NEMO knockout mice, the number of apoptotic IECs was increased, resulting in deterioration of the integrity of the epithelial barrier. Furthermore, these mice exhibited decreased production of the antimicrobial defensin peptides produced by IECs. These changes led to translocation of enteric flora and recruitment of innate immune cells (Nenci et al., 2007).Günther et al. demonstrated that caspase-8 in IECs plays a key role in protecting these cells from TNF-α-induced necroptotic celldeath. Mice with a conditional deletion of caspase-8 in IECs (*Casp8*$^{\Delta IEC}$) spontaneously developed inflammation in the terminal ileum and were highly susceptible to colitis. *Casp8*$^{\Delta IEC}$ mice showed increased TNF-α-induced cell death in the Paneth cell area of small intestinal crypts associated with increased expression of receptor-interacting protein (RIP) 3. They also observed high levels of RIP3 in human Paneth cells and increased necroptosis in the terminal ileum of patients with Crohn's disease (Gunther et al., 2011). Rimoldi et al. reported that thymic stromal lymphopoietin (TSLP) produced by IECs leads DCs toward a suppressive phenotype (IL-10 production, but no IL-12 production) and suppresses excessive Th-1 immune responses and, importantly, that the production of TSLP by IECs may be decreased in patients with Crohn's disease (Rimoldi et al., 2005).

5.2. Macrophage and dendritic cells

Mϕs are the major population of tissue-resident mononuclear phagocytes, and play a key role in bacterial recognition and elimination, as well as in the polarization of innate and adaptive immunity. Besides these classical antibacterial immune roles, it has recently become evident that Mϕs are also important for the maintenance of homeostasis, for example, dampening inflammation via the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Mosser, 2003; Mantovani et al., 2004; Gordon & Taylor, 2005). Recent studies have shown that M1- and M2-Mϕs are functionally polarized in response to microorganisms and host mediators. M1-Mϕs are characterized by the production of pro-inflammatory cytokines such as TNF-α, IL-12, and IL-23, while M2-Mϕs are characterized by an IL-10-producing phenotype (Mantovani et al., 2004). Interestingly, it was previously reported that intestinal Mϕs have immune-regulatory functions. In contrast to splenic macrophages, intestinal Mϕs do not express innate response receptors (Rogler et al., 1998; Smith et al., 2001), and although these cells retain their phagocytic and bactericidal functions, they do not produce pro-inflammatory cytokines in response to several inflammatory stimuli, including microbial components (Kamada et al., 2005; Smythies et al., 2005). Importantly, several independent studies have revealed that murine intestinal Mϕs produce the anti-inflammatory cytokine IL-10 (M2-Mϕ) and contribute to maintaining homeostasis of the intestinal immune system. Hirotaniet al. demonstrated that wild type colonic lamina propria Mϕs (LP-Mϕs) were different from splenic Mϕs, as they produced higher amounts of IL-10 in response to pathogen-associated molecular patterns (Hirotani et al., 2005). Kamada et al. also demonstrated in vitro that Mϕs differentiated by macrophage colony-stimulating factor (M-CSF) and intestinal CD11b^{+} Mϕs produced abundant IL-10 in response to whole bacteria stimulation (Kamada et al., 2005). M-CSF is a key growth factor for the development of intestinal Mϕs, as shown by the number of intestinal Mϕs in M-CSF-deficient *op/op* mice being significantly decreased (Cecchini et al., 1994). These results suggest that intestinal Mϕs in wild type mice are the IL-10 producing M2 type and may contribute to the maintenance of homeostasis. Uniquely, Takada et al. analyzed CD11b^{+}LP-Mϕs from mice and demonstrated they could be divided into two sub-populations (LP-Mϕ1 and LP-Mϕ2) by flow cytometry analysis (Takada et al., 2010). LP-Mϕ2 expressed CCR2 and produced large amounts of IL-10. Interestingly, *Mcp1*$^{-/-}$ mice contained fewer LP-Mϕ2 cells, resulting in the exacerbation of DSS-induced colitis, suggesting that LP-Mϕs contribute to the maintenance of gut immune homeostasis by producing IL-10. LP-Mϕs may have antigen presenting functions and induce the differentiation of FoxP3^{+} Tregs that are dependent on IL-10 and retinoic acid (Denning et al., 2007). Thus, recent studies have suggested that Mϕs located in the intestinal mucosa play an important role in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens and negatively regulating excess immune responses to commensal bacteria (Schenk & Mueller, 2007).

Because of this important role of Mϕs in the intestinal mucosa, immune homeostasis in the gut is disrupted when intestinal Mϕ function is dysregulated, and this may result in chronic intestinal inflammation. IL-10$^{-/-}$ mice develop spontaneous chronic colitis and are widely used as an animal model of human IBD (Kuhn et al., 1993). IL-10$^{-/-}$ mice develop Th1 polarized immunity in response to the intestinal microbiota, as shown by the observation that IL-10$^{-/-}$ mice do not develop intestinal inflammation in germ-free conditions (Sellon et al., 1998). This suggests that enteric bacteria play an essential role in the onset and development of colitis in IL-10$^{-/-}$ mice, which may also be the case in human IBD. Recent studies have demonstrated that antigen presenting cells (APCs), such as Mϕs and DCs, from IL-10$^{-/-}$ mice are potent activators of Th1 responses (Igietseme et al., 2000) and, importantly, depletion of Mϕs prevented chronic colitis in IL-10$^{-/-}$ mice (Watanabe et al., 2003). These data suggest that Mϕs and DCs play a key role in the pathogenesis of colitis in IL-10$^{-/-}$ mice. Kamada et al. demonstrated in vivo that LP-Mϕs from IL-10$^{-/-}$ mice showed a paradoxical overproduction of IL-12p70 upon bacterial stimuli and that endogenous IL-10 is required for inhibition of IL-12p70 production and

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functionally regulates Mϕs towards an anti-inflammatory phenotype (Kamada et al., 2005). These abnormal responses of intestinal Mϕ subsets to enteric bacteria in IL-$10^{-/-}$ mice may contribute to a Th1 cytokine bias and the development of intestinal inflammation. Interestingly, whole bacteria are a potent inducer of IL-12p70 production by Mϕs. Significant repression of IL-12p70 production was achieved by inhibition of phagocytosis, suggesting that intracellular pathogen recognition and signaling are involved in the induction of IL-12p70 in IL-$10^{-/-}$ Mϕs (Naruse et al., 2011). Also of note, histidine, which is one of the most common natural amino acids inhibited LPS-induced TNF-α and IL-6 production by IL-$10^{-/-}$ Mϕs, and dietary histidine ameliorated colitis in an IL-$10^{-/-}$ mouse transfer colitis model (Andou et al., 2009). Indeed, amino acid profiling has revealed decreased levels of plasma histidine concentration in patients with active Crohn's disease (Hisamatsu et al., 2012).

To date, many kinds of IBD animal models have been reported, but none of these completely reflect the characteristics of human IBD pathophysiology. Granuloma formation is the one of the most important pathological characteristics of human Crohn's disease. Mizoguchi et al. demonstrated that F4/80-positive immature $CD11c^+$ DCs produce IL-23 and contribute to granuloma formation in a murine colitis model (Mizoguchi et al., 2007).

A Th1 immune response is predominant in Crohn's disease. Indeed, $CD4^+$ T cells in the LP of Crohn's disease expressed T-bet and produced large amounts of interferon (IFN)-γ (Matsuoka et al., 2004). Sakuraba et al. demonstrated that DCs in the mesentric lymph nodes of patients with Crohn's disease strongly promoted a Th1 and Th17 immune response (Sakuraba et al., 2009). Not only mesentric lymph node DCs, but also LP-Mϕs, contribute to IBD pathogenesis, particularly that of Crohn's disease. The contribution of intestinal Mϕs (that express TREM-1 (triggering receptor expressed on myeloid cells 1)) to disease pathogenesis in a murine experimental colitis model, and patients with IBD, has been reported (Schenk et al., 2007). LP-Mϕs produce large amounts of IL-18 and promote Th1 immune responses in Crohn's disease (Kanai et al., 2001). Kamada et al. observed the infiltration of unique $CD14^+$ intestinal Mϕs ($CD14^+$Mϕs) in the mucosa of Crohn's disease patients (Kamada et al., 2008). This subset expressed both Mϕ (CD14, CD33, CD68) and DC (CD205, CD209) markers and produced larger amounts of pro-inflammatory cytokines, such as IL-23, TNF-α, and IL-6, than typical intestinal resident Mϕs. Mϕ-derived IL-23 strongly enhances IFN-γ production by these cells in Crohn's disease (Kamada et al., 2008) suggesting that Mϕ-derived IL-23 is a key cytokine for the predominance of Th1 responses in Crohn's disease. $CD14^+$ Mϕs also have antigen presenting functions and can stimulate the differentiation and proliferation of naïve $CD4^+$ T cells obtained from peripheral blood (Kamada et al., 2009). Importantly, although in vitro differentiated DCs cannot induce the differentiation of peripheral blood naïve $CD4^+$ T cells to Th17 cells, LP-$CD14^+$Mϕs from Crohn's disease patients can strongly promote T cell differentiation to both Th1 and Th17 cells in response to stimulation by whole bacteria (Kamada et al., 2009). TL1A/TNFSF15, a member of the TNF superfamily, was identified as a susceptibility gene for Crohn's disease, particularly in Japanese patients (Yamazaki et al., 2005; Cho, 2008). $CD14^+$ Mϕs in Crohn's disease express membrane-boundTL1A/TNFSF15 that acts synergistically with IL-23 to promote the production of IFN-γ and IL-17 by LPMCs (Kamada et al., 2010).Thus, $CD14^+$ Mϕ plays a central role in the promotion of an inflammatory cytokine network at mucosal sites of Crohn's disease (Fig. 5).

5.3. NKT and ILC (mucosal NK and LTi)

NKT cells are also a distinct subset of mucosal immune cells and have relationship to IBD. NKT cells respond rapidly to antigens presented on CD1d of APCs and secrete various cytokines, such as IL-13. Oxazolone-induced colitis mice are a Th2-dominant colitis model representing human ulcerative colitis (Boirivant et al., 1998). IL-13 is a key cytokine in the pathogenesis of oxazolone-induced colitis, as shown by the inflammation being prevented by the administration of an IL-13 inhibitor, IL-13Rα2-Fc (Heller et al., 2002). Mucosal NKT cells are the main IL-13-producing cells in oxazolone-induced colitis and drive Th2 inflammation. Importantly, increased numbers of non-classical NKT cells in the LP of ulcerative colitis produced large amounts of IL-13 (Fuss et al., 2004). IL-13 may play a role in the pathogenesis of ulcerative colitis by inducing epithelial cell damage (Kawashima et al., 2006, 2011).

ILC is an emerging category which includes NK cells and lymphoid tissue-inducer (LTi) cells and type 2 helper cells. These ILCs share the transcriptional repressor *Id2* and cytokine signals of IL-2. NK cells are now classified as IFN-γ producing ILCs and play an important role in systemic immune surveillance to protect hosts from neoplasms and infections. NK cells can rapidly detect and dispose of target cells such as tumor cells and infected cells. NK cells also contribute to immunity by producing several types of cytokines such as IFN-γ and TNF-α. Recently, mucosal NK cells were shown to be involved in mucosal homeostasis (Di Santo et al., 2010; Shi et al., 2011). Chinen et al. had previously identified that numbers of mucosal NK cells were increased in the LP of Crohn's disease (Chinen et al., 2007). Furthermore, increased numbers of c-kit^+ lineage markers $(lin)^-$ cells were also observed in human adult intestine. These intestinal immune precursors expressed CD34, CD38, CD33, IL-2Rα, and IL-7Rα. The lin^- c-kit^+ precursors mainly differentiated to $CD56^+$ c-kit^{dim} cells in vitro, corresponding to intestinal NK cells, which are clearly distinguished from peripheral blood NK cells by expression patterns of surface molecules and cytokine production. These cells produced higher amounts of IFN-γ, while their cytotoxic activity was relatively low. Interestingly, both c-kit^{dim} cells and NK cells were increased in the inflamed mucosa of Crohn's disease (Chinen et al., 2007). Later, these findings were confirmed by the evidences that Lin^- $CD45^{int}CD127^{hi}$*RORC*$^+$LTi cells produce IL-17 and IL-22 and generate *RORC*$^+$ $CD127^{hi}$ NK cells which retain the production of IL-17 and IL-22 (Cupedo et al., 2009).Further analysis showed that human intestinal mucosal $CD3^-CD56^+$NK cells could be classified into two subpopulations: $NKp44^+NKp46^-$ ($NKp44^+$) and $NKp44^-$ $NKp46^+$ ($NKp46^+$) cells. In contrast to recent studies in mice, $NKp46^+$ cells expressed RORγt and produced IL-22 (Satoh-Takayama et al., 2008), whereas human intestinal $NKp46^+$ did not express RORC and produced higher amounts of IFN-γ. Importantly, the balance of $NKp44^+$/$NKp46^+$ cells was disrupted in the intestinal mucosa of patients with Crohn's disease, where IFN-γ producing$NKp46^+$ cells (conventional NK (cNK) cells) were dominant and IL-22 producing $NKp44^+$ cells (ILC22 or NK22) are decreased (Takayama et al., 2010). These findings suggest that T cells and mucosal IFN-γ-producing cNK cells may contribute to excessive Th1 immune responses and the pathogenesis of Crohn's disease.

LTi cells are now being classified as RORγt dependent ILCs and several ILC populations have been implicated in promoting innate immunity and intestinal inflammation via production of host protective and/or inflammatory cytokines (Eberl et al., 2004; Satoh-Takayama et al., 2008; Cella et al., 2009; Colonna, 2009; Buonocore et al., 2010; Cua & Tato, 2010; Sawa et al., 2010; Spits & Di Santo, 2011). LTi cells are the first hematopoietic cells recruited to the sites of secondary lymphoid tissue organogenesis during fetal development (Mebius et al., 1997).LTi cells in adult mice and humans constitutively express RORγt, which is a transcriptional factor that regulates the development of LTi cells (Eberl et al., 2004)and these cells also have the capacity to express IL-22 in vitro (Eberl & Littman, 2004; Takatori et al., 2009). Therefore, LTi cells in adults play a role not only in lymphoid tissue generation and maintenance, but also in host innate immunity. In mice infected with *Citrobacter rodentium*, CD4+ LTi cells were a dominant source of IL-22 and played a key role in promoting innate immunity in the intestine (Sonnenberg et al., 2011). Mouse ILCs produce IL-17, IL-22, and IFN-γ in response to IL-23 and this mediates innate colitis. Geremia et al. showed increased expression of IL17A and IL17F among intestinal

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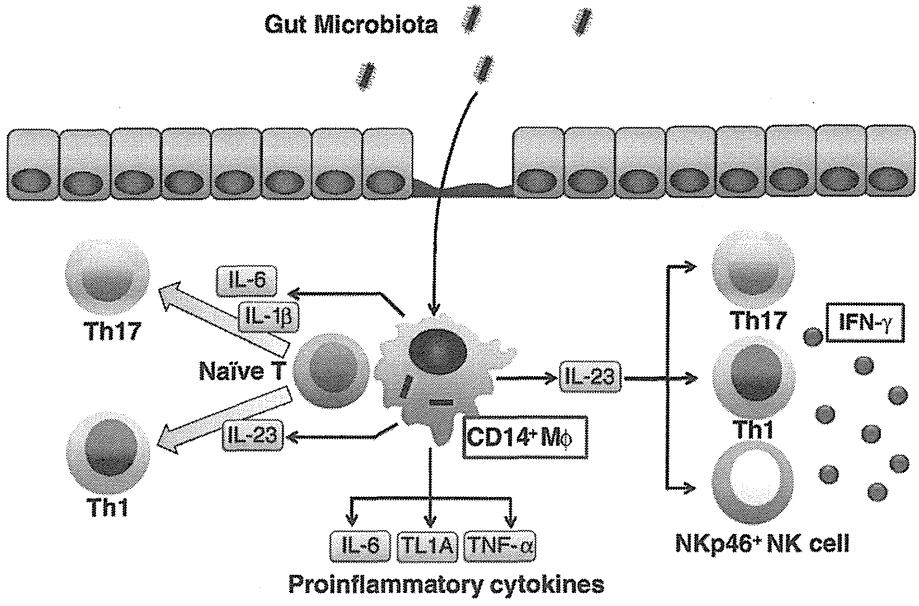

Fig. 5. Contribution of CD14 + Mϕs in the pathogenesis of Crohn's disease. Lamina propria $CD14^+$ Mϕs produce large amount of TNFα, IL-23, and IL-6 resulting in induction of excess Th1 and Th17 responses. $CD14^+$ Mϕs as antigen presenting cells also promote Th1 and Th17 differentiations.

$CD3^-$ cells in IBD. IL17A and IL17F expression is restricted to $CD56^-$ ILCs, whereas IL-23 induces IL22 and IL26 in the $CD56^+$ ILC compartment. Furthermore, a significant and selective increase in $CD127^+$ $CD56^-$ ILCs in the inflamed intestine in Crohn's disease patients, but not in ulcerative colitis patients, has been observed. These results indicate that the selective accumulation of a phenotypically distinct ILC population may play a role in Crohn's disease pathogenesis (Geremia et al., 2011). ILCs also regulate selective containment of lymphoid-resident bacteria to prevent systemic inflammation associated with chronic diseases. Depletion of ILCs has been observed to result in peripheral dissemination of commensal bacteria and systemic inflammation. Disseminating bacteria were identified as *Alcaligenes* species originating from host lymphoid tissues. *Alcaligenes*-specific systemic immune responses were associated with Crohn's disease and progressive hepatitis C virus infection in patients (Sonnenberg et al., 2012).

Collectively, a new category of immune cells, ILCs, may contribute to gut homeostasis and ecology. IL-23 produced by activated DCs and Mϕs stimulates IL-22 production by ILCs. IL-22 may induce an anti-microbial peptide, RegIII-γ, production in IECs. IEC derived factors including IgA, defensins, and RegIII-γ play a protective and homeostatic role in commensal bacteria ecology of gut (Fig. 6). Therefore, abnormal immune responses of ILCs may change intestinal immune environment and cause intestinal inflammation.

5.4. Lamina propria colitogenic T cells

Lamina propria effector memory $CD4^+$ T cells are directory involved in IBD pathogenesis. One of the most important clinical characteristics of human IBD is a pattern of repeated remission and relapse. To date, several factors including a high fat diet, infection, non-steroidal anti-inflammatory drugs, antibiotics, and mental stress have been reported as triggers contributing to the mechanisms of relapse and chronicity. However, these mechanisms remain unclear, in spite of recent research progress in the investigation of IBD pathogenesis. It has been suggested that some triggers (e.g. bacterial antigens) are re-recognized by APCs and the population of antigen-specific effector memory T cells expands, leading to relapse. This hypothesis is quite reasonable, but it raises the question of where the effector memory T cells are "hidden" during remission. Nemoto et al. demonstrated that bone marrow acts as a reservoir for colitogenic $CD4^+$ T cells (Nemoto et al., 2007). In addition, colitogenic $CD4^+$ T cells continuously recirculate in peripheral blood, supporting the efficacy of molecular targeting therapy for adhesion molecules (Tomita et al., 2008a). Interestingly, peripheral lymph nodes are not necessary for recirculation and storage of colitogenic $CD4^+$ T cells. FTY720, a chemical agent that suppresses T cell trafficking at the lymph nodes, also can inhibit recirculation of colitogenic $CD4^+$ T cells at the bone marrow (Fujii et al., 2008). IL-7 plays an important role in the survival of colitogenic $CD4^+$ T cells and the persistence of T cells associated with chronic colitis (Totsuka et al., 2007; Tomita et al., 2008b). Consistent with this observation, IL-7Rα expression in colitogenic $CD4^+$ T cells is significantly up-regulated (Shinohara et al., 2011).

The hypothesis of a Th1/Th2 cytokine balance has been used to explain the pathogenesis of chronic inflammatory disorders such as IBD. However, the recent discovery of a new class of Th cells, Th17 cells, which produce IL-17 family cytokines, has raised a new paradigm: namely, thatTh17 cells are an essential T cell subpopulation in the development of chronic inflammatory disorders in humans and mice. In mice, Th17 cells are generated from naïve T cells in the presence of TGF-β and IL-6 and express a specific transcription factor, retinoic acid-related orphan receptor (ROR)γt (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Bettelli et al., 2008). Recent reports suggest that the combination of IL-6, IL-23, and IL-1β also effectively induces IL-17 production in naive T cells, independent of TGF-β (Ghoreschi et al., 2010). In humans, the mechanism of Th17 development is more complicated. It is difficult to induce Th17 cells from naïve peripheral blood T cells using in vitro differentiated conventional DCs, even in the presence of TGF-β and IL-6. Acosta-Rodriguez et al. reported that IL-1β and IL-6 are essential for the development of Th17 cells inhumans (Acosta-Rodriguez et al., 2007). However, Manelet al. succeeded in inducing Th17 cells from naïve T cells obtained from cord blood in the presence of TGF-β, IL-1β,and IL-21 or IL-23 (Manel et al., 2008).

Several reports have suggested that Th17 cells play a role in the pathogenesis of murine models of colitis. IL-23p19 transgenic mice spontaneously developed chronic colitis (Wiekowski et al., 2001). In $IL\text{-}10^{-/-}$ mice, which develop chronic Th1/Th17 dominant colitis, IL-23 administration exacerbated intestinal inflammation (Yen et al.,

Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

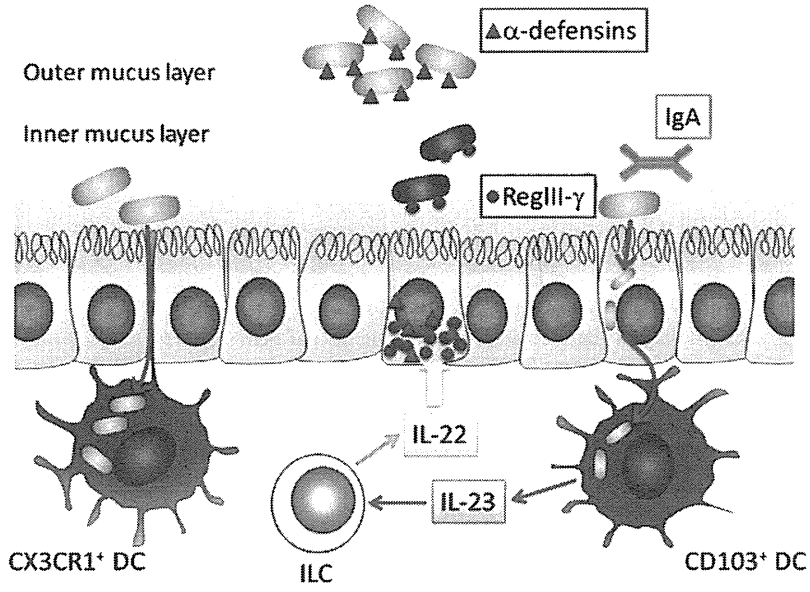

Fig. 6. ILCs protect the host by induction of IEC-derived RegIII-γ. ILCs produced IL-22 which is dependent on IL-23. IL-22 promotes production of IEC-derived RegIII-γ which contributes to intestinal mucosal ecology.

2006). The transfer of IL-17A-producing Th17 cells from C3Bir mice caused severe chronic colitis in SCID mice, which was inhibited by anti-IL-23p19 monoclonal antibody (mAb) treatment (Elson et al., 2007). The transferof $CD4^+$ $CD25^-$ T cells from $ROR\gamma t^{-/-}$ mice into $RAG\text{-}1^{-/-}$ mice did not result in colitis, while IL-17A induced colitis in this model (Leppkes et al., 2009). IL-17 receptor-deficient mice were protected from acute trinitrobenzene sulfonic acid-induced colitis (Zhang et al., 2006). These findings suggest that Th17 cells may contribute to the pathogenesis of chronic intestinal inflammation. However, it is still controversial whether IL-17A plays a pathological role in murine colitis. Administration of neutralizing anti-IL-17A mAb did not reduce the severity of colitis in an adaptive transfer model of $CD4^+CD45RB^{high}$ T cells (Yen et al., 2006) and exacerbated DSS-induced colitis (Ogawa et al., 2004). Furthermore, $RAG\text{-}1^{-/-}$ mice reconstituted with $CD45RB^{hi}$ T cells from IL-17 receptor-deficient mice exhibited an accelerated wasting disease (O'Connor et al., 2009). Thus, the contribution of IL-17 itself, and of Th17 cells, in the pathogenesis of colitis remains unresolved. It has become evident that Th17 cells can be classified into several subpopulations according to their cytokine profile. McGeachy et al. reported that Th17 cells could be divided into two types: Th17 cells producing IL-17 and IL-10, and Th17 cells developed under IL-23 stimulation producing IL-17 and pro-inflammatory cytokines (McGeachy et al., 2007). Furthermore, recent studies have suggested interactions between Th1 and Th17 cells. IL-17A suppressed inflammation in a murine model of colitis by blocking the development of Th1 cells (O'Connor et al., 2009). Mikami et al. demonstrated the existence of interference between colitogenic Th1 and Th17 cells in vivo. In experiments of a co-transferred $CD4^+$ T cells mice model (colitogenic$CD4^+$ T cells from colitic $RAG\text{-}2^{-/-}$ mice transferred with $CD4^+$ $CD45RB^{high}$ cells and colitogenic $CD4^+$ T cells from colitic $IL\text{-}10^{-/-}$ mice). The proportions of IFN-γ and IL-17A producing $CD4^+$ T cells in co-transferred mice were significantly decreased compared with single-cell transferred mice (Mikami et al., 2010). Although to date it has been thought that each Th subpopulation is generated independently, current progress in immunology research has highlighted the plasticity between these Th cell lineages. Lee et al. demonstrated that Th1 cells could be generated from IL-17 F-expressing Th17 cells in the presence of IL-23 in vivo (Lee et al., 2009). Sujino et al. proved the existence of plasticity between Th1 and Th17 cells in vivo using RORγt-GFP reporter mice. They identified a distinct developmental pathway from Th17 to alternative Th1 cells via Th17/Th1 and Th1-like cells during colitis (Sujino et al., 2011). Ono et al. also demonstrated that naturally occurring Th17 cells have regulatory activities in normal mouse intestine, in contrast with colitogenic Th17 and Th17/Th1 cells during inflammation (Ono et al., 2012).

The pathological role of Th17 cells in human disease remains unclear, and there is recent data which show differences in the functional roles of Th17 cells between mice models and human diseases (Steinman, 2008) and the existence of several subpopulations of IL-17 producing T cells in humans. Fujino et al. reported that IL-17A expression is up-regulated in $CD3^+$ T cells and $CD68^+$ Mϕs in the inflamed mucosa of patients with IBD (Fujino et al., 2003). $CD14^+CD68^+$ Mϕs inthe inflamed mucosa of Crohn's disease patients were observed to produce IL-23, IL-6, TNF-α, and TL1A/TNFSF15 in response to stimulation by whole bacteria and to promote the differentiation of naïve T cells to both Th1 and Th17 cells as antigen presenting cells (Kamada et al., 2008, 2009, 2010). Kobayashi et al. also demonstrated that IL-23 from $CD14^+CD68^+$Mϕ enhanced Th17 immunological responses in ulcerative colitis (Kobayashi et al., 2008). Thus, although several observations support a role for Th17 in IBD pathogenesis, to date the contribution of Th17 cells is still controversial. Indeed, the efficacy of anti-IL17A mAb has not yet been proven in patients with IBD.

5.5. Intestinal mucosal B cells

T cell receptor (TCR) α mutants represent a Th2-dominant chronic colitis, often used as a model of human disease ulcerative colitis (Mombaerts et al., 1993). Surprisingly, the spontaneous chronic colitis in $TCR\alpha^{-/-}$ mice (mediated by$CD4^+TCR\alpha^-\beta^+$ T cells) showed more severe inflammation in the absence of mature B cells, suggesting a suppressive role of B cells. Mature B cells play an important role in the development of chronic colitis in $TCR\alpha^{-/-}$ mice by directly regulating the pathogenic $CD4^+TCR\alpha^-\beta^+$ T cells (Mizoguchi et al., 2000). Mizoguchi et al. identified a B cell subset induced in gut-associated lymphoid tissues which is characterized by CD1d up-regulation. This B cell subset produced IL-10, and suppressed intestinal inflammation (Mizoguchi et al., 2002).

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The association of mucosal B cells with human IBD pathogenesis remains unclear. Several sub-populations of mucosal B cells, including pro-inflammatory or immuno-regulatory B cells, may exist in the human intestine. This complexity is reflectedin the results of B cell targeting therapy in ulcerative colitis. A clinical trial of rituximab, which is an anti-CD20 mAb, showed short-term efficacy (Leiper et al., 2011), while in contrast several cases showingexacerbation of ulcerative colitis after rituximab therapy have been reported (Goetz et al., 2007; Ardelean et al., 2010).

Ulcerative colitis is characterized by infiltration of IgG-producing plasma cells. Recently, Uo et al. reported that IgG plasma cells infiltrate the inflamed mucosa via CXCR4, and critically influence pathogenesis of ulcerative colitis by exacerbating mucosal inflammation through the activation of "pathogenic" intestinal $CD14^+$macrophages via IgG-IC-FcγR signaling (Uo et al., 2012).

6. Development of immune regulatory therapy for IBD

As a result of progress in the research of IBD pathogenesis, immune regulatory therapies to effectively manage the symptoms of patients with IBD have been developed. In recent years, molecular targeting therapy has been developed in IBD therapeutics, including monoclonal antibodies (mAbs) and small compounds as specific inhibitors of target molecules.

6.1. Immune regulatory therapy for IBD

Corticosteroids have been used for induction therapy in both ulcerative colitis and Crohn's disease. In steroid dependent and steroid resistant patients, 6-mercaptopurine (6-MP) and azathioprine (AZA) have been used for maintenance therapy (Hanauer & Present, 2003). Several clinical studies of cyclosporine A, a calcineurin inhibitor, for induction or rescue therapy in patients with severe ulcerative colitis refractory to steroid therapy have been reported (Lichtiger et al., 1994; Kobayashi et al., 2010; Mocciaro et al., 2012; Sjoberg et al., 2012). Recently, the efficacy of oral tacrolimus, a newer calcineurin inhibitor, in patients with refractory ulcerative patients has been reported (Ogata et al., 2006, 2012). Thus, therapeutic strategies that regulate immune response have been a central component of IBD therapeutics.

6.2. Development of anti-TNFα mAbs in IBD

One of the most successful examples of immuno-molecular targeting therapies in IBD is an anti-TNFα mAb, which has "opened the door" on the era of biologics. The turning point was a report in 1993 of a 12-year-old female Crohn's disease patient from the Netherlands (Derkx et al., 1993). Following this single case report, the somewhat surprisingly successful results of a clinical trial of anti-TNFα mAb were reported in 1995 (van Dullemen et al., 1995).After several clinical trials that supported the efficacy of a chimeric (75% human, 25% mouse) anti-TNFα mAb, infliximab (Targan et al., 1997; Present et al., 1999; Rutgeerts et al., 1999), a large-scale randomized trial (ACCENT I) was performed and indicated the efficacy of infliximab in induction and maintenance therapy for active Crohn's disease (Hanauer et al., 2002). Following the success of infliximab, other anti-TNFα mAbs were developed. Adalimumab (Hanauer et al., 2006; Colombel et al., 2007), a human anti-TNFα mAb, and certolizumabpegol (Schreiber et al., 2005; Sandborn et al., 2007), a polyethylene-glycolated Fab' fragment of anti-TNFα mAb, also demonstrated efficacy. Anti-TNFα mAbs also showed efficacy in patients with refractory ulcerative colitis (Rutgeerts et al., 2005; Reinisch et al., 2011; Sandborn et al., 2012) suggesting an overlap of pathophysiology between Crohn's disease and ulcerative colitis.

6.3. Novel molecular targeting therapy in IBD

Success of anti-TNFα mAb therapies has encouraged the development of molecular targeting therapy in IBD. An anti-IL-12p40 mAb has been developed and clinical trials are ongoing (Mannon et al., 2004; Sandborn et al., 2008). IL-12p40 is bound to a p35 chain in IL-12 and a p19 chain in IL-23, and these are key cytokines in the pathogenesis of Crohn's disease in terms of promotion of Th1 and Th17 immune responses.

Adhesion molecules have been highlighted as a novel therapeutic target. Natalizumab, a humanized monoclonal antibody against α4 integrin that inhibits leukocyte adhesion and migration into inflamed tissue, has demonstrated some efficacy in Crohn's disease (Sandborn et al., 2005; Feagan et al., 2007; Targan et al., 2007), although the potential for progressive multifocal leukoencephalopathy caused by the reactivation of the JC virus means that caution is required (Van Assche et al., 2005). A clinical trial of GSK-1605786 (CCX-282; Traficet-EN), a selective antagonist of the CC chemokine receptor (CCR9) which also inhibits lymphocyte homing, is ongoing.

Not only cytokines and surface molecules, but signal transduction molecules could also be therapeutic targets for chronic inflammatory disorders. Janus kinase (JAK) inhibitors are expected to be candidates for molecular targeting therapy for rheumatoid arthritis and IBD.

7. Conclusion

We have reviewed the immunological aspects in IBD pathogenesis. Recently identified susceptibility genes strongly suggest a contribution of abnormal immune response to IBD pathogenesis. Advances in research on the microbiota in mice suggest that commensal microbiota can regulate the host gut immune system. Abnormal immune responses to commensal bacteria and/or food antigens may be a central part of IBD pathogenesis. To regulate excess immune responses, immune regulatory therapies have been applied. As a result of newly discovered knowledge about IBD pathogenesis, molecular targeting therapies such as anti-TNFαmAbshave been developed.

As we have described, the pathogenesis seen in patients with IBD varies, and can be very different between individuals. In the near future, it should be possible to establish tailor-made medicine in the field of IBD therapeutics. For example, a combination of a panel of susceptibility genes with analysis of the human gut microbiome may be useful to select the most adequate treatment that can adjust to an individual's pathogenic status. There is still a long way to go, but we confidently expect that recent advances in our understanding of mucosal immunology will contribute to understanding the etiology of IBD, and therefore the development of new therapeutics.

"The authors declare that there are no conflicts of interest."

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Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

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Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

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Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

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Please cite this article as: Hisamatsu, T., et al., Immune aspects of the pathogenesis of inflammatory bowel disease, *Pharmacology & Therapeutics* (2012), http://dx.doi.org/10.1016/j.pharmthera.2012.10.008

nature COMMUNICATIONS

OPEN

ARTICLE

Received 12 Apr 2012 | Accepted 27 Jul 2012 | Published 4 Sep 2012

DOI: 10.1038/ncomms2023

Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors

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Mast cells are known effector cells in allergic and inflammatory diseases, but their precise roles in intestinal inflammation remain unknown. Here we show that activation of mast cells in intestinal inflammation is mediated by ATP-reactive P2X7 purinoceptors. We find an increase in the numbers of mast cells expressing P2X7 purinoceptors in the colons of mice with colitis and of patients with Crohn's disease. Treatment of mice with a P2X7 purinoceptor-specific antibody inhibits mast cell activation and subsequent intestinal inflammation. Similarly, intestinal inflammation is ameliorated in mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, and reconstitution with wild-type, but not $P2x7^{-/-}$ mast cells results in susceptibility to inflammation. ATP-P2X7 purinoceptor-mediated activation of mast cells not only induces inflammatory cytokines, but also chemokines and leukotrienes, to recruit neutrophils and subsequently exacerbate intestinal inflammation. These findings reveal the role of P2X7 purinoceptor-mediated mast cell activation in both the initiation and exacerbation of intestinal inflammation.

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Both active and quiescent immunity occur simultaneously to achieve immunological homoeostasis in the harshest of environments—namely, the intestine. Aberrant immune responses in the gut lead to the development of intestinal immune diseases such as colitis and food allergies[1,2]. Mast cells (MCs) are generally recognized as major effector cells of type 1 allergic diseases, as well as of inflammation, host defenses, innate and adaptive immune responses and homoeostatic responses[3–5]. Histological analyses of patients with, and murine models of, colitis have implicated the involvement of MCs in intestinal inflammation[4,6], but the factors responsible for MC activation are not fully understood.

Several lines of evidence have demonstrated that release of extracellular ATP and ADP from injured, dying or activated cells acts as a danger signal by modulating various cellular functions via the activation of P2 purinoceptors[7,8]. P2 purinoceptors comprise P2X ($P2X_{1-7}$) and P2Y receptors ($P2Y_{1, 2, 4, 6, 11-14}$). $P2X_{1-7}$ receptors are ATP-gated ion channels and specific for ATP, whereas P2Y receptors are G protein-coupled receptors that are specific for ADP, UTP and ATP[7,8].

Stimulation by ATP or ADP through the P2 purinoceptors of macrophages and dendritic cells (DCs) results in the production of inflammatory cytokines; this can lead to the development of asthma, contact hypersensitivity or graft-versus-host disease[9–11]. MCs also express several P2 purinoceptors and release histamine, cytokines and chemokines upon nucleotide stimulation[12]. Although MCs are thought to be involved in intestinal inflammation, it is unclear whether extracellular nucleotides are required for this process.

Here, we used a newly established anti-MC monoclonal antibody (mAb) to identify activated MCs and found that extracellular ATP mediates MC activation through P2X7 purinoceptors to initiate and amplify intestinal inflammation. Consequently, obstruction of the ATP-P2X7 purinoceptor cascade could be used to inhibit gut inflammatory diseases.

Results

Activated MCs in intestinal inflammation. Using a 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis model, we first examined whether MCs were involved in intestinal inflammation. To assess MC activation *in vivo*, we established an mAb (clone: 5A9) specific for CD63, a marker of activated MCs[13]. We confirmed that our anti-CD63 mAb was reactive specifically to MCs activated by immunoglobulin (Ig)E plus relevant allergen or a calcium ionophore, and not to naïve and CD63-knocked down MCs (Supplementary Fig. S1). In the colons of TNBS-treated mice, increased numbers of $CD63^+$-activated MCs were noted until day 3 post administration; the numbers then gradually decreased and reached a basal level on day 6 (Fig. 1a,b), indicating that MC activation was associated with the initiation phase of colitis development, as previously reported in a murine model and in patients with inflammatory bowel disease[6,14]. It has generally been accepted that the mechanistic basis of ulcerative colitis (UC) and Crohn's disease (CD) are different. Indeed, the pathogenic cytokines involved in the development of UC and CD are different[2] and the genetic polymorphisms specific for UC and CD are also different[15]. In addition, the cytokines required for the development of MCs differ between humans (stem cell factor) and mice [interleukin (IL)-3 and stem cell factor][4]. Thus, these different pathological environments may have led to differences in the requirement for, and involvement of, MCs in the development of inflammation. Therefore, we analysed MC numbers in both UD and CD patients, although we focused on the TNBS-induced colonic inflammation model. We detected increased numbers of MCs in the colons of patients with CD or UC (Fig. 1c,d). Thus, increased numbers of MCs in the colon is a characteristic of intestinal inflammation.

To directly show the involvement of MCs in the development of intestinal inflammation, we used MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. We confirmed that immunological and inflammatory symptoms induced by TNBS treatment were identical in $Kit^{W\text{-}sh/+}$ heterozygous and $Kit^{+/+}$ homozygous mice; however, inflammatory symptoms, such as body weight loss, massive inflammatory cell infiltration and colon shortening, were restored in $Kit^{W\text{-}sh/W\text{-}sh}$ mice but not in $Kit^{W\text{-}sh/+}$ heterozygous and $Kit^{+/+}$ homozygous mice (Fig. 1e–h). Similarly, our histological and immunological analyses revealed that destruction of the colonic epithelial layer and infiltration by inflammatory cells—especially neutrophils, which were stained neutral pink and had lobulated nuclei,—were reduced in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 1f,h,i). Moreover, inflammatory signs were ameliorated in $Kit^{W\text{-}sh/W\text{-}sh}$ mice when we used other well-known inflammatory bowel disease models, such as the dextran sodium sulphate (DSS) colitis model (Fig. 2a–c). As the use of $Kit^{W\text{-}sh/W\text{-}sh}$ mice as an MC-deficient model is controversial[16,17], we also used the MC-specific enhancer-mediated toxin receptor-mediated conditional cell knockout (TRECK) system (Mas-TRECK mice)[18]. We confirmed that specific depletion of MCs ameliorated the inflammation in this DSS-induced colitis model (Fig. 2d–h). Our data indicate that activated MCs participate in the aggravation of intestinal inflammation.

Establishment of an inhibitory MC-specific mAb. IgE plus a relevant allergen induces MC activation; however, $Rag\text{-}1^{-/-}$ and $Tcr\beta^{-/-}\delta^{-/-}$ mice showed inflammatory responses comparable to those in TNBS-induced intestinal inflammation (Supplementary Fig. S2a–d)[19] and had increased numbers of $CD63^+$-activated MCs in their colons (Supplementary Fig. S2e), suggesting that T and B cells are not involved in MC activation during colitis. We also found no increase in CD63 expression on MCs after stimulation with IL-18 and IL-33, which are known to be involved in colitis (Supplementary Fig. S2f)[20,21].

We next tried to establish an anti-MC mAb that could ameliorate activated MC-mediated intestinal inflammation. We immunized rats with purified murine-activated colonic MCs, established hybridomas, performed flow cytometry to select hybridomas that produced mAbs that preferentially recognized colonic MCs and examined the hybridomas' ability to inhibit ovalbumin-induced food allergy[22] or TNBS-induced intestinal inflammation (Supplementary Fig. S3). Among 2,000 clones, we obtained an anti-MC mAb (designated clone 1F11; rat IgG2b) that was strongly reactive to colonic MCs (Fig. 3a; Supplementary Fig. S3). In addition to colonic MCs, 1F11 mAb bound efficiently to peritoneal cavity-, lung- and bone marrow (BM)-derived MCs, but not to skin MCs (Fig. 3a). When tested with other immunocompetent cells in the colon, 1F11 mAb was weakly reactive to some $CD3^+$ T cells, $CD11c^+$ DCs and $F4/80^+$ macrophages, but was not reactive to $Gr\text{-}1^+$ granulocytes, IgA^+ plasma cells or epithelial cells (ECs) (Fig. 3b).

To show the inhibitory function of 1F11 mAb in intestinal inflammation, mice were given 1F11 mAb (0.5 mg day^{-1} in a single dose) for 3 days, beginning 1 day before intrarectal administration of TNBS. 1F11 mAb treatment reduced the intestinal inflammation (Fig. 3c–g) and decreased the number of $CD63^+$-activated MCs in 1F11 mAb-treated mice (Fig. 3h).

Targeting P2X7 receptors reduces intestinal inflammation. Mass spectrometry analyses of immunoprecipitants of MC cell lysates with 1F11 mAb showed that the P2X7 purinoceptor is recognized by 1F11 mAb (Supplementary Fig. S4a). The specificity of 1F11 mAb for the P2X7 purinoceptor was confirmed by its specific reactivity to cells transfected with P2X7 receptors but not with other types of P2X receptor (for example, P2X1 and P2X4; Supplementary Fig. S4b). MCs derived from $P2x7^{-/-}$ mice, however, were not recognized by 1F11 mAb (Supplementary Fig. S4c). Western blot and flow cytometric analysis showed that, among the several variants of P2X7 purinoceptors[23], 1F11 mAb bound to variant a (full-length; Supplementary Fig. S4d,e). In contrast, variant c (possessing

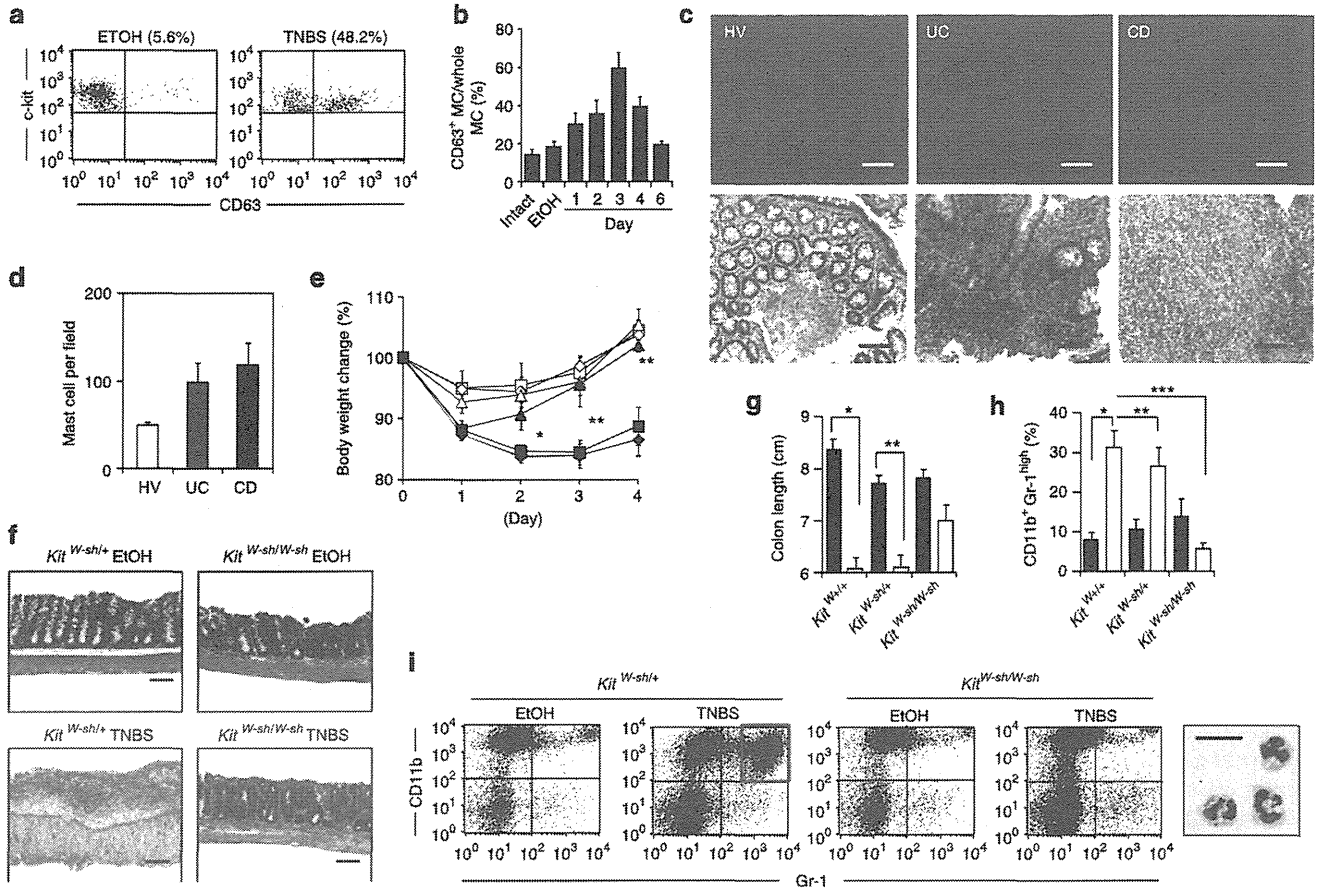

Figure 1 | Role of activated intestinal MCs in the development of intestinal inflammation. (**a**) CD63 expression on colonic MCs was examined with flow cytometry. Cells were gated on c-kit$^+$ and FcεRIα$^+$ cells. (**b**) The percentage of CD63$^+$ MCs in all c-kit$^+$ FcεRIα$^+$ MCs was determined with flow cytometry at various time points after TNBS administration ($n=3$ for day 6, $n=5$ for day 3, $n=7$ for intact, EtOH, day 1 and 2, $n=14$ for day 4). Control mice were analysed 4 days after EtOH administration (EtOH; $n=7$). Data are shown as means±s.e.m. (**c**) Colonic tissue sections from a healthy volunteer (HV) and UC and CD patients were stained with 4′,6-diamidino-2-phenyl indole (blue) and MC tryptase (red) or haematoxylin and eosin (H&E) (bottom). Scale bars, 100 μm. (**d**) Tryptase-positive MCs were counted in the fields of the tissue sections (four fields for each section). Data are means±s.e.m. ($n=6$). (**e**) Body weight changes were monitored after TNBS administration to *Kit* $^{W\text{-}sh/W\text{-}sh}$ MC-deficient mice (*Kit* $^{W\text{-}sh/W\text{-}sh}$ EtOH; open triangles: $n=4$, *Kit* $^{W\text{-}sh/W\text{-}sh}$ TNBS; closed triangles: $n=9$), *Kit* $^{+/+}$ control mice (*Kit* $^{+/+}$ EtOH; open diamonds: $n=4$, *Kit* $^{+/+}$ TNBS; closed diamonds: $n=13$) and *Kit* $^{W\text{-}sh/W+}$ control mice (*Kit* $^{W\text{-}sh/W+}$ EtOH; open squares: $n=4$, *Kit* $^{W\text{-}sh/W+}$ TNBS; closed squares: $n=11$). Data are shown as percentages of baseline weights and are means±s.e.m., $^*P<0.0001$ (two-tailed Student's *t*-test); $^{**}P=0.0024$ (two-tailed Student's *t*-test). (**f**) The colon was isolated 4 days after TNBS treatment for H&E staining. Data are representative of at least three independent experiments. Scale bars, 100 μm. (**g**) Colon length was measured 4 days after colitis induction. EtOH, closed column; TNBS, open column. $^*P<0.0001$ (two-tailed Student's *t*-test), $^{**}P=0.0024$ (two-tailed Student's *t*-test). Data are shown as means±s.e.m. (**h**) The percentage of CD11b$^+$ Gr-1^{high} cells in the colonic lamina propria was calculated, as measured with flow cytometry. EtOH, closed column; TNBS, open column. $^*P=0.0003$ (two-tailed Student's *t*-test), $^{**}P=0.0029$ (Welch's *t*-test) and $^{***}P<0.0001$ (Welch's *t*-test). Data are shown as means±s.e.m. (**i**) Colonic mononuclear cells were isolated 4 days after TNBS administration and stained with anti-CD11b and anti-Gr-1 antibodies. CD11b$^+$ Gr-1^{high} cells were sorted and then stained with May-Giemsa stain. Scale bar, 20 μm. Data are representative of three experiments.

the ATP-binding portion but lacking the C-terminal region) was detected by western blot, but its surface expression was not detected by flow cytometry because of its defect in extracellular expression (Supplementary Fig. S4d,e)[24]. In addition, neither western blot nor flow cytometry detected variant d (lacking the ATP-binding portion; Supplementary Fig. S4d,e). These data strongly suggest that 1F11 mAb recognizes P2X7 receptors, specifically the ATP-binding portion. We also confirmed that 1F11 mAb had similar reactivity to that of a commercially available anti-P2X7 mAb (clone: Hano43; Supplementary Fig. S4f,g).

To evaluate whether 1F11 mAb directly affects MCs during ATP-mediated activation, we treated MCs with ATP in the presence of 1F11 mAb *in vitro*. 1F11 mAb treatment reduced the number of CD63$^+$-activated MCs induced by ATP in a dose-dependent manner (Fig. 4a). High concentrations of extracellular ATP increased the cell permeability of the MCs[12]. Thus, uptake of Lucifer yellow was observed in ATP-stimulated MCs but was substantially impaired in 1F11 mAb-treated and *P2x7*$^{-/-}$ MCs (Fig. 4b,c).

As many cell types (MCs, T cells and DCs) express P2X7 receptors (Fig. 3b), we then asked whether the P2X7 receptors on MCs were responsible for the MC-mediated intestinal inflammation *in vivo* by analysing MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *P2x7*$^{+/+}$ or *P2x7*$^{-/-}$ MCs. We confirmed that reconstituted MCs were present in the colon and maintained P2X7 expression (Supplementary Fig. S5). Like wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *P2x7*$^{+/+}$ MCs showed severe inflammatory responses when treated with TNBS. However, these inflammatory responses were ameliorated when *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were reconstituted with *P2x7*$^{-/-}$ MCs; the amelioration included inhibition of neutrophil infiltration and MC activation (Figs 1 and 5a–f).