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PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE: THE BACTERIAL CONNECTION

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SUMMARY

The inflammatory bowel diseases are complex, idiopathic disorders whose pathogenesis is beginning to be understood largely due to the generation and investigation of many experimental models over the past decade. In most of these models, the enteric microbial bacteria are obligatory for disease expression, and in most, $CD4^+$ T-cells are the effector cells mediating chronic intestinal inflammation. Thus these disorders appear to represent disorders of host - microbial interactions in the intestine. One model, C3H/HeJBir mice that are a high susceptibility phenotype for spontaneous colitis, has been particularly informative regarding the 'bacterial connection' to IBD. C3H/HeJBir T-cells are highly reactive to enteric bacterial antigens, and such Th1 cells can mediate disease upon transfer to immunodeficient SCID recipients. Sera from C3H/HeJBir mice has been used to identify and clone 60 antigens that stimulate their pathogenic T-cells and B-cells. The adaptive response to the microbiota is thus highly selective, even in the setting of chronic intestinal inflammation. Interestingly enteric bacterial flagellins comprised the major group of these antigens, and about half of patients with Crohn's disease have IgG antibodies to them as well. There are emerging data from both experimental models and humans that the innate immune system plays a major role in the host interaction with the microbiota and that defects in innate immune cells, such as epithelial cells, dendritic cells, and macrophages, direct and shape the abnormal T-cell and B-cell immune responses to enteric microbial antigens that result in IBD.

INTRODUCTION

The term "inflammatory bowel diseases (IBD)" comprise two idiopathic chronic inflammatory diseases of the intestine, ulcerative colitis and Crohn's disease. Similar to other chronic inflammatory and autoimmune conditions, IBD appears to involve complex interactions among environmental, immune, and genetic factors. Over the past decade many new experimental models of chronic intestinal inflammation have been developed that are providing insights into the pathogenesis of these disorders (Table 1). Many of these models have resulted from either deletion of a gene or to insertion a gene into a mouse to generate an "induced mutant" strain. Hundreds of immu-

Table 1: Selected experimental models of inflammatory bowel disease.

Excess T-cell effector function	Deficient T-cell regulation	Defective innate immune response	Spontaneous
STAT4 Tg	CD45RB^{hi} transfer	mdr1a$^{-/-}$	C3H/HeJBir
IL-7 transgenic	IL-10$^{-/-}$	Keratin-8$^{-/-}$	SAMP1/Yit ileitis
TNF-α "knock-in"	IL-10Rß$^{-/-}$	Gai1$^{-/-}$	
CD40L transgenic	Macrophage-PMN	Conditional STAT3$^{-/-}$	
	STAT3$^{-/-}$	NF-κB p65$^{+/-}$ p50$^{-/-}$	
	IL-2$^{-/-}$	A20$^{-/-}$	
	IL-2Ra$^{-/-}$		
	BM-->Tge26		
	TGFß$^{-/-}$		
	TCRa$^{-/-}$		

nologic genes have been selectively deleted or transgenically over-expressed in mice. A small subset of these induced mutants have developed IBD in the absence of further manipulation. Acommon feature in these induced mutant mice that develop colitis has been that the normal intestinal microbiota is the stimulus driving the inflammatory disease and that CD4$^+$ T-cells are the effector cell mediating disease in most of them (for review see: *Elson* and *Weaver*, 2003). These experimental models have provided strong support for the immunologic hypothesis that IBD is due to a dysregulated mucosal immune (CD4$^+$) T-cell response to enteric bacterial antigens in a genetically susceptible host.

The intestine is the major interface between the host and the external environment. There have been major advances in revealing the mechanisms by which pathogens interact with the immune system, but little is known about how commensal bacteria in the intestine interact with the host and how the host response to them is regulated. It is clear that the microbiota has profound effects on the intestine and on the host. Such effects were highlighted by a recent study in which germfree mice were mono-colonised with *Bacteroides thetaiotaomicron*, a commensal that resides in the gut lumen and does not approach the epithelium itself. Such colonisation resulted in dramatic changes in gene expression in the epithelium and in the lamina propria. The concept is emerging that there is a circuit of interactions among the microbiota, the epithelium, and the immune system in the intestine, and that the communication continues throughout life. Moreover, abnormalities of this circuit can result in chronic intestinal inflammation.

One of the barriers to investigation of the interaction of the host with the microbiota is the latter's sheer complexity, comprising an estimated 500-1000 species. The aggregate number of genes associated with the microbiota, the "microbiome", has been estimated at 2-4 million genes (*Xu* et al., 2003). The microbiota has profound effects on the development of the intestine, including the epithelial layer (*Hooper* and *Gordon*, 2001; *Hooper* et al., 2001), the mucosal immune system (*Cebra*, 1999), and the enteric nervous system. In regard to the mucosal immune system, certain strains such as segmented filamentous bacteria are highly stimulatory and others are not (*Klaasen* et al., 1993; *Umesaki* et al., 1995). The reasons for these differences are presently unknown. One concept is that microbes that are resident in the

mucus layer are more stimulatory to the immune system, but data supporting this idea are lacking. Clearly bacteria that reside only in the lumen can have profound effects on the intestine, particularly on epithelial cells (*Hooper* et al., 2001). Information about the effects of the anaerobic microbiota is particularly lacking because of the difficulty in culturing them; yet these are the majority of the intestinal flora.

The molecular basis of the immune recognition of microbes is being defined (*Janeway* and *Medzhitov*, 2002). Innate immune and other cells have on their cell surface a set of receptors that recognise microbial products. These receptors are called pattern recognition receptors (PRR) in that they bind to and are triggered by selected microbial structures that form a molecular pattern (*Underhill* and *Ozinsky*, 2002). These microbial structures have been termed "pathogen-associated molecular patterns" (PAMPs) but the same molecular patterns are present on non-pathogenic commensal bacteria. The best studied of these PRRs is the Toll-like receptor (TLR) family which is comprised of at least 10 members (*Akira* and *Hemmi*, 2003). PRR are of ancient origin and appear to be conserved in insects, plants, and animals. The molecular patterns that they discern include lipopolysaccharide, bacterial DNA, bacterial peptidoglycan, flagellins, etc. Different TLRs can distinguish among different types of bacteria, for example TLR2 responds to Gram-positive bacterial ligands, whereas TLR4 responds mainly to endotoxin of Gram-negative bacteria (*Takeuchi* et al., 1999; *Yoshimura* et al., 1999; *Hirschfeld* et al., 2001; *Re* and *Strominger*, 2001). The TLRs are clearly important in host defence against pathogens but are also likely crucial in the host interaction with the intestinal microbiota (*Cario* and *Podolsky*, 2000; *Cario* et al., 2000; *Ortega-Cava* et al., 2003). NOD1/ CARD4 and NOD2/ CARD15 are intracytoplasmic PRRs that bind to the muramyl dipeptide component of bacterial peptidoglycan (*Girardin* et al., 2003; *Inohara* et al., 2003). When PRRs bind their ligand, they activate the NF-κB signalling pathway, which, in turn, activates many immune system genes. For most of the TLR family an important adapter protein, MyD88, is required for initiation of the signalling cascade through IRAK-1 (*Barton* and *Medzhitov*, 2003).

C3H/HeJBir AS MODEL SYSTEM TO STUDY INNATE AND ADAPTIVE IMMUNE RESPONSE TO THE MICROBIOTA

An experimental model that has provided insight into the host interaction with the microbiota is the C3H/ HeJBir mouse, a sub-strain of the common C3H/HeJ strain. The C3H/ HeJBir mouse was generated by a program of selective breeding for peri-anal ulceration and soft faeces (diarrhoea) that was observed sporadically in C3H/ HeJ mice due to focal chronic inflammation in the caecum and right colon. These lesions developed by 2-4 weeks of age, coinciding with bacterial colonisation, and largely resolved by 8-12 weeks of age (*Sundberg* et al., 1994).

Analysis of immune cells and function in colitic C3H/HeJBir mice vs. the parental C3H/HeJ strain has revealed few differences between the two strains. Both strains have mutations in TLR4 rendering them unresponsive to the effects of bacterial endotoxin. The major difference is that the C3H/ HeJBir substrain has increased levels of S-IgA in the intestine, high levels of serum IgG antibodies to commensal bacterial antigens, and increased T cell responses to intestinal microbial antigens.

B-cell response to microbiota

C3H/HeJBir mice do not respond to food or epithelial cell antigens, but have high titre IgG antibodies, mainly IgG2a, to antigens of the commensal bacterial flora (*Brandwein* et al., 1997). Serum from control C3H/HeJ mice has no reactivity to the enteric bacteria under the same conditions. A striking feature of the C3H/HeJBir serum antibody reactivity to the flora is that it is highly selective: Only a very small subset of antigens of the enteric bacterial flora is detected out of the thousands of bacterial proteins that are present. Trypsin treatment of the microbial preparation abolishes reactivity indicating that these are proteins or glycoproteins. The pattern of bands on Western blots was highly reproducible within different cohorts of C3H/HeJBir mice. When specific species of enteric bacteria were tested by Western analysis, there was no relationship between the relative abundance of a bacterial species and the number of bands detected. Quantitative analysis of antibody reactivity against *E. coli* outer membrane antigens demonstrated more than a 100,000-fold increase in reactivity in C3H/HeJBir serum IgG compared to C3H/HeJ. These data are consistent with an abnormal B-cell adaptive immune response to antigens of the enteric bacterial flora in the C3H/ HeJBir strain. The timing of the appearance of antibody relative to the development of caecal or colon lesions was not consistent with these antibodies playing a pathogenic role, however they did identify the antigens stimulating the pathogenic adaptive response (*Brandwein* et al., 1997).

T-cell response to microbiota

C3H/HeJBir $CD4^+$ T-cells failed also to respond to food or epithelial antigens but did respond vigorously to antigens of the commensal bacteria with both proliferation and cytokine production. The kinetics of the response was typical of an antigen-specific rather than a mitogenic or super-antigen response. The precursor frequency of bacterial-reactive, IL-2 producing $CD4^+$ T-cells was 1:2000 in C3H/HeJBir mice compared to 1:25,000 in normal C3H/ HeJ mice. Bacterial-reactive $CD4^+$ T-cells were detectable by 4 weeks of age in C3H/HeJBir mice, concomitant with the age of onset of disease. The cytokine pattern of these $CD4^+$ T-cells, mainly IL-2 and IFN-γ, was consistent with a Th1 subset response to the enteric bacterial antigens (*Cong* et al., 1998).

Transfer of disease by $CD4^+$ T-cells

To determine the pathogenic potential of these bacterial-reactive C3H/ HeJBir T-cells, adoptive transfer experiments were done. C3H/HeJBir $CD4^+$ T-cells were activated with commensal antigen-pulsed APCs for 4 days *in vitro*, then adoptively transferred into histocompatible C3H/HeSnJ $Prkdc^{scid}/Prkdc^{scid}$ (SCID) recipients. The SCID mice developed a focal colitis over the ensuing 2 months, lesions similar to that observed in the C3H/ HeJBir donor. Adoptive transfer of bacterial antigen-activated control C3H/HeJ $CD4^+$ T-cells, or of anti-CD3-activated C3H/HeJBir $CD4^+$ T-cells did not result in colitis in the SCID recipients, indicating that non-specific activation was insufficient. This was the first formal demonstration that $CD4^+$ T-cells reactive with conventional antigens of the commensal bacterial flora can mediate chronic inflammatory bowel disease.

A series of $CD4^+$ T-cell lines reactive with commensal bacterial antigens was derived subsequently from C3H/ HeJBir (Bir) mice by repeated cycles of stimulation with antigen-pulsed APCs followed by intervals of rest. All of these $CD4^+$ T-cell lines produce mainly Th1-type cytokines when re-stimulated *in vitro* and all are pauciclonal based on analysis of the TCRßV repertoire util-

ised. Most of these memory T-cell lines induced focal colitis uniformly after transfer to SCID recipients with increased levels of IL-12p40 and IFN-γ mRNA and protein detected in the lesions. Administration of anti-CD40L to SCID recipients of pathogenic Bir CD4 T-cell lines blocked the development of colitis. Thus interactions between CD40L on pathogenic $CD4^+$ T-cells with CD40 on mucosal APCs endogenously loaded with commensal bacterial antigens is critical for a sustained increase in IL-12 and thus progression to colitis (*Cong* et al., 2000).

Tr1 regulation of pathogenic bacterially-reactive T-cells *in vivo*

The above studies on T-cell reactivity to commensal antigens in C3H/ HeJBir mice, including the derivation of the long-term cell lines, utilised mice 10-12 weeks old that no longer had any evidence of gut inflammation. The presence of pathogenic $CD4^+$ T-cells in the absence of lesions implied that regulatory T-cells might be preventing the expression of pathogenic T-cell function *in vivo*. This would be compatible with results in other model systems such as the $CD4^+$ $CD45RB^{high}$ T-cells adoptive transfer system in which regulatory T-cells can prevent the effects of potentially pathogenic T-cells *in vivo*, at least in part by production of IL-10 (*Groux* et al., 1997; *Asseman* et al., 1999).

We asked whether T regulatory cells could be detected in adult C3H/HeJBir mice, and if so what were their properties. A C3H/HeJBir $CD4^+$ T-cell line, named Bir 8, was generated in the presence of IL-10 (*Cong* et al., 2002). The Bir 8 line produced high levels of IL-10, low levels of IL-4 and IFN-γ, and no IL-2, consistent with the phenotype of Tr1 cells (*Groux* et al., 1997). The Tr1 cells proliferated poorly to caecal bacterial antigen (CBA) stimulation compared to Th1 or Th2 lines with similar specificity, but proliferation of all three types was dependent on CD28-B7 interactions and was MHC class II restricted. CD40 blockade did not change IL-10 production significantly. Transfer of Bir 8/Tr1 cells into SCID mice did not result in colitis, although transfer of a pathogenic CBA-reactive $CD4^+$ Th1 cell line did induce colitis. Co-transfer of Bir 8/Tr1 cells with pathogenic $CD4^+$ Th1 cells prevented such colitis. Bir 8/Tr1 cells also inhibited the proliferation and IFN-γ production of a CBA-specific Th1 cell line *in vitro*. Addition of anti-IL-10 or anti-IL-10R mAb partially reversed this inhibition. Thus, CBA reactive Tr1 $CD4^+$ T-cells can be generated from C3H/HeJBir mice. These Tr1 cells inhibited pathogenic Th1 cells *in vivo* and *in vitro*. $CD4^+$ T-cells were isolated from the lamina propria of normal C3H/HeJ mice had properties of Tr1 cells, producing IL-10 only. These lamina propria $CD4^+$ T-cells inhibited a pathogenic Th1 cell line in the presence of CBA-pulsed APC, but not in the presence of anti-CD3 mAb, indicating the inhibition was CBA specific. Addition of anti-IL-10 or anti-IL-10R mAb partially reversed the inhibition. Thus, enteric bacterial antigen-specific T-cells with activity similar to Tr1 cells are present in the murine lamina propria, and can inhibit pathogenic $CD4^+$ T-cells, at least partially through production of high amounts of IL-10.

IDENTIFICATION OF THE ANTIGENS STIMULATING PATHOGENIC ADAPTIVE IMMUNE RESPONSES

A major limitation to progress has been the lack of information on the identity of the microbial products stimulating pathogenic T-cells and B-

cells. We have recently resolved this problem using serologic expression cloning. This strategy was based on our earlier observations that C3H/HeJBir mice develop IgG2a (Th1 dependent) antibodies to a limited, but reproducible set of bacterial protein antigens (*Brandwein* et al., 1997). Serum from C3H/HeJBir mice was used to probe a caecal bacterial DNA phage lambda library. A small number of immunodominant antigens was identified, cloned and sequenced. Unexpectedly, the major class of antigen was commensal bacterial flagellins, representing 25% of the 56 proteins cloned (*Lodes* et al., 2004). Two of these flagellins, CBir1 and FlaX, were studied in detail. Serum IgG anti-flagellin was identified in three different mouse models and in roughly half of patients with Crohn's disease, but not in patients with ulcerative colitis or in normal controls. CBir1 flagellin stimulated pathogenic Th1 cells in two mouse models and CBir1-reactive Th1 cells were able to induce colitis upon transfer to SCID recipients. Subsequently a similar serologic expression cloning has been done using serum antibodies from mdr1a$^{-/-}$ mice (unpublished data), and again, 25% of the antigens identified were bacterial flagellins. These data are consistent with the hypothesis that there is a limited set of immunodominant antigens in the microbial flora that activate pathogenic effector T cells and thus induce IBD. Some of these antigens may be peculiar to one strain or model, but others such as the flagellins appear to cross both strains and species.

Flagellins are bacterial proteins that assemble in long polymers to form the bacterial flagellum (*Samatey* et al., 2001). Flagellins have conserved amino and carboxy domains which are connected by a hypervariable region of variable length (*Eaves-Pyles* et al., 2001a). The conserved amino and carboxy domain form the polymerisation site that is necessary for bacterial motility (*Smith* et al., 2003). The amino and carboxy domains are sufficiently conserved as to allow phylogeny trees to be developed showing relatedness of different bacteria (*Winstanley* and *Morgan*, 1997). Flagellins are strong antigens and the immune responses to them are protective in certain intestinal infections such as with *Salmonella* (*McSorley* et al., 2000). Flagellins are the ligand for TLR5 (*Hayashi* et al., 2001) and have potent effects on the host, including a sepsis-like syndrome (*Eaves-Pyles* et al., 2001b). Because of their potent effects on innate immune cells they can serve as adjuvants for other antigens (*das Gracas Luna* et al., 2000; *McSorley* et al., 2002). Thus flagellins have both adjuvant activity and are strong immunogens, properties that might account for their strong representation in the expression cloning.

INNATE IMMUNE INTERACTIONS WITH THE MICROBIOTA SHAPE THE ADAPTIVE IMMUNE RESPONSE

The innate immune system appears to play a major, if not predominant, role in host interaction with the microbiota. For example, mice that have innate immunity but lack T-cells and B-cells such as SCID or RAG$^{-/-}$ mice are able to live in harmony with the commensal flora. MacPherson and colleagues have identified a T-cell-independent pathway stimulating S-IgA responses to commensal bacteria that involves direct interactions between bacteria bearing dendritic cells and mucosal B-cells (*Macpherson* et al., 2000; *Macpherson* and *Uhr*, 2004). The latter interaction does not stimulate serum IgG or spleen T cell responses. We have completed a study of the immune response to 20 re-

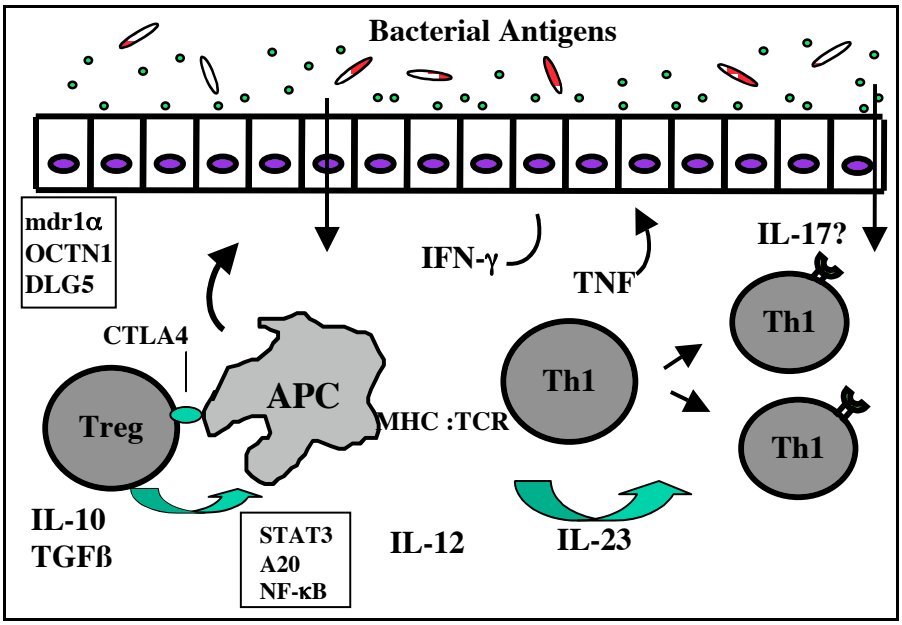

Figure 1: A schematic representation of the cells, cytokines and other factors that maintain normal intestinal homeostasis in the face of the huge challenge of the microbial flora. Genes important in maintaining homeostasis in the innate immune system of the epithelium or antigen presenting cells are shown in the boxes. (IL: interleukin; IFN-γ: Interferon-γ).

combinant proteins randomly derived from the microbiota (see Preliminary Data). Although S-IgA responses were detected to many of these proteins, no serum IgG or systemic T-cell response was identified to any of them in normal C3H mice, nor was there any evidence of immune tolerance to them (*Konrad* et al., 2003), similar to the results of Macpherson. Thus, the concept is emerging that there is a mucosal compartmentation of immune interactions with the microbial flora in normal hosts (*Becker* et al., 2003; *Uhlig* and *Powrie*, 2003), which effectively avoids inflammation or activation of systemic T-cells or B-cells. In colitic mice this innate pathway is subverted and microbial-reactive T-cells and IgG B-cells are activated to a set of immunodominant antigens of the microbiota. Microbial flagellins form a substantial fraction of this small number of immunodominant antigens, perhaps because they act as both adjuvant and antigen. It is well known that the innate immune system activates and thus directs the T-cell response to vaccine antigens (*Bendelac* and *Medzhitov*, 2002) and the same is likely true for endogenous priming to antigens of the microbiota (*Kapsenberg*, 2003).

The epithelium forms an important part of the host innate immune response to the microbiota, and it has been hypothesised that defects in the epithelium might result in IBD. Indeed, there are several mouse models where this appears to be the case, the most compelling of which is the multidrug resistance gene 1 alpha knockout (mdr1a$^{-/-}$) mouse. The mdr1a$^{-/-}$ gene encodes the P-glycoprotein transport protein that is expressed in the epithelium and in some lymphoid cells. Deficiency of this gene in mice results in colitis. Interestingly, bone marrow chimeras have demonstrated that colitis develops in mice that have an mdr1a$^{-/-}$-deficient epithelium but a normal bone

marrow compartment (*Panwala* et al., 1998). Although an abnormality of the epithelium appears to be the primary abnormality, this epithelial abnormality translates somehow into a pathogenic CD4 T-cell effector response to the microbiota and it is these T-cells that directly mediate the colitis. A single nucleotide polymorphism of the *mdr1a* gene has been linked to ulcerative colitis in humans (*Ho* et al., 2003). In addition, two recent reports have identified epithelial related genes causing susceptibility to IBD. The *OCTN1* cation transporter gene on Chr.5 was linked to Crohn's disease (*Peltekova* et al., 2004), and the DLG5 gene on Chr.10, which encodes a scaffolding protein involved in maintenance of epithelial integrity, was linked to IBD (*Stoll* et al., 2004). Interestingly, the *OCTN1* gene appears to interact with the *CARD15/NOD2* gene previously identified as a major susceptibility gene for Crohn's disease (*Hugot* et al., 2001). The epithelium is now recognised as a crucial component of the innate immune system and as such likely directs the adaptive immune response but the detailed mechanisms involved in how this happens remain undefined.

There are certain genes that are important in innate immune interactions with the microbiota, including the STAT3 transcription factor expressed in myeloid cells (*Kobayashi* et al., 2003; *Welte* et al., 2003) and PPARg (*Kelly* et al., 2004) (Figure 1). *Kobayshi* et al. (2003) have shown recently in mice with a myeloid cell-specific deletion of STAT3 that LPS stimulation of IL-12p40 via TLR4 on innate immune cells then induces a vigorous Th1 response and IBD. An important concept that is arising from recent data is that genetic defects that impair the innate immune system's ability to deal with the microbial flora can result in a more vigorous adaptive immune response to them and thus lead to inflammation. Supporting this concept is the discovery that a loss of function mutation in the NOD2/CARD15 PRR in humans results in susceptibility to Crohn's disease (*Bonen* et al., 2003). This mutation is likely to impair the innate immune response to as yet undefined microbes. We have recent data that indicates that the innate immune response to TLR ligands is impaired in colitis-susceptible C3H/HeJBir mice as compared to the more colitis-resistant C57BL/6 strain. Moreover, a colitis susceptibility gene locus on Chr3, Cdcs1 (*Farmer* et al., 2001), appears to regulate the innate immune response to TLR ligands as well as the $CD4^+$ T-cell response to microbial antigens. These data support the notion that impaired innate immune responses to the microbial flora may be a common pathogenic factor in both experimental and human IBD.

CONCLUSION

The inflammatory bowel diseases are complex disorders with genetic, immune, and environmental components that interact to generate disease. In the past decade, many experimental models have been generated that have allowed insights into these various components. Data from these models has shown that a dysregulated immune response to the enteric microbial flora can result in IBD. Studies in humans are converging with what has been learned in mouse models. For example, the CARD15/NOD2 susceptibility gene for Crohn's disease is a bacterial pattern recognition receptor. This report has focused on this 'bacterial connection', particularly the recent identification of flagellins as dominant antigens of the microbial flora that stimulate adaptive immune responses in

multiple mouse models and in patients with Crohn's disease. We expect our knowledge about these bacterial connections to IBD will expand substantially in the near future.

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