



## Progress in basic inflammatory bowel disease research

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A modern approach to inflammatory bowel disease (IBD) research has been under way for little over one-half century, but only during the last two decades has progress accelerated and finally generated tangible results that have been translated into practical and better therapeutic strategies. The areas where progress has been more evident are those currently believed to be the key components of IBD pathogenesis, and include the environment, genetics, enteric microbiology, and immune reactivity. Progress in these different areas has been somewhat uneven, yielding a better understanding of the mechanisms behind gut inflammation and tissue injury rather than of specific etiological agents or predisposing factors. However, with the rapidly increasing utilization of novel methodological approaches like genetics, genomics, proteomics, and pharmacogenomics, it is reasonable to anticipate that the etiopathogenesis of IBD will be unveiled in the next couple of decades and more definitive, perhaps disease-modifying, approaches will be uncovered and implemented.

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The two main forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), still pose major clinical and therapeutic challenges, as their exact etiology remains obscure and the mechanisms of inflammation appear exceedingly complex. It is now well established that CD and UC represent two distinct forms of chronic inflammation and have different causes and discrete mechanisms of tissue damage.<sup>1,2</sup> However, several factors underlying both CD and UC are shared, including a temporal association with environmental changes, an intrinsic genetic predisposition, the enteric flora, and an abnormal immune reactivity responsible for causing gut inflammation and local damage, and ultimately inducing clinical manifestations. A new notion that is being increasingly accepted is that IBD may be different in children and adult patients, and that pediatric IBD may represent an entity on its own, with

distinct susceptibility and inflammatory mechanisms.<sup>3</sup> In this review, the major factors leading to CD and UC will be presented and discussed in the general context of IBD and pediatric IBD in particular.

### Environmental factors

The well-documented increase in the incidence and prevalence of IBD is part of a world-wide emergence of chronic autoimmune and inflammatory diseases, a phenomenon closely linked to social and economical development.<sup>4</sup> Initially noted in Northern Europe and North America, this increase has been documented in the rest of Europe, Japan, and South America, and most recently in the Asian Pacific Region.<sup>5</sup> An explanation for the higher frequency of IBD has been linked to the "hygiene hypothesis," which postulates that there has been a fundamental lifestyle change from one with high to one with low microbial exposure.<sup>6</sup> Exposure to fewer microbial antigens early in life would lead to a less robust immune system,

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ill prepared to tackle a variety of exogenous challenges later on in life, and mounting immune responses unable to eliminate offending agents and thus resulting in chronic inflammation.

Numerous environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography and social status, stress, the enteric flora, altered intestinal permeability, and appendectomy.<sup>7</sup> Among them, cigarette smoking is the best documented, having a detrimental effect in CD and a protective effect in UC, suggesting distinct pathogenic pathways in each form of IBD.<sup>8</sup> Oral contraceptives and nonsteroidal antiinflammatory drugs have also been investigated, but a direct causal relationship with IBD has not been firmly established.<sup>9</sup> Particularly relevant to the pediatric and young adult population is appendectomy, which has also been proposed as protecting against UC, whereas favoring the appearance of CD.<sup>10,11</sup>

The epidemiological evidence linking environmental factors to IBD is convincing, but environmental factors alone cannot directly cause CD or UC, and an intrinsic predisposition must also be present, and this is based on genetic susceptibility.

## Genetic susceptibility

### Association with specific gene mutations

During the last 6 years, a number of significant genetic associations have been identified in IBD. The initial breakthrough came in 2001, with the identification of the first susceptibility gene for CD on chromosome 16.<sup>12,13</sup> This gene is *CARD15/NOD2*, and its product is a cytoplasmic protein involved in microbial sensing. Three mutations (R702W, G908R, and 1007fsinsC) are independent risk factors for CD in Caucasians, but they are absent in Asians, Arabs, Africans, and African Americans.<sup>14</sup> Even among white CD patients, a great amount of genetic heterogeneity exists, and individuals with one of the disease-associated alleles have a 2- to 4-fold increased risk for developing CD, whereas homozygous or compound heterozygous carriers have an up to 40-fold increase in genotype-relative risk. Despite their strong association with CD, genetic alterations of the *CARD15/NOD2* gene are neither sufficient nor necessary for the development of CD. In fact, most CD patients do not carry *CARD15/NOD2* mutations, and its estimated penetrance (the probability to develop the disease) is only about 5% to 10%.

The IBD5 locus on chromosome 5 was first identified in a genome-wide linkage scan, and its association with CD was subsequently confirmed and narrowed to an 11-SNP (single nucleotide polymorphism) haplotype.<sup>15,16</sup> Two novel polymorphisms in the solute carrier family 22A4/22A5 genes were identified and proposed as two CD-associated alleles encoding the organic cation transporters 1 and

2, respectively. Interestingly, the IBD5 locus was initially associated with childhood CD, raising the hope that IBD5 may represent an early onset CD gene. However, recent studies from pediatric onset CD cohorts showed that the IBD5 risk is only modest and lower compared with adult onset CD.<sup>17</sup>

Stoll and coworkers described three IBD-associated genetic variations in the disk large homolog 5 (*DLG5*) gene, which encodes a scaffolding protein involved in the maintenance of epithelial integrity/proliferation.<sup>18</sup> Subsequent report suggested that *DLG5* does confer IBD susceptibility, indicating only a modest susceptibility risk for CD (OR 1.5). More interestingly though was the finding of a marked increase in risk for men (OR 2.49) but not women (OR 1.0).<sup>19</sup>

Several other IBD loci (IBD3, IBD4, and IBD6–9) and alleles have been proposed and expected to confer relatively low susceptibility risks. Modifying loci on chromosomes 4q23–25 and on 7q have been described, suggesting a role of the *NF-κB1* gene expression and of the multidrug resistance 1 gene (*MDR1*), respectively.<sup>14</sup> Intriguingly, *MDR1*<sup>-/-</sup> mice have been shown to develop spontaneous colitis.<sup>20</sup> Given the pharmacogenetic significance of *MDR1* combined with its importance in mediating intestinal epithelial homeostasis, which unveils the role of this transporter gene, could provide important insights into the pathogenesis and treatment of IBD.

Using a whole genome association approach, a highly significant association of CD susceptibility (although also UC) with variation in the gene encoding the interleukin-23 receptor (IL-23R) has been recently reported.<sup>21</sup> IL-23 is a pro-inflammatory cytokine member of the IL-12 family and is known to drive pathologic inflammatory responses. Interestingly, the initial study cohort was limited to ileal CD, and IL-23 is known to be overproduced in the terminal ileum and upregulated in CD.<sup>22</sup> Previous work in mouse models has highlighted the role of IL-23 in activating both the innate immune response and effector T cells, as well as in perpetuating organ-specific inflammatory responses.<sup>23</sup>

### Clinical implications of genetic discoveries in IBD

The currently proposed genetic model for IBD phenotypes emphasizes complex interactions between environmental factors and promoting/modifying genetic determinants, resulting in the clinical expression of disease in the gastrointestinal tract of genetically predisposed individuals.

*CARD15/NOD2* mutations have been studied extensively in genotype–phenotype correlation studies. *CARD15/NOD2* variants are consistently associated with younger age at onset, ileal involvement, and tendency to develop strictures or fistulas.<sup>24</sup> A significant gene dosage effect has been observed for CD site and complications. For instance, at least 95% of the patients homozygous for *CARD15/NOD2* mutations present with ileal lesions.<sup>14</sup> Existing data still are conflicting as to whether *CARD15/NOD2* carriage is

associated with a more severe disease course, suggesting that this feature depends on modifying genes and/or environmental risk factors. Structuring complications leading to early surgery were found more frequently in children with the *CARD15* 1007fs mutation compared with children without mutations, conferring a 6.6-fold increased risk.<sup>25</sup>

The results with *CARD15/NOD2* have shown a gene-dosage effect consistent with a recessive model and a predisposition to the development of lesions in the terminal ileum where Paneth cells are abundant. Mice lacking *CARD15/NOD2* mount a defective defense against *L. monocytogenes* accompanied by a decreased expression of Paneth cell-derived cryptdins.<sup>26</sup> Consistent with these findings, CD patients homozygous for *CARD15/NOD2* mutations show decreased expression of Paneth cell  $\beta$ -defensins HD-5 and HD-6.<sup>14</sup> Further work could clarify how such immunodeficiencies affect the gut flora and promote intestinal infection and inflammation.

### Future of genetic testing in IBD

At present, we have few, if any, objective markers to predict the future course of IBD at the time of diagnosis. It is apparent now that reclassification of IBD into subgroups on the basis of genetics and genomics, complemented by proteomics and other biomarkers, will provide the tools to identify at-risk individuals, so they may be treated with "individualized" therapies. This may be particularly important for children who may be considered as representing the early stages of IBD. It is likely that such tools will not just involve single gene tests, but a panel of genetic (DNA) markers (IBD chip technology) and gene expression (RNA) profiles, which are more dynamic biomarkers than DNA. At present, few advances in IBD genetics are ready for "prime time" clinical applications. Such tests are currently being developed, with the hope that this will help understand IBD pathogenesis and, ultimately, prevent disease.

## Microbial factors

### Pathogenic organisms

That infectious agents may be the cause of IBD has been a popular hypothesis for many years, and several microorganisms, such as *L. monocytogenes*, *C. tracomatis*, *E. coli*, *Cytomegalovirus*, and *S. cerevisiae*, have been proposed as potential pathogens. *M. paratuberculosis* is the agent that has received the most attention as a possible cause of CD. Initially isolated from CD tissues,<sup>27</sup> the vast majority of follow-up studies failed to confirm its presence by histological examination, culture of tissue homogenates, genomic identification, and serum antibodies. Controlled therapeutic trials also failed to show a consistent beneficial effect of antituberculous therapy in CD patients.<sup>28</sup> More recently, an

entero-adhesive/invasive strain of *E. coli* has been described as being associated with ileal CD,<sup>29</sup> but its potential etiological role remains unclear.

The measles virus has also been implicated as possible cause of CD based on initial findings of paramyxovirus-like particles in affected tissues, where the virus would cause a chronic vasculitis in the mucosa.<sup>30</sup> An association between perinatal measles and an increased probability to develop CD was also proposed,<sup>31</sup> but never fully substantiated. Overall, the worldwide decline of measles infection with the concomitant rise of CD during several decades speaks against an etiological role of measles in CD.

### Commensal bacteria

Instead of specific infectious agents, evidence continues to accumulate that the indigenous commensal flora of the gut is the target of the chronic immune response in IBD.<sup>32</sup> Data from IBD animal models leave little doubt that the enteric flora is necessary to develop experimental colitis, as gut inflammation fails to develop in a germ-free environment, and an immune response against enteric bacteria seems essential to disease pathogenesis.<sup>33</sup> The paradigm "no bacteria, no colitis" has been created to underscore the central role of the intestinal microbiota in IBD, and is supported by a variety of clinical observations in IBD patients. An increased number of bacteria is found in close contact with the mucosa in IBD patients<sup>34</sup>; IBD lesions occur preferentially in segments with the highest concentrations of bacteria; fecal stream diversion prevents reappearance of CD, whereas restoration induces disease recurrence<sup>35</sup>; antibiotics and probiotics attenuates inflammation; the pouchitis that develops in postcolectomy UC patients is associated with a bacterial dysbiosis likely induced by the contact of the once near sterile small bowel with the rich colon-like flora repopulating the pouch.<sup>36</sup>

The majority of IBD patients show an enhanced immunological reactivity against gut bacterial antigens. Bacterial flagellin has been recently reported as a dominant antigen in CD, apparently defining a population of patients with complicated CD.<sup>37</sup> This broad antibacterial reactivity has been proposed to be caused by a "loss of tolerance" toward the autologous enteric flora, resulting in an inappropriate immune response manifested by the chronic inflammation typical of CD and UC.<sup>38</sup> In health, there is an intimate and tightly regulated interaction between commensal intestinal bacteria and the mucosal immune system, a complex interplay resulting in the development of immune tolerance.<sup>39</sup> Why tolerance is lost and an abnormal response to otherwise normal gut bacteria develops in IBD is still unclear. The discovery that CD is associated with mutations of the *NOD2/CARD15* gene, whose product is a bacteria-sensing cytoplasmic protein, suggests that the ability of the immune system to normally recognize the gut flora may be genetically altered in IBD.<sup>40</sup>

## Immune factors

Inflammation is the most common type of reaction that the body mounts against external or internal offending agents. The gut is particularly susceptible to inflammation because, even under normal circumstances, a baseline “physiological inflammation” is present in the mucosa, representing a controlled immune response against dietary and microbial antigens.<sup>41</sup> When this physiological response becomes excessive and chronic, it invariably leads to injury, resulting in anatomical and functional abnormalities. Major advances have occurred during the past few decades in our understanding of the cellular and molecular mechanisms mediating mucosal immunity and the alterations that lead to chronic gut inflammation.<sup>32</sup>

## Innate immunity

The discovery that CD patients with small bowel and stricturing disease display mutations of the *NOD2/CARD15* gene, whose product is found primarily in cells mediating innate immunity, such as macrophages and dendritic cells, and recognizes bacteria-derived muramyl dipetide (MDP), has drawn attention to the role of innate immunity in IBD pathogenesis. Of interest, the protective function of *NOD2/CARD15* as an antibacterial factor is lost when cells express the 3020insC mutant associated with CD,<sup>42</sup> suggesting a defective capacity to limit bacterial invasion in IBD. Dendritic cells are potent antigen-presenting cells pivotal to the balance between tolerance and active immunity and may control whether or not inflammation follows recognition of commensal bacteria.<sup>43</sup> Dendritic cells display evidence of activation in IBD mucosa, with increased levels of toll-like receptor (TLR) 2 and TLR4 (which mediate recognition of bacterial lipoproteins and lipopolysaccharide, respectively) and CD40, and enhanced production of IL-12 and IL-6,<sup>44</sup> suggesting a prominent role of these antigen-presenting cells in IBD pathogenesis. Epithelial cells are also involved in innate immunity. Interestingly, ileal Paneth cells also express the *NOD2* protein, and their production of mucosal  $\alpha$ -defensins is decreased in CD patients with *NOD2/CARD15* mutations.<sup>45</sup> However, whereas the expression of human  $\beta$ -defensins (HBD) increases in the presence of gut inflammation, this does not occur in CD in regard to HBD-2 and -3.<sup>46</sup> The combined defects of *NOD2* mutations and diminished defensin expression could lead to defects in the resistance against enteric microorganisms and possibly result in bacteria-induced mucosal inflammation.

Another crucial component of innate immunity are the TLRs, cell surface molecules that also detect normal and pathogenic microbial agents and can trigger antimicrobial host defense responses.<sup>47</sup> TLRs are abundantly expressed on the surface of monocytes, macrophages, and dendritic and epithelial cells, and recognize not only pathogenic microorganisms, but are also essential to identify the commensal microflora and maintain intestinal homeostasis.<sup>48</sup> Con-

sidering their strategic importance, abnormalities of TLR expression or function may be critically involved in the development or persistence of gut inflammation. TLR4 is overexpressed in CD epithelial cells and TLR3 is down-regulated, whereas the expression of TLR2 and TLR5 remains unchanged.<sup>49</sup> Polymorphisms have also been described in IBD in regard to the *TLR4* and *TLR9* genes, but their functional significance is still unclear.<sup>50,51</sup>

Because both NOD2 and TLRs are involved in recognition of and response to bacteria, they may function in a mutual biological interrelationship that could be dysfunctional in IBD. Macrophages of CD patients carrying homozygous mutations of NOD2 show defects of IL-1 $\beta$  and IL-8 production when stimulated by MDP or TNF- $\alpha$ .<sup>52</sup> Moreover, peripheral blood mononuclear from CD patients with double mutant genotypes fail to show synergism between MDP and TLR ligands that should result in a substantial upregulation of TNF- $\alpha$  and IL-1 $\beta$  production as observed in normal controls.<sup>53</sup> Thus, generalized defects of innate immune responses mediated via pattern recognition receptors may contribute to IBD, CD in particular.

## Adaptive immunity

One of the first abnormalities of intestinal immunity reported in IBD was that production of systemic and mucosal antibodies was drastically increased, and that the relative proportions of immunoglobulin classes and subclasses were altered as a consequence of chronic gut inflammation.<sup>34-36</sup> Some studies showed that IgG1 antibodies against colonic epithelial cells were selectively overproduced in UC, but not CD, perhaps translating an autoimmune pathway in this condition.<sup>54</sup> Definitive proof for the existence of tissue injury-inducing autoantibodies in UC is still missing, and whether this condition represents a form of autoimmunity is unclear.

For over a decade, the focus of adaptive immunity in IBD has shifted from humoral to cell-mediated immunity, particularly T helper (Th) cell subsets and their soluble mediators. A large number of cytokine abnormalities have been described, including pro-inflammatory and immunoregulatory molecules.<sup>55</sup> In CD, intestinal CD4+ T cells produce large amounts of INF- $\gamma$  and display marked overexpression of the Th1-cell-specific transcription factor, T-bet,<sup>56</sup> whereas mucosal macrophages produce large amounts of IL-12 and IL-18.<sup>57,58</sup> CD mucosal T-cells are resistant to apoptosis and proliferate faster than normal T-cells.<sup>59,60</sup> Different immune abnormalities are found in UC, where NKT-cells produce increased amounts of IL-13, and mucosal T-cells produce more IL-5, proliferate less, and die more than control cells.<sup>60-62</sup> Based on these observations, the two main forms of IBD are associated with distinct immune profiles, characterized by a typical Th1 response in CD and an atypical Th2 response in UC. This situation, however, may be changed by the very recent discovery of a new class of IL-17-producing Th-17 cells, which are the product of a completely different lineage of

CD4+ cells and display potent immunoregulatory and pro-inflammatory activities.<sup>63,64</sup> Th-17 cells are essential to the development of murine colitis,<sup>65</sup> and their number is increased in both CD and UC mucosa.<sup>66</sup> Active investigation is currently underway to define the role of Th-17 cells in IBD pathogenesis.

Another active area of investigation of adaptive immunity in IBD is the study of possible immunoregulatory defects. Different types of immunoregulatory cells exist, the best defined being CD4+CD25+ T-cells, which are critically important in preventing autoimmunity and suppressing excessive immune reactivity. In one report, the number of CD4+CD25+ T-cells in IBD patients was decreased in the circulation with only a moderate expansion detected in the inflamed intestine, suggesting the presence of defective immunoregulation during active disease.<sup>67</sup> Another report, however, found that the number of CD4+CD25+ T-cells was increased in IBD mucosa,<sup>68</sup> underscoring the need of additional studies in the area.

## Other cellular factors

In addition to immune cells, other cell types are involved in IBD pathophysiology, including epithelial, mesenchymal, and endothelial cells, and platelets.

Intestinal epithelial cells (IEC) inappropriately express class II antigens in the mucosa of UC and CD patients,<sup>69</sup> fail to induce CD8+ suppressor cells and instead activate CD4+ T-cells,<sup>70</sup> and improperly express members of the B7 family of costimulatory molecules.<sup>71</sup> All of these represent alterations that can potentially contribute to intestinal inflammation. These findings, together with the above-mentioned altered expression of TLRs in IBD,<sup>49</sup> provide support of the notion that IEC play a role in IBD pathogenesis, but additional investigation is required to better understand the mechanisms involved.

Intestinal fibroblasts are also involved in gut inflammation and injury because they represent a source of matrix metalloproteinases (MMPs), proteolytic enzymes responsible for tissue degradation.<sup>72</sup> T-cells interact with and activate fibroblast causing MMP production, a process linking fibroblast function, adaptive immunity, and gut tissue injury.<sup>73</sup> Activation of fibroblasts through the CD40 pathway induces the upregulation of cell adhesion molecules and production of chemokines which, in turn, induce the migration of T-cells through local microvascular cells.<sup>74</sup> Therefore, mucosal fibroblasts are obviously active participants of the IBD inflammatory process.

Endothelial cells normally function as “gatekeeper of inflammation” by controlling the quality and quantity of leukocytes that transmigrate from the vascular into the interstitial space, a complex process mediated by cytokines, chemokines, and cell adhesion molecules. Human intestinal microvascular endothelial cells (HIMEC) isolated from CD and UC mucosa exhibit a significantly higher cytokine-

mediated leukocyte binding capacity compared with endothelial cells from normal mucosa, probably because of their chronic exposure to the inflammatory milieu of the IBD mucosa.<sup>75</sup> Deficient production of inducible nitric oxide (NO) synthase by IBD HIMEC may also contribute to enhanced leukocyte adhesiveness.<sup>76</sup>

Platelets have acquired a strong immunological connotation because they play an initiator or amplificatory role in both normal immunity and inflammation, mostly mediated through the CD40/CD40 ligand pathway.<sup>77</sup> In IBD patients, platelets circulate in an activated state, and the elevated levels of soluble CD40 ligand present in their systemic circulation are mostly of platelet origin, apparently due to platelet activation in the inflamed intestinal microvascular bed.<sup>78</sup> Platelets can trigger a CD40-dependent inflammatory response in the microvasculature of IBD patients,<sup>79</sup> and contribute to mucosal angiogenesis, a novel component of IBD pathogenesis.<sup>80</sup>

## Pediatric versus adult IBD: children with early onset IBD are a better population to study IBD

Studying gene–gene and gene–environmental interactions, the immune effector mechanisms of inflammation, and eventually devising etiologically and mechanistically based therapeutic interventions is a monumental task requiring substantial resource allocation. Therefore, selecting the ideal population to study gene–environmental interactions for IBD susceptibility and define the major inflammatory pathways before they are masked or confounded by disease chronicity is critically important. Although the basic pathogenesis of CD may be the same in pediatric and adult populations, pediatric onset IBD is not compounded by numerous and prolonged environmental exposures, interventions, and complications, thereby providing a more pure and early study population. For example, the prevalence of active or passive smoking—a major confounding variable in studies of IBD—is substantially lower in the pediatric population. If incident cases of IBD are studied, environmental factors are relatively easy to track compared with adult IBD, where long-standing disease with multiple environmental exposures is common. In addition, family members of pediatric index cases, particularly parents and siblings, are often more readily available for enrollment into studies than family members of adult subjects. These family-based studies, in particular transmission disequilibrium testing (TDT), a gold standard test for genetic associations, can be performed easily when DNA from family trios and siblings are available. This type of analysis eliminates the problems of traditional case controls studies such as spurious associations and genetic heterogeneity. Lastly, pediatric patients are usually followed prospectively from the time of diagnosis, so that environmental exposures and evolving phenotypes can be more accurately monitored and recorded.

## Conclusions

For several decades, the investigation of IBD has gone through repeated cycles of new hopes, new knowledge, and new realities. Infectious, allergic, dietary, psychosocial, environmental, microbial, vascular, metabolic, immune, and other theories have been put forward, but few of them have resisted the test of time.<sup>81</sup> At present, most investigators have embraced the view that IBD results from complex interactions between evolving environmental changes induced by society's progress, a still undefined number of predisposing genetic mutations, an incredibly complex gut microbiota that may be constantly changing, and the intricacies of our own immune defenses.<sup>82</sup> The ability to integrate all of these variables into cohesive and logical etiological and pathogenic mechanisms explaining all aspects of IBD still escapes us at the moment. On the other hand, the progress recently achieved in our overall understanding of IBD has been remarkable, and has clearly modified the routine clinical approach to the management of children affected by IBD.<sup>83</sup> Whereas intervening at the genetic level is not practically feasible yet, the way we control inflammation has become far more effective, using approaches that are rationally based on state-of-the-art knowledge of immune-mediated inflammatory pathways and flora modulation.<sup>84,85</sup> So far, this progress has helped proportionally more adults than children with IBD. However, now that pediatric IBD has become finally recognized as an entity with specific pathogenic and clinical features,<sup>3</sup> progress will quickly reach and benefit this expanding population of suffering young patients.

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