Published in final edited form as:

Inflamm Bowel Dis. 2013 August ; 19(9): 2011–2020. doi:10.1097/MIB.0b013e318281f590.

The Role of the Innate and Adaptive Immune System in Pediatric Inflammatory Bowel Disease

Lee A. Denson, MD*

Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH

Abstract

Recent translational studies have provided new insights into the pathogenesis of pediatric-onset Inflammatory Bowel Disease (IBD). Registry studies have identified distinct clinical phenotypes with increasing age of onset; this has led to a revision of the clinical phenotyping system, now termed the Paris classification system. It is recognized that there are infantile (age <1 years), very early onset (VEO, age 1-10), and early onset (EO, age 10-17) forms of disease. Rare genetic mutations affecting anti-microbial and anti-inflammatory pathways have been discovered in infantile and VEO forms, while genetic pathways identified in EO disease have been similar to adult-onset IBD. An increasing incidence in the infantile and VEO forms has suggested an important environmental influence. This is likely ultimately expressed via alterations in the enteric flora (dysbiosis) and dysregulated immune responses to the flora which are recognized as a critical trigger for mucosal inflammation. These data should ultimately guide new pathogenic models of disease which will inform both therapy in individual patients, and disease prevention in their at-risk family members.

Keywords

Pediatric Crohn disease; pediatric Ulcerative Colitis; infantile colitis; mucosal immunity; pathogenesis

Introduction

While the key structural components of the mucosal immune system are present at birth, these do not reach complete functional majority until early adolescence. In contrast to the systemic immune system, the mucosal immune system in the gut must be able to mount

*to whom correspondence should be addressed: MLC 2010, 3333 Burnet Avenue Cincinnati, OH 45229 Tel: 513-636-7575 Fax: 513-636-5581 lee.denson@cchmc.org.

Financial disclosures: The authors have no financial arrangement(s) with a company whose product figures prominently in the submitted manuscript or with a company making a competing product.

Writing assistance: not applicable

Competing Interest: None to declare

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vigorous responses to pathogens, while maintaining tolerance to a tremendous variety of non-pathogenic antigens from the diet and the commensal flora. This is accomplished in part via compartmentalization of inductive and effector functions, and in part via a more “tolerigenic” skewing of cell populations, cell surface receptors, and local cytokine production. The inductive compartments are the organized lymphoid structures including the Peyer's Patches (PP) in the small bowel and isolated lymphoid follicles (ILF) in the small bowel and colon. The effector compartments include the T and B cell populations distributed throughout the lamina propria and in the intra-epithelial location. Individual variation in development of the mucosal immune system in the post-natal period is critically dependent upon environmental influences. The most important environmental influence is likely the mode of bacterial colonization, which may have a profound and lasting effect upon the functional properties of the mucosal immune system. During puberty, rapid changes in circulating growth factors and sex hormones also influence the function of the systemic and mucosal immune systems. These environmental influences may then combine with genetic susceptibility and discrete intrinsic stages in the development of the mucosal immune system to create windows of susceptibility for developing sub-types of Crohn's Disease (CD) or Ulcerative Colitis (UC). This review will address the role of the innate and adaptive immune systems in pediatric Inflammatory Bowel Disease (IBD), in the context of the development of the mucosal immune system, in infants, children and adolescents.

Clinical Sub-Types of IBD Relative to Stages of Mucosal Immune Development

A report which included 1370 patients from six centers in North America defined important clinical and demographic differences in pediatric IBD relative to adult-onset disease¹. The mean age at diagnosis was 10 years, with 54% of patients being male. 15% of the patients presented before the age of 6, 48% between the ages of 6 and 12, and 37% at age 13 to 17. Twenty-nine percent had one or more family members with IBD. The highest frequency of affected first-degree family members, 44%, was observed in the youngest (< age 3) children with UC. This is in contrast to the relatively low concordance (10%) for UC in adult monozygotic twins, and illustrates the importance of considering the effects of genetic variation upon the development of IBD during specific periods of immune development.

In contrast to adults, colonic involvement is predominant in children under the age of 8 with CD, while ileal involvement becomes more common above this age, reaching levels comparable to adulthood by adolescence. In the Heyman series, 63% of the children with IBD less than age 8 had isolated colonic disease, while only 35% age 8 and older had isolated colonic disease, when considering CD, UC, and indeterminate colitis (IC) combined¹. The frequency of UC was comparable to the frequency of CD in children less than 6 years of age, with the frequency of CD exceeding UC in older children. Importantly, the majority of pediatric UC patients have pan-colitis (>80%), while this represents a minority of adult UC patients (25%)². Data from a European series have been quite similar, with the probability of ileal involvement at the age of 8 years being equal to 0.19 (0.07-0.39), increasing to a maximum of 0.61 (0.54-0.68) at age 16³. The overall probability of ileal disease appeared to plateau at approximately age 12. A concurrent *NOD2* mutation

at age 16 years in this study increased the probability of ileal involvement, from 0.46 (0.34-0.58) to 0.75 (0.55-0.89), and reduced the age of ileal involvement by approximately two years. These clinical observations support the concept that susceptibility to the different sub-types of IBD in childhood and adolescence is likely influenced by an interaction between IBD risk alleles (eg *NOD2*) and stages of development of the mucosal immune system.

As illustrated in Figure 1, humoral mucosal immune responses are largely mature by age 5-6, while the full range of inductive and effector cellular mechanisms are not fully mature until age 10-12. Moreover, the overall helper T cell (Th) Th1/Th2/Th17 skewing of the mucosal T cell response also varies over this timeframe, with Th2 responses more common at younger ages. If colonic IBD, Crohn's colitis and UC, are more dependent upon defects in epithelial function, Th2 responses, and activation of humoral immunity, while small bowel CD is more dependent upon activation of cellular immunity and Th1/Th17 responses, this may contribute to the different types of IBD observed at different ages. Pro- and anti-inflammatory effects of estrogen, progesterone, and androgens may then further modify these responses during puberty, ultimately leading to the frequency and gender dependence of IBD sub-types observed in adulthood.

Genetic Polymorphisms Influencing Risk for Pediatric-onset IBD Sub-Types

The genetic polymorphisms and pathways associated with increased susceptibility for adult-onset IBD have recently been extensively reviewed, and so will not be covered in detail here⁴. This section will be limited to evidence for any specific effects upon early-onset disease, or sub-types of disease observed with different frequencies in the pediatric age group. Genome-wide-association studies of CD and UC in EO (all patients < age 17) with IBD did not yield unique susceptibility genes compared to similar studies of adult-onset patients⁵⁻⁸. Collectively, these studies have suggested that common variants with a modest effect upon overall disease risk are largely independent of age of onset between the EO (10-17) and adult onset groups, and associated pathogenic mechanisms are likely quite similar (see Figure 2). As a whole, loss of function polymorphisms in genes involved in the innate response to the enteric flora, including *CARD15* and *ATG16L1*, have been implicated in increased risk for CD with small bowel involvement^{9, 10}. By comparison, a rare protective polymorphism in *IL23R*, which would be predicted to influence primarily adaptive T cell responses, has been associated with both CD and UC¹¹. Patients with small bowel CD are also more likely to exhibit increased serologic responses to the enteric flora, and expansion of effector T cells directed against components of the flora such as flagellin (see Figure 2). Taken together, these studies have suggested that the development of small bowel CD may require defects in innate immunity which promote induction of adaptive T cell responses to the enteric flora, mediated by interactions between antigen presenting cells (APC) and naïve T cells in Peyer's Patches, and subsequent recruitment of these activated/memory T cells to the ileal lamina propria. Interestingly, the inductive and effector components of mucosal immunity needed to mount this response are not fully developed until early adolescence, when this form of CD reaches a frequency comparable to that observed in adulthood (see Figure 1).

While not replicated to the same degree as the multiple studies examining *NOD2/CARD15* and CD, studies in UC have identified susceptibility related to genes involved in regulating colon epithelial cell function. These have included *ABCB1*, an epithelial transport gene, and *MYO9B*, a component of the epithelial cytoskeleton⁵. Importantly, targeted deletion of *abcb1/mdr1* in the mouse leads to spontaneous colitis resembling human UC¹². These studies have suggested that alterations in the epithelial barrier or response to injury may be fundamental causes of UC, and so could conceivably play a role at the early age (3-8) when UC occurs at a comparable frequency to CD in children. However, an association between *CARD15* SNP carriage and disease in children less than age 6 with UC has been reported (27.6% in very early onset UC versus 7.8% in UC diagnosed after age 6, and 9.2% in controls)¹³. This intriguing result awaits confirmation in additional cohorts. If confirmed this could indicate a fundamental difference in the pathogenesis of UC in younger children. It would also be important to determine whether this sub-group in fact develops small bowel disease and is re-classified as CD with increasing age.

Pathways Identified by Genetic Studies of Infantile and VEO IBD

In contrast to genetic studies in older pediatric patients, recent case:control analyses of infantile and VEO IBD have identified rare loss-of-function mutations in critical immune genes leading to severe disease (see Table I and Figure 3). In some cases these may represent true monogenic forms of human IBD. For example, mutations in the IL-10 receptor (IL-10) which abrogate anti-inflammatory IL-10 signaling have been described specifically in colitis presenting in the first year of life¹⁴. IL-10 ordinarily suppresses bacterial product dependent production of pro-inflammatory cytokines by monocytes; this anti-inflammatory effect is profoundly suppressed in infants with IL-10R mutations and colitis (see Figure 3). This leads to high production of cytokines including TNF α and a severe disease course refractory to medical therapy. Allogeneic stem cell transplantation may be required to induce disease remission in these patients. Currently, screening can be performed via research assays involving IL-10 stimulation of peripheral blood mononuclear cells (PBMC), followed by sequencing for the *IL-10R* mutations¹⁴. However, it should be noted that a recent comprehensive analysis of 75 children with IBD, including 13 infants, confirmed loss of IL-10 signaling due to IL-10R mutations in only 2 of the infants, suggesting that this will be a rare pathogenic mechanism restricted to some infantile forms of IBD¹⁵.

Mutations in the *XIAP* gene also have recently been reported to be associated with infantile or VEO colitis that may mimic idiopathic IBD¹⁶. Up to 20% of patients with XIAP deficiency will develop IBD; this may be due to combined defects in ant-microbial responses and regulatory T cell functions (see Figure 3). Allogeneic stem cell transplantation may be required to induce disease remission and prevent mortality from hemophagocytic lymphohistocytosis. Testing for this condition is via a flow cytometry-based assay that measures expression of the XIAP protein on white cells (<http://www.cincinnatichildrens.org/service/c/cancer-blood/hcp/diagnostic-lab/tests/>).

A series of very young patients with Dyskeratosis Congenita have recently been described who initially presented with lymphopenia, hypogammaglobulinemia, recurrent infections,

and VEO severe enterocolitis¹⁷. This disorder is characterized by premature telomere shortening, which may lead to a reduction in the stem cell compartment supporting lymphocytes and intestinal epithelial cells, and thereby produce the clinical phenotype of immune deficiency and IBD (see Figure 3, *DKC1* genetic variants). Screening can be accomplished via measurement of telomere length in PBMC (<http://repeatdiagnostics.com/>). Allogeneic stem cell transplantation is indicated for bone marrow failure and recurrent infections, but may not alleviate symptoms of IBD.

A recent study limited to VEO CD identified a novel rare variant in the *NCF2* gene that would be predicted to affect the NADPH oxidase complex, and thereby phagocyte killing of microbes (see Figure 3)¹⁸. Remarkably, this is the same complex in which genetic mutations cause the primary immune deficiency disorder chronic granulomatous disease (CGD), which is in turn often associated with VEO IBD. This information has important implications for future adequately powered studies in the VEO sub-group, and for therapies designed to stimulate microbial killing in those with mutations reducing NADPH oxidase complex function. Collectively, these studies have demonstrated that for at least infantile forms of disease, severe loss-of-function mutations in genes regulating the epithelial barrier and monocyte and T lymphocyte function may be sufficient to cause IBD.

A More Common Cause for Reduced Innate Function in Pediatric CD: Granulocyte-Macrophage Colony Stimulating Factor Auto-antibodies (GM-CSF Ab)

While rare genetic mutations recently identified in infantile and VEO IBD have provided valuable insights into fundamental mechanisms of disease, these are less likely to contribute to pathogenesis in the more common cases with older age of onset. Because mild reductions in neutrophil function had been described in adult-onset CD, and high titers of neutralizing GM-CSF auto-antibodies (Ab) had been shown to impair phagocyte antimicrobial function in the rare lung disease pulmonary alveolar proteinosis, we asked whether more modestly elevated titers of GM-CSF Ab would be present in IBD^{19, 20}. We discovered that GM-CSF Ab are present in a subset of CD patients, and that titers increase with increasing age of onset, from VEO to EO pediatric disease, and further with adult-onset disease²¹. Overall, approximately fifty percent of CD patients exhibit high levels of GM-CSF Ab which are associated with reduced neutrophil phagocytic capacity, increased intestinal permeability and reactivity to the enteric flora, and expansion of effector T cells expressing the gut homing receptor CCR9²¹⁻²³. This is of potential therapeutic interest, as an oral small molecule inhibitor of CCR9 is entering phase 3 clinical trials for CD, and we have found that CD patients with elevated GM-CSF Ab who are treated with current therapies progress more rapidly to stricturing behavior requiring surgery²⁴. Moreover, as GM-CSF itself has been tested as a therapy in adult and pediatric-onset CD, with some patients achieving remission, this raises the possibility that GM-CSF Ab titers might be used to predict which CD patients would derive the greatest benefit from GM-CSF administration^{25, 26}. Overall, the concept that endogenous cytokine auto-antibodies regulate inflammation in IBD is new and highly relevant, given the advent of therapeutic monoclonal anti-cytokine antibodies (see Figure 2).

Bacterial Colonization and the Development of Mucosal Immunity

The principal components of the mucosal immune system initially develop within the sterile prenatal environment. The mode of feeding and bacterial colonization following birth plays a critical role in the ensuing maturation of the mucosal immune system over the next 1-3 years. This section will review what is currently known regarding the effects of breast versus formula feeding upon colonization of the intestine in humans, and the effect of bacterial colonization in turn upon development of the mucosal immune system.

Breast feeding has been shown to have a profound influence upon bacterial colonization in infancy. Prebiotic oligosaccharides present in breast milk such as inulin-type fructans are fermented in the colon to produce SCFA, which in turn promote colonization with bacteria including lactobacilli and bifidobacteria²⁷. By comparison, formula feeding leads to a relative reduction in these species, and an increase in enterococci and enterobacteria. Both patient-based and animal studies have shown that bacterial species including lactobacilli and bifidobacteria may promote tolerogenic gut immune responses. For example, one study showed that PP lymphocytes produced significantly less IFN γ and TNF α after treatment with bifidobacteria²⁷. Similar results have been shown when CD colon biopsies have been co-cultured with lactobacilli species²⁷. If bacterial colonization is stable over time, this may thereby influence the likelihood of developing IBD during critical windows of susceptibility.

Studies in gnotobiotic mice have shown that bacterial colonization is essential for the development of a normal mucosal immune system. Colonization with the enteric flora has a net anti-inflammatory effect in the developing intestine²⁸. In the absence of gut bacteria, both the inductive and effector compartments of the mucosal immune system are substantially underdeveloped. The PP are small and lack germinal centers, and the numbers of IgA plasma cells, CD4+ lamina propria (LP) T cells, and CD8+ intra-epithelial lymphocytes (IEL) are greatly reduced²⁹. The gut-associated lymphoid tissue (GALT) of germ free mice also resembles that of neonates in terms of exhibiting a Th2 bias³⁰. Reconstitution with a relatively limited flora is sufficient to restore development of the mucosal immune system, including the development of the organized lymphoid tissues, the expansion of IgA plasma cells, and a shift towards a more balanced Th1/Th2 cytokine profile.

However, studies using germ free (GF) mice have also shown that the enteric flora are required for the development of experimental IBD, and by extension, human disease. Interestingly, the pattern of neonatal bacterial colonization has been shown to differ between normal and colitis-prone mice, suggesting that genetic variation in the host may influence early colonization, development of the mucosal immune system, and the later emergence of clinically apparent IBD³¹. Moreover, intriguing animal studies have shown that mono-association of genetically susceptible IL-10 deficient mice with different bacterial strains will lead to disease in discrete segments of the small intestine or colon³².

Taken together, these patient-based and animal studies suggest that parameters which influence the composition of the enteric flora in humans, such as the mode of feeding during infancy, the use of antibiotics, the relative “cleanliness” of the environment, and the

frequency of childhood infections, are likely to interact with genetic variation to have a profound influence upon the development of specific sub-types of IBD at older ages.

Alterations in the Microbiota in Pediatric IBD

Studies performed in mice genetically susceptible to developing spontaneous colitis demonstrated that colitis would not occur in the absence of the enteric flora. This finding suggested that antigens derived from the enteric flora could drive the development of IBD in humans. Intestinal epithelial cells (IEC) are glycosylated. During infancy, most oligosaccharides attached to the epithelial surface contain terminal sialylation²⁸. This changes to predominately terminal fucosylation and galactosylation with maturity; in rodents, this change occurs after weaning²⁸. These changes in IEC glycosylation patterns may in turn affect bacterial colonization. For example, colonization with *Bacteroides thetaiomicon*, which exerts anti-inflammatory effects in the healthy gut, is facilitated by fucosylation because it uses L-fucose as a nutrient source²⁸. In this regard, several recent studies have identified differences in bacterial colonization of the gut in IBD patients{Papa, 2012 #2120}. Whether this is due in part to alterations in IEC glycosylation during infancy is not known.

The identification of genetic variation in *FUT2* as a risk factor for pediatric and adult CD, and for increased mortality in premature infants with necrotizing enterocolitis, was therefore of great interest^{33, 34}. The *FUT2* gene product, fucosyltransferase 2, is known to regulate bacterial colonization of the gut; thus the *FUT2* risk variant may influence CD and necrotizing enterocolitis pathogenesis by leading to altered intestinal microbiota (see Figure 2). Alpha-defensins are paneth cell products which are important for host defense in the gut. Alpha-defensin-5 (HD-5) has now been shown to be suppressed in the ileum of pediatric CD patients, independent of inflammation³⁵. This suppression may contribute to local invasion of commensal bacterial species. Conversely, the degree of local inflammation was associated with the level of up-regulation of paneth cell HD-5 in the colon of pediatric CD patients.

Several recent studies using modern molecular techniques have begun to define differences in the microbiome (“dysbiosis”) in children with IBD. Most studies have shown evidence for altered flora in CD, but not UC. An expansion of adherent-invasive *E. Coli* (AIEC) has been consistently noted in patients with CD, together with a reduction in *F. prausnitzii*³⁶⁻³⁸. In tissue culture and animal models, colonization with AIEC has been shown to promote epithelial injury and colitis, suggesting that it may also drive disease in humans³⁹. In animal studies, flagellin has been identified as a critical virulence factor for colitis, and a potent antigen for elicitation of effector T cell responses in both animal models and patients with IBD⁴⁰. *In vitro* studies using cell lines have shown that AIEC strains will induce pro-inflammatory TNF α and IL-8 expression, and damage the epithelial barrier^{36, 39}. Remarkably, the oral microbiome in children with CD, but not UC, has recently been shown to exhibit reduced diversity, suggesting that clinical sampling of this more accessible site may provide important insights into dysbiosis in IBD⁴¹. It will be important in future studies to account for genetic and acquired differences in host innate immunity which may promote dysbiosis.

Dysregulated Immune Responses to the Normal Enteric Flora in CD

In contrast to the evidence for dysbiosis in IBD, many IBD patients may exhibit enteric flora similar to that of healthy individuals, but a dysregulated host response to these normal commensals. Two recent studies have illustrated aberrant inflammatory responses to commensals specifically in pediatric CD patients. In the first, IL-8 secretion was induced in ileal or colon biopsies of CD patients exposed to *B. thetaiotaomicron*; there was no effect on biopsies from healthy controls⁴². Similarly, candida induced both IFN γ and IL-17 production by CD, but not UC or control, peripheral blood mononuclear cells⁴³. Collectively, these studies provide a rationale for the use of antibiotics, probiotics, or prebiotics to regulate components of the enteric flora which may be driving mucosal immune responses in selected IBD patients. However, clinical tools to define elements of the flora which are driving disease in individual patients are currently lacking, making rationale use of these approaches challenging.

Serologic Responses to the Enteric Flora and Complicated Disease Behavior in CD

A large body of work now supports the concept that a sub-set of pediatric CD patients develop long-lasting IgA and IgG antibodies targeting commensal flora, and that high titers of these antibodies are associated with more rapid progression to stricturing or internal penetrating behavior (see Table II)⁴⁴. Recent studies have shown that genetic mutations that reduce neutrophil antimicrobial function in patients with CGD or glycogen storage disease 1b (GSD1b) are sufficient to lead to high titers of anti-microbial serologies (AMS)^{45, 46}. These serologies include the AMS previously linked to complicated CD, including ASCA, OmpC, I2, and CBir1 (see Table II). Remarkably, high titers of AMS were observed even in CGD or GSD1b patients who lacked intestinal inflammation. Consistent with this, we have found that AMS are also elevated in CD patients with high GM-CSF auto-antibodies (Ab) which are in turn associated with reduced neutrophil antimicrobial function²¹. Studies in animal models have shown that GM-CSF Ab promote more severe NSAID-induced ileitis, while immunization to flagellin (e.g., CBir1 Ab) promotes more severe dextran sodium sulfate-induced colitis^{21, 47}. Therefore, at least the GM-CSF Ab and CBir1 Ab may be directly involved in disease pathogenesis, as well as serving as biomarkers for more aggressive disease. Interestingly, CBir1 titers tend to be higher in children with CD younger than 10 years old, while GM-CSF Ab and ASCA titers are increased in the 10-17 age group⁴⁸, suggesting distinct microbial and immune mechanisms of disease in the VEO and EO groups.

A large pediatric cohort study has confirmed the strong association between high titers of AMS and more rapid progression to stricturing or internal penetrating behavior previously reported in adults⁴⁴. Importantly, AMS in this prospective cohort study were measured close to the time of diagnosis, and so were predictive of the progression from the initial inflammatory phenotype to the stricturing/penetrating phenotype. The CCFA-sponsored RISK study will seek to validate this association in 1100 children with CD enrolled at the time of diagnosis, and to identify additional factors which will improve the positive-predictive value of the current AMS panel^{21, 44}.

A recent adult study confirmed the prior observation in pediatric-onset disease that the presence of a *NOD2* mutation increases the risk for complicated disease behavior for each level of AMS titers, and that the ANCA biomarker is associated with reduced risk for stricturing/penetrating behavior^{49, 50}. For each predicted level of risk, there was excellent agreement between the modeled and actual outcomes ($r=0.973$), with the area-under-the-curve for the receiver operator curve being equal to 0.801. Therefore, a model for stricturing/internal penetrating behavior, which includes the *NOD2* genotype, AMS titers, and ANCA, provides sufficient accuracy for use in clinical practice.

The implication is that earlier use of immune modulator or anti-TNF therapy may reduce the rate of progression in these high risk patients; however, that has not yet been conclusively established. A prospective cohort study of 796 pediatric CD patients using a novel system dynamics analysis of clinical outcomes and AMS suggested that early use of immune modulators or anti-TNF therapy would reduce the risk of complications in high risk patients defined by serologic responses⁵¹. Importantly, this project also developed a patient decision aid that can be used to review an individual patient's risk factors and the potential effect of specific therapies. However, as AMS titers vary with age within the pediatric age range, it will be important to validate this model in younger children enrolled in RISK. Once that has been accomplished, it is quite likely that this will become a useful clinical tool.

T Cell Polarization in Pediatric IBD

Chronic inflammation in IBD is believed to be ultimately driven by polarized T cells reactive in part to enteric antigens. These T cells can include Th1 cells which produce IFN γ ; Th17 cells which produce IL-17; or Th2 cells which produce IL-4, IL-5, or IL-13 (see Figure 2). A recent pediatric study has identified intriguing variation in expression of Th1 and Th17 cytokines as a function of both intestinal inflammation and location⁵². IL-17A was shown to be highly expressed in healthy ileum. With the onset of inflammation, both IL-17A and IFN γ were induced in CD ileum, while IL-17A was induced in UC colon, and IFN γ in CD colon. These findings may have implications for treatment of extensive disease involving the ileum and colon, which is typically the case in pediatric CD, using monoclonal antibodies targeting only Th1 or Th17 pathways. Moreover, several studies found an atypical Th2 polarization in UC, with up-regulation of IL-13 leading to epithelial dysfunction (see Figure 2)⁵³. This IL-13 upregulation may have implications for development of new therapies targeting IL-13 in UC, as was recently reported for a clinical trial of IFN β -1a, which suppressed IL-13 and improved symptoms in adults with UC⁵⁴. It is likely that clinical assays to measure tissue cytokine profiles will be required to translate this knowledge of intestinal T cell polarization to the clinic, and ultimately to guide use of specific biologic therapies.

Th2 Bias and Maturation of Mucosal Humoral Immunity Relative to the Development of UC and Colonic CD in Young Children

The infant normally exhibits a Th2 bias in the cytokine profile of potential effector cells in the gut, as well as a relatively high level of tolerogenic IL-10 and TGF β production. This likely contributes to the much higher frequency of allergic gastro-intestinal disease in this

age group, and the reduced frequency of IBD. Preferential production of IL-4 and TGF β during this period supports humoral IgG1 and IgE responses to exogenous antigens⁵⁵. With a susceptible genetic background and environmental stimuli, this Th2 bias with IgE production will lead to the development of food allergies and atopic dermatitis in this age group.

With increasing age, infants and young children then normally evolve to a more balanced Th1/Th2 phenotype in the gut, which is felt to limit the development of allergic gastrointestinal disease. The important Th17 population is not yet well understood in this regard, although recent animal studies have shown that alterations in post-natal colonization can result in early Th17 expansion⁵⁶. While results have been mixed, most early studies in UC demonstrated a relative increase in the production of Th2 cytokines including IL-5 and IL-13, while most studies in CD have demonstrated an increase in Th1 cytokines including IL-12 and IFN γ . However, more recent studies have suggested a more mixed profile which may change over time, as well as what is likely a significant role for the recently characterized IL-23 dependent Th17 pathway. Nevertheless, a persistent skewing of the mucosal immune profile towards Th2 beyond age 3-5 would be anticipated to promote the persistence of food allergy or development of UC, while a skewing towards a Th1/Th17 pathway would be expected to promote the development of CD. These intrinsic age dependent differences in skewing of the Th1/Th2 pathways in the gut may therefore contribute to the increased frequency of UC in the youngest IBD patients, and the consistent increase in the relative frequency of CD at ages above 6-8.

In UC, an increase in IgG1 secreting B cells and increased IL-5 production by mucosal CD4+ T cells has been observed⁵⁵. IgG1 coating of epithelial cells has in turn been shown to lead to complement activation, which likely contributes to epithelial injury⁵⁷. While data have been mixed, a reasonable number of studies have demonstrated that the IgG-producing cells in UC may target true auto-antigens. Tropomyosin isoform 5 has been identified as a putative UC autoantigen in this regard⁵⁸. Importantly, the same group has recently reported that autoantibodies against tropomyosin isoform 5 in UC do mediate antibody and complement mediate lysis of colon epithelial cells⁵⁹. Anti-neutrophil cytoplasmic antibodies (ANCA) have also been identified in the majority of UC patients, and a substantial fraction of patients with Crohn's colitis. However, whether these exert a pathogenic effect is not known. A functional reduction in the production and secretion of IgA by tissue plasma cells and epithelial cells, which would be expected to promote increased exposure of the mucosal adaptive immune system to bacterial products, may also contribute to the chronic inflammatory response in UC. This type of mucosal humoral immune response would be expected to be fully mature by age 6, and so could account for the relative increased frequency of UC in younger children.

Organized Lymphoid Follicles and Induction of Th1/Th17 Adaptive Immune Responses Relative to the Development of Ileal CD in Older Children and Adolescents

In contrast to the systemic immune system, the inductive sites in the organized lymphoid tissue (PP, ILF) in the intestine are anatomically separated from the effector sites (LP, epithelium) ²⁹. Peyer's patches contain B cell lymphoid follicles and interfollicular populations of CD4 and CD8 T cells. Following activation by mucosal dendritic cells, these T cells can then re-circulate to the intestinal lamina propria, where they may mediate both antigen specific protective responses against pathogens, and in the case of CD, inappropriate responses directed against the enteric flora. In this regard, mucosal DC provide instruction to naïve PP T cells for both antigen specificity of responses, and subsequent homing to the intestinal lamina propria. Both an intact thymus and bacterial colonization are required for complete development of the PP ²⁸. Microfold cells, or M cells, are specialized epithelial cells which overlie the organized lymphoid follicles in the gut and mediate antigen uptake to these tissues. These cells differentiate in parallel with the development of the underlying PP and ILF, due to the combined effects of the enteric flora and the germinal center B cells ²⁸.

Current data suggests that the organized lymphoid follicles (LF) in the gut represent critical sites for activation of adaptive immune responses to the enteric flora in CD. As recently summarized by Meinzer et al, these data include: 1) the most frequent sites of involvement for CD are in the segments (ileum, Peyer's Patches (PP) and colon, isolated lymphoid follicles (ILF)) where the LF are most frequent; 2) CD lesions are discontinuous, as is the distribution of the LF; and 3) detailed morphological analyses have localized early CD lesions to areas where LF are most abundant ³. Of course, the ileum and colon also contain the highest concentrations of enteric flora, and therefore potential antigenic load, in the body. Importantly, a recent animal study has demonstrated increased numbers of M cells, specialized epithelial cells overlying FAE and designed for antigen uptake, combined with increased CD4+ T cells, in PPs of *NOD2/CARD15* deficient mice ⁶⁰. These data have for the first time provided a reasonable mechanistic basis for increased antigen uptake and T cell activation in patients with CD and *NOD2/CARD15* SNP carriage.

While the principal inductive sites for antigen processing in the small bowel, the Peyer's Patches, are present in infants, these do not reach adult levels of frequency and size until adolescence ³. Van Kruiningen et al compared the frequency of PP and the incidence of CD, and found these to be quite similar, with the peak in PP formation preceding the peak in CD incidence by 5-10 years ⁶¹. Term infants have approximately 80-120 PP, this increases to a maximum of 250 in mid-adolescence, and then progressively decreases to 100 by age ninety ⁶². By comparison, the frequency of ILF in the colon is relatively constant with age ³. It has been speculated that this overall difference in the abundance of inductive sites may contribute to the higher frequency of colonic CD in younger children, and the increasing frequency of ileal involvement after age 8-10. It will be important in the future to define the relative IL-23/Th17 versus IL12/Th1 bias in PPs from individuals with CD and healthy controls, and to determine whether data support increased antigen exposure and T cell activation during the age range when PP reach a maximal size. This will have implications

for future strategies to restore mucosal tolerance, and potentially prevent disease in genetically susceptible individuals.

Gender Differences, Puberty and the Mucosal Immune System

Prior to puberty, CD, but not UC, is more common in males than females. Population-based studies have demonstrated up to a 2:1 male:female (M:F) ratio in pediatric onset cases. After puberty, the gender frequency approaches that observed in adulthood. Overall, data have suggested a somewhat more severe course for females with pediatric CD, with higher rates of surgery ^{63, 64}. This has suggested that circulating levels of growth factors and sex hormones which change around the time of puberty might influence mucosal immunity and susceptibility to CD. Both patient-based and animal studies have demonstrated that estrogen exerts a pro-inflammatory effect in this regard, while androgens including testosterone may exert an anti-inflammatory effect. Moreover, in some autoimmune diseases such as SLE, both male and female patients have been shown to have increased levels of biologically potent estrogen, and lower levels of androgens ⁶⁵. Whether this is also the case with IBD during puberty is not known.

Non-pregnant women tend to exhibit more pronounced Th1 responses to infection and other stimuli, compared to men or pregnant women. Prior to puberty, young females actually have low levels of estrogen compared to males, while after puberty, this is reversed ⁶⁶. Greater post-pubertal Th1 responses in females may in part involve effects of estrogen upon TLR4 activation of NFkB and production of pro-inflammatory cytokines including IL-12. These effects of estrogen are antagonized by androgens. Moreover, estrogen in other settings has been shown to promote expansion of auto-reactive CD4+ effector cells, and the production of IgG class auto-antibodies. A recent animal study demonstrated that post-pubertal female mice exhibit an increased expression of genes regulating adaptive immunity, while male mice increase expression of genes involved in innate responses. In this study, estrogen was shown to promote CD8+ T cell-derived B cell help, and increased immunoglobulin production in post-pubertal females ⁶⁷. By comparison, androgens have been shown to promote IL-2 production by Th1 cells, which reduces Th2 activity and drives expansion of CD8+ suppressor T cells, with a generalized anti-inflammatory effect ⁶⁵. In this regard, it is intriguing to note that estrogen increases the expression of *BCL-2*; this anti-apoptotic gene has now been implicated in the resistance of mucosal effector T cells to apoptosis in CD ^{65, 68}.

Demonstration of direct effects of pubertal hormones upon the mucosal immune system has largely been limited to animal studies. The severity of colitis due to TNBS in rats has been shown to vary during the menstrual cycle, with more severe disease when progesterone levels are highest. This has been linked to increased expression of MIF in the colon, and increased pro-inflammatory cytokine levels ⁶⁹. Susceptibility to TNBS colitis in female mice has been linked to TLR4 signaling genetically, while this has not been the case for male mice ⁷⁰. Whether alterations in estrogen or progesterone production around the time of puberty directly regulate pro-inflammatory TLR4 or MIF dependent pathways in the gut in humans is not known.

Effectiveness and Potential Adverse Effects of IBD Therapy Relative to Stages of Mucosal Immune Development

The stage of development of the mucosal immune system might in theory influence the effectiveness of specific therapies, although to date this has not been evident. However, as younger children are diagnosed and treated at earlier stages of the disease, this may become more apparent. In general, it would be expected that therapies which target the epithelial barrier or the mucosal humoral immune response might be fully effective in younger children age 5-6 and above, as these aspects of the mucosal immune system are fully mature at this stage. By comparison, therapies specifically targeting the inductive and effector aspects of cellular immunity might not fully match the maturational stage of the mucosal immune system until age 10. Likewise, therapies targeting modulation of mucosal inflammation by growth factors or sex steroids, or immune pathways regulated by sex steroids, might be most applicable in post-pubertal patients. However, current therapies used to induce or maintain remission, including corticosteroids, enteral nutrition, immunomodulators, and/or anti-cytokine monoclonal antibodies, do not act in a manner which is anticipated to have an age dependent window of maximal efficacy. This will become more of an issue as strategies to induce mucosal tolerance enter clinical trials, in which specific aspects of mucosal immunity may be required to observe a maximal effect.

Of more immediate concern is the potential for these agents to interact during critical windows of mucosal immune development to incur long term adverse events including malignancies. It would appear that the incidence of lymphoma in conjunction with the use of thiopurines in children with IBD is likely comparable to that observed in adults. By comparison, the increasing reports of hepatosplenic T-cell lymphoma primarily in adolescent and young adult males with CD treated with both thiopurines and the monoclonal anti-TNF α antibody infliximab may represent the emergence of a rare malignancy during a vulnerable period of immune development ⁷¹. Further study and increased vigilance will be required to determine whether this is in fact the case.

Conclusions

Data from large prospective registry studies have recently led to revision of the clinical phenotyping guidelines for children with IBD. This new approach, termed the Paris Classification system, accounts for VEO (< age 10) and EO (age 10-17) age of onset, and recognizes the unique features of extensive small bowel disease with the L4b modifier ⁷². It will be important to utilize this system when designing and reporting results from future translational studies, outcomes registries, and clinical trials. A tremendous amount of new information regarding the specific etiology of infantile forms of IBD has become available over the past few years. Much as has occurred for neonatal cholestasis, this new information has broadened the differential diagnosis and specific evaluation of these patients (see Table I). Importantly, if a single immune gene mutation is identified in a particular patient, this may support the use of allogeneic stem cell transplantation. A large body of literature now supports the use of prognostic panels including the *NOD2* genotype, AMS, and pANCA in estimating risk for progression to stricturing or penetrating behavior in pediatric-onset CD

(see Table II). Validation of a model which includes the effects of very early age of onset and specific therapies offered early in the disease course is eagerly awaited from RISK. It is important to note that the frequency of AMS titers may differ in children younger than 10 years old compared to older patients, and so the currently available models may not be applicable to these youngest patients. While recent studies to develop predictive models for induction therapies are more preliminary, it is anticipated that as these models are validated in large prospective cohort studies, they will enter clinical practice as well.

Acknowledgments

Grant Support: This work was supported in part by NIH grant R01 DK078683 (LAD)

Abbreviations

Ab	auto-antibody
CI	confidence interval
B1	inflammatory behavior
B2B3	stricturing or internal penetrating behavior
CD	Crohn Disease
GM-CSF Ab	Granulocyte-Macrophage Stimulating Factor auto-antibodies
EO	early onset
IL	interleukin
IBD	Inflammatory Bowel Disease
L1	ileum-only location
L2	colon-only location
L3	ileocolonic location
MIF	macrophage migration inhibitory factor
OR	odds ratio
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
VEO	very early onset
UC	Ulcerative Colitis

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Figure 1. Maturation of Mucosal Immunity and Development of IBD

Pediatric-onset IBD may be classified based upon age-of-onset as infantile, very early onset (< age 10), or early onset (age 10-17). The frequency of isolated colitis and disease involving the ileum and colon as a function of age is shown, together with the time span over which the humoral and cellular components and Th1/Th2 skewing of the mucosal immune system become fully mature. Most components of the mucosal immune system mature over the first three years of life, under the influence of diet and bacterial colonization. During this period, the mucosal immune system exhibits a Th2 bias. Following this, a more balanced Th1:Th2 bias develops. The humoral immune system, including IgG and IgA producing lamina propria plasma cells, is fully mature by age five. By comparison, specific inductive (eg ileal Peyer's Patches) and effector (eg oligoclonal TCR repertoire) components of the cellular immune system are not fully mature until age 10-14, when rates of ileal involvement equal that of adult-onset disease.

Figure 2. Activation of Mucosal Immunity in Pediatric-onset IBD

Critical pathogenic components driving chronic mucosal inflammation in pediatric-onset IBD are illustrated. These include: 1) maladaptive responses to intestinal injury and the enteric flora, 2) T cell activation and polarization which may vary between CD and UC, and between early and late phases of disease, 3) pro-inflammatory cytokine and chemokine production, and 4) recruitment of activated effector neutrophils, monocytes, and T lymphocytes which drive ongoing tissue injury. Genetic variation in *FUT2* contributes to abnormal bacterial colonization (dysbiosis), while genetic variation in *IL-27* promotes effector T cell proliferation. Auto-reactive B cells producing GM-CSF auto-antibodies and CBir1 Ab may contribute to more severe disease.

Figure 3. Activation of Mucosal Immunity in Infantile-onset IBD

Susceptibility genes (blue ovals) associated with infantile-onset IBD are shown, at the presumptive site in the pathogenic cascade where these may exert an effect. Variants in *DKC1* and *XIAP* may both impair barrier function/wound healing and promote effector T cell proliferation. *NCF2* variants impair phagocyte antimicrobial function and response to injury. *IL10R* variants disrupt IL-10 suppression of monocyte pro-inflammatory cytokine production, and potentially regulatory T cell (Treg) function. *FOXP3*, *IL-10*, and *WASP* variants impair Treg function and thereby promote effector T cell proliferation. In some cases loss-of-function in these genes appears to be sufficient to cause infantile-onset IBD.

Table I Pathogenesis and Diagnostic Tests for Infantile Enterocolitis

Disorder	Presumed IBD Pathogenesis	Screening Test
Common Variable Immune Deficiency	Defective cellular and/or humoral immunity	Lymphocyte subsets, IgA, IgM, IgG, IgE, vaccine titers
CGD	Defective phagocyte microbial killing	Neutrophil oxidative burst
Dyskeratosis Congenita	Premature telomere shortening leading to stem cell dysfunction	Telomere length in PBMC
IPEX Syndrome	Defective regulatory T cell function	FOXP3 expression by flow cytometry
IL10R Mutations	Defective IL-10 anti-inflammatory action	IL-10 signaling in PBMC (research assay)
Wiskott-Aldrich Syndrome	Effector & regulatory lymphocyte defects	WASP expression by flow cytometry
XIAP deficiency	Combined microbial response and regulatory T cell defects	XIAP expression by flow cytometry

Table II Genetic and Serologic Predictors of Disease Behavior

Predictor	Associated Disease Behavior
<i>NOD2</i> genotype	Ileal CD location & stricturing behavior. Increases risk of stricturing for each level of anti-microbial serology (AMS)
ASCA	Stricturing and internal penetrating behavior. Lower titers in CD < age 10 at onset
pANCA	Colon-only CD location & reduced risk of stricturing behavior.
CBir1	Stricturing and penetrating behavior. Relatively higher titers in CD < age 10 at onset.
GM-CSF Ab	Ileal CD location & stricturing behavior. Increasing titers with increasing age of onset.
I2	Stricturing & penetrating behavior.
OmpC	Stricturing & penetrating behavior.