Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn’s disease

Andrew T. Gewirtz,1 Matam Vijay-Kumar,1 Steven R. Brant,4 Richard H. Duerr,3 Dan L. Nicolae,2 and Judy H. Cho2

1Department of Pathology and Laboratory Medicine, Epithelial Pathobiology Unit, Emory University School of Medicine, Atlanta, Georgia; 2Departments of Medicine, and Statistics, The University of Chicago, Chicago, Illinois; 4Department of Medicine, Johns Hopkins School of Medicine Baltimore, Maryland; and 3Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania

Submitted 29 November 2005; accepted in final form 24 January 2006

Crohn’s disease (CD) has long been associated with elevated immune responses to the enteric microflora. Whether such immune responses are a healthy adaptation to an aspect of this disorder or are inappropriate, perhaps resulting from immune dysregulation, is unclear. Mechanistic studies in mice have observed that a variety of engineered deletions in immunoregulatory genes result in spontaneous colitis and that, in such immune-dysregulated mice, immune responses to normal gut microflora (or associated antigens) are necessary and sufficient to drive colitis, suggesting that immune responses to commensal microbes may drive CD (2). However, the results of studies in CD patients have been less clear. Specifically, that a number of studies have associated specific microbes (or functional classes therein) with CD suggests that CD may actually be an immune deficiency and that CD-associated immune responses may not in fact be inappropriate but rather are a healthy adaptive response (5, 11, 29). The discovery that individuals carrying frame shift mutations in nod2 have an increased chance of developing CD initially seemed to support this concept in that nod2 serves as an intracellular receptor for gram-negative peptidoglycan (specifically muramyl dipeptide) and thus is thought to function in host defense against intracellular microbes (7, 16, 17, 26). However, a recently generated murine model of the nod2 frame shift mutation indicates that it may in fact be a “hyperactive” rather than nonfunctional allele (23). Furthermore, considering the role of innate immunity in general, and nod2 in particular of regulating adaptive immunity (20, 30, 31), CD-associated immune responses may yet prove to be a detrimental consequence of immune dysregulation rather than a potentially helpful adaptation. Thus, in light of the ambiguity of whether CD-associated immune responses protect patients or drive/exacerbate their disease, it is not surprising that therapeutic approaches to both promote immune responses (e.g., granulocyte macrophage colony-stimulating factor) or suppress immune responses (e.g., anti-tumor necrosis factor-α) are being explored or utilized to treat CD.

Although evidence that commensal microflora are the target of the CD-associated adaptive immune response has been accumulating for some time, the specific molecular targets have only recently begun to be elucidated. One such recently identified target of the CD-associated immune response is bacterial flagellin, the molecular subunit of bacterial flagella (21, 28). Flagellin is considered a dominant CD antigen in that a random identification of antigenic targets in spontaneously colitic mice found that 25% of antigenic targets were flagellins (21). Although, like other proteins, flagellin can be recognized by T cells (4), it is a unique antigen in that it is the only known protein that is recognized by germ line-encoded pattern recog-
nition receptors of the innate immune system. Specifically, at picomolar concentrations, flagellin is recognized by toll-like receptor 5 (TLR5), resulting in the potent induction of pro-inflammatory gene expression (10, 14). Although gut epithelial cells are relatively unresponsive to a number of TLR ligands, they are exquisitely responsive to flagellin, consistent with their expression of TLR5, and thus flagellin may not only be an antigenic target of CD but may also drive acute flares of inflammation in this disorder (8, 9). Considering the emerging appreciation for innate immunity in regulating adaptive immunity (15), and our demonstration that flagellin is a potent T cell adjuvant (4) we speculated that the innate and adaptive immune responses to flagellin are not disparate but rather that flagellin elicits potent adaptive immune responses because of its ability to activate innate immunity, thus serving as its own adjuvant. Although this appears to be true using mice injected with a bolus of highly purified flagellin (32), whether or not this would also be the case for humans in their natural acquisition of adaptive responses to flagellin, likely to occur in context of many other microbial products that may also have adjuvant ability, is a very different matter.

Recent pioneering work by Tom Hawn and colleagues (12) has provided a means to test the importance of TLR5 in human health and disease. Specifically, these investigators discovered the existence of a relatively common (5% allele frequency) TLR5 polymorphism referred to as TLR5-stop in which the stop codon preceding the signaling region of this receptor (referred to as the toll/interleukin-1 receptor or TIR domain). TLR5-stop is unable to activate nuclear factor (NF)-κB in response to flagellin and furthermore acts as a dominant-negative receptor in that its expression in vitro blocks flagellin-induced NF-κB activation mediated by wild-type (WT) TLR5. TLR5-stop also acts as a dominant-negative receptor in vivo in that monocytes from heterozygous carriers of TLR5-stop do not exhibit cytokine secretion upon stimulation with flagellin. Lack of innate ability to detect flagellin resulting from carriage of TLR5-stop leads to increased susceptibility to Legionella pneumophila infection (12). Herein, to gain insight into the role of TLR5 in CD and in regulating adaptive immunity to flagellin, we analyzed the frequency of TLR5-stop in CD patients and measured whether persons carrying TLR5-stop displayed altered levels of flagellin-specific immunoglobulins. We observed that, in unaffected persons, carriage of TLR5-stop retarded acquisition of adaptive immunity to flagellin and that carriage of TLR5-stop was negatively associated with CD in Jewish subjects.

METHODS

Subject patient ascertainment. In all cases, informed consent was obtained for molecular genetics studies approved by the local Institutional Review Boards, and genomic DNA was obtained from peripheral blood leukocytes. Inflammatory bowel disease (IBD) families were ascertained at the Johns Hopkins, University of Pittsburgh, and University of Chicago Hospitals and an Ashkenazi Jewish population-based control cohort through the New York Cancer Project. Diagnoses were confirmed by review of primary medical records, including radiologic, endoscopic, and pathology reports.

Genotyping. rs5744168 at nucleotide 1174 of TLR5 was determined to be either C (WT) or T (TLR5-stop; see Ref. 12) by allele-specific PCR using two-tailed allele-specific primers GAAG- GTGACCAAGTTGCTATGTA-ATGGTTGTAAGGACATT-GTCTCA and GAAGGCCAGTCAAGGGATTG-ATGTTGTAAGGACATTGTCCG and the common untailed reverse primer AAATTCTGGAAAAATTACGACCTTGGAT.

Serum analysis. Levels of flagellin-specific, lipopolysaccharide (LPS)-specific, bacterial-specific, and total serum IgG and IgA in serum were measured by ELISA and SDS-PAGE immunoblotting (for bacterial-specific), as previously described (28). The flagellin used for this purpose was purified from Escherichia coli, and purity verified as previously described. Briefly, such flagellin does not stimulate any other human TLR (1–4 nor 6–10; see Ref. 10). Furthermore, when such flagellin is SDS-PAGE immunoblotted with sera from mice or rabbits immunized with this flagellin, only the band corresponding to flagellin (22) is observed, indicating that flagellin itself is the only immunoreactive component of this preparation of purified flagellin. Although all assays used some common control samples to correct for any day-to-day or plate variance (which was never >10% of the values), all comparisons within individual panel in Figs. 1 and 2 and values in Tables 1–3 were performed as direct side-by-side comparisons.

Statistical analysis. The difference in the TLR5-stop allele frequency between two groups [for example, Jewish ulcerative colitis (UC) patients and Jewish CD patients] was tested using a statistic based on the difference in the frequencies estimated using a likelihood procedure. The likelihood calculations are based on conditional probabilities similar to those needed in linkage analysis, and take into account the dependence between the genotypes of related individuals. P values were calculated for two-sided alternative hypotheses. Determination of statistical significance for values of antibody measurements was performed via Student’s t-test, which assumes normally distributed data, and the Mann-Whitney test, which does not. Similar P values were obtained (results for the t-test are shown).

RESULTS

To investigate whether TLR5 might be a physiological regulator of adaptive immunity to flagellin, as naturally acquired by humans, we measured serum antibody responses in persons carrying or lacking TLR5-stop, a recently described relatively common polymorphism that encodes a dominant-negative TLR5 allele (12). To avoid measuring immunoreactivity that might potentially result from CD, we used sera from clinically confirmed healthy first-degree relatives of IBD patients. The ethnicity of this cohort was reflective of the IBD population seen by our centers; i.e., largely European Americans, mostly (>80%) non-Jewish. We genotyped >1,000 such healthy subjects and observed a carriage rate of TLR5-stop of 9.4%, with TLR5-stop homozygosity being observed in 0.4% of these subjects. Retrospective analysis of available health records of the subjects homozygous for TLR5-stop (and presumably having no TLR5 function) did not show any unusual health characteristics of these subjects. Our observed rate of TLR5-stop heterozygosity is very similar to the carriage rates published for Caucasian Dutch (12) and Vietnamese (6) and also similar to the values reported for both African Americans and European Americans (12.1 and 12.5%, respectively, based on 50 subjects) on the openly accessible Innate Immunity web site jointly maintained by the University of Arizona and The Channing Labs (www.innateimmunity.net). Total immunoglobulins, IgG and IgA, and those specific for flagellin and LPS (purified from E. coli) were measured by ELISA. As shown in Fig. 1, we observed that persons carrying TLR5-stop exhibited significantly lower levels of both flagellin-specific IgG and IgA. This difference in immunoreactivity was at least some-
what specific for flagellin in that TLR5-stop subjects and control subjects did not exhibit significant differences in LPS-specific immunoglobulins nor did they display any differences in total levels of either Ig. In light of the ethnic differences observed below, we considered whether ethnic stratification (i.e., Jewish vs. non-Jewish) might affect these results but did not observe such stratification to affect the relative reduction in levels of anti-flagellin antibodies associated with carriage of TLR5-stop (data not shown). To further explore whether TLR5-stop globally affects immune responses to bacterial products or rather specifically affects responses to flagellin, we used sera from control and TLR5-stop subjects to Western blot total extracts of a flagellated and aflagellate E. coli strain. Use of a flagellin monoclonal antibody allows identification of the flagellin band. Although this method is substantially less quantitative than ELISA, we could nonetheless still consistently see reduced flagellin-specific immunoreactivity in TLR5-stop subjects compared with WT controls (Fig. 2). However, beyond this difference, although there were differences in the immunoreactivity of individual serum samples in terms of their overall recognition of the bacterial extracts and the specific bands they detected, there was no consistently discernable difference in the patterns of immunoreactivity from sera of TLR5-stop and control subjects. Thus healthy persons carrying
TLR5-stop may have similar overall levels of immunoreactivity to gut microbes but display reduced immunoreactivity to the dominant CD antigen flagellin.

To investigate whether TLR5 plays a role in IBD, we examined the presence of TLR5-stop in IBD. Because of the relatively low frequency of this polymorphism, transmission disequilibrium testing had little statistical power, and thus we simply compared the carriage rate of TLR5-stop in CD, healthy controls, and UC, an IBD not associated with altered immune responses to flagellin (21, 28). We focused on a cohort of Jewish IBD patients, their unaffected relatives, and unrelated Jewish control subjects to reduce genetic heterogeneity and because our preliminary results in this ethnic group suggested differences between controls and patients. We genotyped 1112 Jewish subjects (215 CD patients, 185 UC patients, 296 unaffected relatives, and 416 unrelated Jewish control subjects) drawn from the Chicago, Baltimore, Pittsburgh, and New York metropolitan areas (Table 1). The carriage rate of TLR5-stop in the control Jewish subjects of 6.5% was, interestingly, reduced compared with other analyzed populations, including non-Jewish European ancestry cohorts. However, compared with unaffected relatives and unrelated Jewish control subjects, CD patients displayed a marked reduction in the frequency of TLR5-stop with only 0.9% of CD patients being positive for TLR5-stop (allele frequency 0.93%; \( P = 0.037 \) by likelihood calculations). In contrast, UC patients displayed a carriage rate of 6.0% (allele frequency 3.5%) that did not significantly differ from their first-degree relatives nor the unrelated Jewish control population, which was significantly higher than that observed in CD \( (P = 0.043) \). Persons homozygous for TLR5-stop were, as expected, quite rare, thus precluding any attempts to make any associations with this genetic state. Thus, among Jewish subjects, heterozygous carriage of TLR5-stop is negatively associated with CD but not UC.

We next analyzed the presence of TLR5-stop in a more genetically heterogeneous group of IBD patients (i.e., non-Jewish IBD patients and their unaffected relatives). We did not observe a statistically significant reduction of TLR5 carriage in CD patients in this cohort (carriage rates were 11.1, 10.4, and 11.7% for unaffected relatives, CD, and UC, respectively; \( n = 841, 543, \) and 300, respectively, for unaffected relatives; respective allele frequencies were 5.8, 5.2, and 5.9%). Furthermore, among non-Jewish CD patients, those with and without TLR5-stop exhibited identical mean ages of onset (Table 2), indicating that TLR5-stop did not delay disease development in this cohort. Modest differences in disease location among CD patients carrying TLR5-stop were observed in that such patients showed a trend toward slightly reduced incidence of colorectal involvement (where flagellin would be expected to be in greatest abundance) and modestly increased incidence of upper gastrointestinal/jejunal involvement (Table 2), but these differences are not statistically significant. Thus, although TLR5-stop may have functional consequences in CD, consistent with the notion that complex interrelationships of the multiple genetic loci differentially affect the susceptibility of various ethnic groups to CD (1), the negative association of TLR5-stop with CD appears to be restricted to Jewish ethnicity consistent with the emerging results that the well-defined CD risk allele, IBD5, differs markedly in Jewish and non-Jewish subjects with regard to its association with IBD (Cho, unpublished observation).

To gain insight into the reason for this ethnic difference, we compared levels of flagellin antibodies between and within these cohorts (Table 3). We first measured among non-Jewish CD patients whether carriage of TLR5-stop still resulted in reduced levels of flagellin-specific antibodies. No significant difference, or even a consistently suggestive trend, was observed, suggesting that the impediment imposed by TLR5-stop for making adaptive immune responses to flagellin can be overcome in CD. Next, we compared levels of flagellin-specific antibodies between our Jewish and non-Jewish subjects among healthy relatives and persons with CD. Among healthy relatives, Jewish subjects exhibited moderately elevated levels of flagellin immunoreactivity compared with non-Jewish subjects, but no difference in this parameter was observed between these cohorts in CD. Note that the relative elevation in flagellin-specific antibodies among CD patients in general is somewhat less than that previously observed (to E.

### Table 1. Occurrence of TLR5-stop in inflammatory bowel disease patients and controls

<table>
<thead>
<tr>
<th>Subject Type</th>
<th>Total No.</th>
<th>TLR5-stop hets</th>
<th>TLR5-stop Allele Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD patients</td>
<td>215</td>
<td>0.9*</td>
<td>0.93*†</td>
</tr>
<tr>
<td>UC patients</td>
<td>185</td>
<td>6.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Related controls</td>
<td>296</td>
<td>5.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Unrelated Jewish controls</td>
<td>416</td>
<td>6.5</td>
<td>3.2</td>
</tr>
</tbody>
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<td>416</td>
<td>6.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

TLR5, toll-like receptor 5; CD, Crohn’s disease; UC, ulcerative colitis. All DNA samples from Jewish persons to which we had access were genotyped for TLR5-stop as described in METHODS. Statistical significance differences are indicated as follows: *\( P = 0.037 \) compared with unrelated controls. †\( P = 0.043 \) compared with UC patients.

### Table 2. Comparison of disease characteristics of WT and TLR5-stop heterozygous non-Jewish CD patients

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total No.</th>
<th>Age Dx ± SD</th>
<th>Upper, %</th>
<th>Jejunal, %</th>
<th>Ileal, %</th>
<th>Colorectal,</th>
<th>Perianal, %</th>
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<tr>
<td>WT</td>
<td>486</td>
<td>22.6±9.6</td>
<td>6.4</td>
<td>7.8</td>
<td>78.8</td>
<td>55.3</td>
<td>31.0</td>
</tr>
<tr>
<td>TLR5-stop</td>
<td>57</td>
<td>22.5±10.2</td>
<td>10.5</td>
<td>15.7*</td>
<td>78.9</td>
<td>47.3</td>
<td>28.0</td>
</tr>
</tbody>
</table>

WT, wild type. Existing data of disease characteristics were compared among patients carrying or not carrying the TLR5-stop polymorphism. Dx, Results are shown as the percentage of each group of subjects with reported involvement of indicated locale: *\( P \) value = 0.08.
TLR5-STOP NEGATIVELY ASSOCIATES WITH CROHN’S DISEASE

Table 3. Levels of flagellin-specific immunoglobulins in CD cohorts and unaffected first-degree relatives

<table>
<thead>
<tr>
<th></th>
<th>TLR5-stop</th>
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</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1:100</td>
<td>1.15±0.35</td>
<td>1.15±0.46</td>
<td>NS</td>
<td>0.86±0.25</td>
<td>0.93±0.30</td>
<td>NS</td>
<td>1.11±0.46</td>
<td>1.02±0.46</td>
<td>NS</td>
<td>0.67±0.24</td>
</tr>
<tr>
<td>IgA</td>
<td>1:100</td>
<td>0.59±0.39</td>
<td>0.68±0.30</td>
<td>NS</td>
<td>0.31±0.24</td>
<td>0.42±0.30</td>
<td>NS</td>
<td>0.34±0.25</td>
<td>0.35±0.22</td>
<td>NS</td>
<td>0.36±0.18</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>0.44±0.17</td>
<td>0.34±0.27</td>
<td>NS</td>
<td>0.29±0.13</td>
<td>0.42±0.17</td>
<td>0.05</td>
<td>0.39±0.31</td>
<td>0.39±0.22</td>
<td>NS</td>
<td>0.34±0.17</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.13±0.20</td>
<td>0.19±0.12</td>
<td>NS</td>
<td>0.05±0.05</td>
<td>0.13±0.09</td>
<td>0.02</td>
<td>0.08±0.07</td>
<td>0.12±0.06</td>
<td>NS</td>
<td>0.19±0.07</td>
</tr>
</tbody>
</table>

Data are shown as mean values of optical density (at 650 nM) ± SD. Sera from Jewish (J) or non-Jewish (NJ) patients with CD or their unaffected relatives with wild-type TLR5 or heterozygous carriers of TLR5-stop were diluted as indicated, applied to microtiter plates coated with flagellin, and probed with horse radish peroxidase-labeled anti-IgG or IgA. P values refer to the horizontal comparisons, all of which were performed in side-by-side analysis. The comparison unaffected WT vs. unaffected TLR5-stop is simply tabular values of the data used to generate Fig., provided here for ease of comparison.

coli and Clostridia flagellin; see Refs. 21 and 28). Such studies utilized “environmental controls” (i.e., spouses), whereas this study used first-degree relatives, who are known to have elevated risk for developing CD. Together, these results suggest the possibility that Jewish subjects may receive a greater protective benefit of TLR5-stop because of a greater tendency to acquire immune responses to flagellin. However, these ethnic differences in flagellin responses are ablated by the development of CD. Nonetheless, at least in some genetic backgrounds, persons carrying TLR5-stop in the heterozygous state are less likely to develop CD than persons carrying two WT alleles.

DISCUSSION

Although a number of studies of clinical immunology have associated seemingly aberrant immune responses with particular disease states, we hold the view that all immune responses should be presumed beneficial until proven detrimental. By this guideline, we interpreted the identification of inactive nod2 as a CD susceptibility gene (16, 26) to indicate that the CD-associated adaptive immune response was likely a beneficial means of correcting for a germ line-encoded innate immune deficiency. However, in contrast to this notion, we herein observed that, at least in some genetic backgrounds, specifically Jewish ethnicity, persons deficient in TLR5 and thus unable to make normal innate and adaptive immune responses to flagellin were in fact protected from developing CD. The negative associations of TLR5-stop with flagellin immunoreactivity and TLR5-stop with CD could reflect a role for flagellin immunoreactivity promoting CD development and/or could result from flagellin immunoreactivity serving as a general marker of the adaptive immune responses promoted by TLR5 (to flagellin and other bacterial products). In either case, it indicates that at least some CD-associated immune responses may not be beneficial adaptations merely associated with CD but rather may promote disease development.

That deficiency in TLR5 (i.e., carriage of TLR5-stop) correlated with reduced levels of flagellin Ig indicates that adaptive immunity to flagellin is regulated by innate immune recognition of this molecule. Although this conclusion is consistent with Janeway’s (18) notion that adaptive immunity is dependent on innate immunity, it is to our knowledge among the first demonstrations of this concept in humans and the only case where human germline polymorphisms regulating the innate response to a protein product quantitatively affects the adaptive response to that same product. We speculate that the relative protection against developing CD in Ashkenazi TLR5-stop carriers is observed because flagellins represent such an immunodominant class of antigens stimulating the pathogenic, acquired intestinal immune response. Although the reduction in flagellin antibodies in persons carrying TLR5-stop is significant, it should be noted that such persons still have one functional copy of this gene and, in spite of TLR5-stop’s dominant-negative activity, are likely not totally deficient in TLR5 function. In agreement with this notion, the one TLR5-stop homozygote from whom we could attain serum had the lowest levels of flagellin immunoreactivity of all serum samples analyzed herein. Thus the role of TLR5-stop in regulating adaptive immunity to flagellin may in fact be greater than that observed using TLR5-stop heterozygotes.

Because the research subjects studied herein were never specifically treated with flagellin or bacteria, it seems likely that the responses they exhibited were acquired over their lifetimes as a result of exposure to bacteria and their products at their mucosal surfaces, with exposure through the gastrointestinal tract seeming particularly likely because of the large bacterial load in this tissue, which includes a variety of seemingly nonpathogenic flagellated E. coli serotypes. For most persons, exhibiting such apparently TLR5-mediated development of adaptive immunity to flagellin does not seem to have any negative consequences and probably provides mucosal protection against a variety of flagellated pathogens. Why immune responses to flagellin exceed these levels and are possibly detrimental in CD remains unclear. Although TLR5 promotes such responses, its presence alone is likely not normally sufficient to generate a robust antibody response to flagellin, since most persons (90%) are WT for TLR5 but do not exhibit elevated levels of flagellin Ig. One reasonable possibility is that elevated levels of flagellin antibodies may result from a dysregulated immune system because of a variety of potential causes such as lack of Nod2 signaling, which has recently been demonstrated to play an important role in down-regulating TLR2-mediated cytokine expression, thus resulting in overproduction of Th1 cytokines, the same cytokine pattern seen in CD patients (31). Conversely, despite being potentially detrimental, such elevated immune responses to flagellin may still result from a normal immune system properly responding to increased exposure to flagellin, which could occur as a result of altered species or location of gut bacteria or as a consequence of altered epithelial barrier function, consistent with association of polymorphisms in the epithelial-expressed multi-drug resistance protein Mdr with some forms of IBD (27).
Because other bacterial products, notably LPS, are also potent adjuvants (19), one might have reasonably expected that innate recognition of flagellin might be dispensable for adaptive immune recognition of this molecule. The fact that, among CD patients, carriage of TLR5-stop was not associated with reduced flagellin-specific Ig suggests that this may indeed be the case under inflammatory conditions. The inability of these other products to fully rescue acquisition of responses to flagellin in healthy persons could be caused by a potential requirement of flagellin to directly activate the antigen-presenting cell that will present it and/or respond to the presented peptide major histocompatibility complex, a mechanism consistent with the fact that TLR5 is expressed and functional on human dendritic cells (25). Alternatively, it may reflect a potentially very important role for flagellin as being the major activator of innate responses in the mucosa because of its potent ability to activate pro-inflammatory gene expression by epithelial cells (33), which are by far the most numerous cells in the intestinal mucosa. Because this ability to potently directly activate epithelial cells does not appear to be shared by some other bacterial components (e.g., LPS and lipopeptide; see Ref. 8), these products may be less effective mucosal adjuvants than flagellin. In light of such a potentially important role for flagellin as being the major activator of innate responses in the mucosa and pharmacological inhibition of TLR5 may be a potential therapeutic target for treating and/or preventing diseases associated with seemingly aberrant mucosal immune responses such as CD. Interestingly, TLR5-stop has also been negatively associated with systemic lupus erythematosus (13), suggesting a potentially broad role for flagellin in driving aberrant immune responses that underlie chronic inflammatory diseases.

ACKNOWLEDGMENTS

We gratefully acknowledge Peter Gregersen for providing unrelated control Jewish DNA samples and thank Eric Swanson and Daniel Moore for technical support.

GRANTS

This work was supported by National Institutes of Health Grants R24 DK-064399 (Emory Digestive Diseases Research Center), R01DK-061417 (A. T. Gewirtz), U01DK-62429 (J. H. Cho), U01DK-062422 (J. H. Cho), and DK-42086 (J. H. Cho) and by the Broad Medical Research program (A. T. Gewirtz and J. H. Cho) and the Burroughs Wellcome Fund (J. H. Cho).

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