Genetic Variants Synthesize to Produce Paneth Cell Phenotypes That Define Subtypes of Crohn’s Disease

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BACKGROUND & AIMS: Genetic susceptibility loci for Crohn’s disease (CD) are numerous, complex, and likely interact with undefined components of the environment. It has been a challenge to link the effects of particular loci to phenotypes of cells associated with pathogenesis of CD, such as Paneth cells. We investigated whether specific phenotypes of Paneth cells associated with particular genetic susceptibility loci can be used to define specific subtypes of CD. METHODS: We performed a retrospective analysis of 119 resection specimens collected from patients with CD at 2 separate medical centers. Paneth cell phenotypes were classified as normal or abnormal (with disordered, diminished, diffuse, or excluded granule phenotypes) based on lysozyme-positive secretory granule morphology. To uncover the molecular basis of the Paneth cell phenotypes, we developed methods to determine transcriptional profiles from whole-thickness and laser-capture microdissected, formalin-fixed, paraffin-embedded tissue sections. RESULTS: The proportion of abnormal Paneth cells was associated with the number of CD-associated NOD2 risk alleles. The cumulative number of NOD2 and ATG16L1 risk alleles had an additive effect on the proportion of abnormal Paneth cells. Unsupervised clustering analysis of demographic and Paneth cell data divided patients into 2 principal subgroups, defined by high and low proportions of abnormal Paneth cells. The disordered and diffuse abnormal Paneth cell phenotypes were associated with an altered transcriptional signature of immune system activation. We observed an inverse correlation between abnormal Paneth cell presence and granuloma. In addition, high proportions of abnormal Paneth cells were associated with shorter time to disease recurrence after surgery. CONCLUSIONS: Histologic analysis of Paneth cell phenotypes can be used to divide patients with CD into subgroups with distinct pathognomonic and clinical features.

Keywords: Pathogenesis; Prognostic Factor; Diagnosis; Inflammatory Bowel Disease.
clinical variability in natural history and response to therapy is likely, in part, a consequence of the genetic heterogeneity that underlies these conditions. Major challenges to genotype–phenotype association studies are the lack of robust and reproducible criteria to define end points as well as sufficient numbers of genotyped patients. Recent genome-wide association studies have extended the number of known IBD susceptibility loci to >160. These studies and others have implicated multiple pathways in IBD pathogenesis, including epithelial barrier homeostasis, innate immune response, antigen presentation, autophagy, Paneth cell de- 

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Materials and Methods

Description and Genotyping of Patient Cohort

Full methods are provided in the Supplementary Detailed

Methods. Patients were recruited at Barnes-Jewish Hospital, St Louis between 2005 and 2013 or at Cedars-Sinai Medical Center, Los Angeles between 1999 and 2013. Patient DNA samples were genotyped for ATG16L1 T300A and the CD-associated NOD2 variants.10,20,21 Patients from the Barnes-Jewish Hospital cohort were genotyped by the Digestive Disease Research Core Center using matrix-assisted laser desorption ionization-time of flight mass spectrometry and by the Genome Technology Access Center using the Human OmniQuad SNP genotyping arrays (Illumina, San Diego, CA). Patients from the Cedars-Sinai cohort were genotyped using the Immunochip (Illumina). The study protocol was approved by the Institutional Review Boards of Washington University-St Louis and Cedars-Sinai Medical Center. Written informed consent was obtained from all study participants.

Morphological Analysis of Paneth Cells

For each resection case, an H&E-stained tissue section of the proximal margin (terminal ileum) was identified by pathologists (T.S.S. and T.C.L.). Cases were included for Paneth cell analysis if the section contained at least 100 well-oriented intestinal crypts and exhibited absent or minimal acute and/or chronic inflammation (Supplementary Figure 1). Lysozyme distribution was quantified as described previously.17 For each case, a pathologist (T.C.L.) who was blinded to the characteristics of the cases scored a minimum of 200 Paneth cells (range, 206–2702) in well-oriented crypts. Paneth cells located within Peyer’s patches were excluded.

Transcriptional Analysis

RNA was procured from the set of archived formalin-fixed paraffin-embedded surgical resection samples used for histo-

logical analysis. Microarrays were performed as described pre-

viously.8 Data are deposited at ArrayExpress (http://www.ebi.

ac.uk/arrayexpress/) with accession number E-MTAB-1281.

Statistical Analyses

For analysis of lysozyme quantification, permutation tests were performed to determine the association between NOD2 variants and the percentage of abnormal Paneth cells using R statistical software (version 2.13.1, R Foundation for Statistical Computing). Mann-Whitney tests were used to demonstrate statistical difference between cases with 1 or 2 NOD2 risk variants and controls. Linear regression was used to analyze the cumulative number of risk variants. For correlation analyses, Pearson correlations were calculated using GENE-E,22 which were then used as the distance measure for unsuper-

vised, hierarchical clustering of the patients. A marker selection strategy based on signal-to-noise ratios23 was used to identify clinical variables associated with patient subtypes. A χ² test and a log-rank test were performed for the analysis of granuloma incidence and time to disease recurrence, respectively (Prism GraphPad software). P < .05 was considered significant.

Results

Association of NOD2 CD Susceptibility Variants With Abnormal Paneth Cell Phenotype

We performed a retrospective analysis of Paneth cell pheno-

types in genotyped CD patients (N = 119) using resection specimens. In order to study tissue that might exhibit early pathologic and molecular changes associated with disease pathogenesis, we examined ileal tissue samples that demonstrated no evidence of active/chronic disease. Paneth cell analysis was performed using our previously developed system for robust, quantitative scoring of Paneth cell phenotypes based on high-resolution localization of lysozyme protein,17 a highly expressed antimicrobial protein that is normally pack-

aged into Paneth cell secretory granules.17 A pathologist (T.C.L.) who was blinded to the individuals’ characteristics scored a minimum of 200 Paneth cells per case as normal or as abnormal (including Paneth cells scored as disordered, diminished, diffuse, or “excluded granule”) (Figure 1A and Supplementary Figure 2). The excluded granule abnormal Paneth cell phenotype was identified in this study and is characterized by granule shapes that have low/absent lysozyme staining, but also contain diffuse cytoplasmic lysozyme staining and occasionally a few lysozyme-positive granules (Figure 1B). For most cases, Paneth cells with normal morphology were predominant. When present, Paneth cells with an abnormal phenotype were found interspersed among those with normal morphology.

In light of the established effect of the ATG16L1 T300A CD susceptibility variant on Paneth cell phenotype,17 we initially excluded patients with this variant. The ATG16L1 T300A CD risk allele is common, with a risk allele carriage
rate >80% in people of European descent\(^20\) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2241880). Therefore, we included cases from both the Washington University School of Medicine and the Cedars-Sinai Medical Center patient cohorts. Confounding effects due to specimen source were not apparent. In this combined cohort ATG16L1 “safe” cases (cases 1–59, Supplementary Table 1), we first demonstrated that the group of cases without NOD2 risk variants (n = 29) and the group of cases with 1 or more of the common and/or rare\(^21\) CD-associated NOD2 variants (n = 30) had similar demographic compositions (Table 1). In control cases without NOD2 CD risk alleles, an average of 87% of the total number of Paneth cells counted per case had a normal phenotype based on lysozyme localization. In cases with one or more NOD2 CD susceptibility alleles, the average percent of Paneth cells scored as normal was significantly lower than controls (76% with 1 variant allele and 69% with 2 variant alleles) (Figure 1C). Accordingly, cases with one or more NOD2 CD susceptibility variants had significantly increased proportions of disordered, diminished, and diffuse abnormal Paneth cell phenotypes compared with the control cases (Figure 1C).

The NOD2 locus contains 3 common CD susceptibility alleles, namely R702W, G908R, and L1007fsXinsC. Several

**Figure 1.** CD-associated NOD2 risk alleles are associated with abnormal Paneth cell morphology. (A–C) Lysozyme immunostaining (red) was performed to visualize and score Paneth cell secretory granule morphology from cases with 0 (n = 29), 1 (n = 25), or 2 (n = 5) CD-associated NOD2 risk variants. Paneth cells were scored as normal if they contained numerous small (~1 μm), lysozyme-positive apically located granules. Disordered Paneth cells contained lysozyme-positive granules of normal size and quantity, but had some basally located granules. Diminished Paneth cells contained <10 granules, with the remaining granules frequently enlarged or fused. Diffuse Paneth cells did not contain any secretory granules and had diffuse lysozyme staining throughout their cytoplasm. (A) Representative images of lysozyme immunostaining. Nuclear counterstain, 4',6-diamidino-2-phenylindole (blue). Well-oriented Paneth cells are outlined in white and the scored phenotype is indicated: normal (N), disordered (Dis), diminished (Dim), diffuse (Diff), or excluded granule (Exc). Bars = 10 μm. (B) Representative images of Paneth cells with the excluded granule phenotype (arrowheads). (C) Quantification of Paneth cell phenotypes according to the number of CD-associated NOD2 risk variants. (D) Quantification of the percent of abnormal Paneth cells in cases with 0 CD-associated NOD2 susceptibility alleles (n = 29) or in cases that carried one allele of R702W (n = 10), G908R (n = 4), or L1007fsXinsC (n = 9). Data are presented as the mean ± SEM of the percent of Paneth cells with the indicated phenotype out of the total number of Paneth cells counted for each case. Mann–Whitney tests were used to demonstrate statistical difference between the presence of 1 or 2 NOD2 risk variants and controls (\(P < .05; \, \, \, **P < .01\)).
studies have suggested that each of these variants attenuate NOD2 function based on an impaired cellular response after exposure to the NOD2 ligand, bacterial peptidoglycan.24–27 We investigated whether these variants were similarly associated with abnormal Paneth cell phenotype in the ATG16L1 safe cohort (Figure 1D). We found that the R702W and L1007fsXinsC variants were associated with increased proportions of abnormal Paneth cells. Of note, 2 groupings of cases were observed for each of these alleles, one with <20% abnormal Paneth cells and the other with ≥20% abnormal Paneth cells. This observation suggests that the NOD2 variant alone is not sufficient to drive the abnormal Paneth cell phenotypes, but rather additional genetic variants or host/environmental factors also contribute to this phenotype. We did not observe an association between NOD2 G908R and abnormal Paneth cell phenotypes in this study. However, we were able to obtain only a small number of patients with this allele and cannot exclude the possibility of such an association. Similarly, potential associations of abnormal Paneth cell phenotypes and compound NOD2 variants (eg, R702W + L1007fsXinsC), rare CD-associated NOD2 variants or homozygous variants were not interpretable due to the rarity of cases with these genotypes (Supplementary Figure 3).

**Paneth Cell Phenotypes Define CD Patient Subtypes**

As our previous studies showed that the CD risk variant ATG16L1 T300A was associated with increased proportions of abnormal Paneth cell phenotypes, we next wanted to test for a potential interaction between this variant and the CD-associated NOD2 variants. For this analysis, we used a larger CD cohort that included both ATG16L1 T300A safe and risk cases (cases 1–119, Supplementary Tables 1 and 2). Similar to our previous study, we observed an association between ATG16L1 T300A and abnormal Paneth cells in this expanded cohort (Supplementary Figure 4). We observed a significant, positive correlation between the cumulative number of ATG16L1 T300A or CD-associated NOD2 risk alleles and the proportion of abnormal Paneth cells, demonstrating an additive effect of these alleles (Figure 2A).

As a second experiment to test the association between the CD-associated NOD2 and ATG16L1 T300A variants and Paneth cell phenotype, we performed a patient–patient comparison using the same expanded cohort. Unsupervised analysis of Paneth cell phenotypes, genetics, documented environmental exposures, and demographic information was used to cluster the CD cases according to similarity (Figure 2B). Interestingly, the resulting heat map showed 2 principal subtypes of cases. A minor number of cases did not fit either subtype. Using a marker selection strategy,23 we identified the factors that defined the 2 CD patient subtypes: the abnormal Paneth cell phenotype categories of diminished, diffuse, and excluded granule Paneth cells and the total percent of abnormal Paneth cells (Figure 2C). The cumulative number of NOD2 risk variants and NOD2 R702W genotype were also defining factors for the 2 CD patient subtypes, supporting our initial finding that CD-associated NOD2 variants are associated with abnormal Paneth cell phenotypes. We also performed the inverse experiment and looked for potential correlations between Paneth cell phenotypes and the other experimental factors (genetics, documented environmental exposures, and demographic information). Significant correlations were again observed between the abnormal Paneth cell phenotype categories and NOD2 genotype, but we did not observe significant correlations between the abnormal Paneth cell phenotypes and the environmental/demographic parameters (Supplementary Figure 5). Taken together, these data demonstrate that Paneth cell phenotype is strongly linked to particular genetic alleles and can more clearly define subtypes of CD patients than other demographic parameters (treatment, disease behavior, disease location, etc).

**An Activated Immune Response Gene Signature Is Associated With Diffuse Paneth Cell Phenotype**

To investigate the molecular basis of the Paneth cell phenotypes, we developed a method whereby we could obtain RNA from the same archival formalin-fixed,
paraffin-embedded tissue samples used to morphologically phenotype Paneth cells. Transcriptional profiles of the CD patient material were generated by performing microarray analysis with one set of RNAs procured by laser capture microdissection of ileal crypt bases (enriched for Paneth cells, n = 15 samples) and a second set of RNAs procured from whole, unstained ileal tissue sections scraped from glass microscope slides (n = 40). To analyze these data...
(Supplementary Figure 6), we first compared the 2 sets of transcriptional profiles to identify transcripts enriched in the Paneth cells of CD patients. Next, we performed a correlation analysis between the expression of the Paneth cell-enriched genes and the quantitative Paneth cell phenotype data for the same 40 patients using the whole ileal tissue dataset to identify distinct sets of Paneth cell—enriched transcripts with expression values that were significantly correlated with each Paneth cell phenotype (Supplementary Table 4).

We performed gene ontology term analysis for each transcript set that was correlated with a particular Paneth cell phenotype (Figure 3 and Supplementary Figures 7–9). This analysis showed that the transcript sets that correlated with the disordered and diffuse Paneth cell phenotypes were both enriched for immune system-related biological processes. We previously observed a gene signature of cytokine stimulation in the Paneth cells of mice with hypomorphic expression of Atg16l1 (which have increased proportions of Paneth cells with the disordered and diffuse phenotypes). These data suggest that altered immune activation might be occurring in human and mouse intestinal tissue that exhibits abnormal Paneth cell phenotypes, although the underlying causes of these transcriptional profiles remains unclear. The disordered and diffuse Paneth cell phenotypes appear to be histological readouts of specific types of inflammatory responses occurring in a subset of CD patients.

**Abnormal Paneth Cell Phenotype Inversely Correlates With Granuloma Incidence**

We next examined whether other more classic morphological features of CD, such as the presence of granuloma, were correlated with the Paneth cell phenotypes. Granulomas are a distinguishing feature of CD that is not present in all patients. For this analysis, pathologists (T.C.L. and D.D.) reviewed the entire case for each patient, including both involved and noninvolved intestinal regions (n = 107, cohort from patients 1–119 with available material, Supplementary Tables 1 and 2). We found that cases with low proportions of abnormal Paneth cell phenotypes (arbitrarily defined as <20% based on this study and our previous in vivo studies) had a higher incidence of granuloma than cases with high proportions of abnormal Paneth cell phenotypes (43.8% vs 19.4%, respectively; P = .0160) (Figure 4A).

We then examined whether the presence of granuloma was associated with a particular abnormal Paneth cell phenotype. We found that the excluded granule phenotype exhibited the strongest inverse correlation with granuloma incidence, with 39.4% of cases with low proportions of excluded granule Paneth cells (<1%) having granuloma compared with 17.9% of cases with high proportions of excluded granules (≥1%) (Figure 4B; P = .0344). A similar finding was observed with the diminished granule Paneth cell phenotype (46.5% vs 26.8% incidence in cases with low (<10%) and high (≥10%) proportions of diminished granule Paneth cells, respectively; P = .0347) (Figure 4C). Finally, cases with low proportions of diffuse granule Paneth cells (<5%) had 37.0% granuloma incidence compared with 14.3% granuloma incidence observed in cases with high proportions of diffuse granules (≥5%), although this correlation was not significant (P = .4159; Figure 4D). In accordance with previous reports, we did not find a significant association between NOD2 status (including individual risk alleles) and granuloma incidence in our cohort (data not shown). In summary, we identified that abnormal Paneth cell phenotypes are inversely associated with the presence of granuloma.
Discussion

Here we have provided the first evidence that a defined cellular phenotype (in Paneth cells) is linked to multiple CD genetic susceptibility loci and subdivides patients into 2 groups. In addition, we defined the molecular consequences of this phenotype in human Paneth cells and found an association with immune activation. We demonstrated that Paneth cell phenotypes are associated with the presence of granuloma, a classic histological finding associated with CD. We also showed that the Paneth cell phenotypes are associated with a specific clinical outcome in the current cohort, ie, time to disease recurrence. Although there clearly is a genetic basis for this phenotype, because of the potential complexity of the genetics and environmental interactions, we propose that the Paneth cell phenotypes would be a readily accessible, integrative readout of this information (rather than genetics alone) and should be further evaluated for their ability to stratify CD patients in a clinically meaningful way.

We demonstrated that CD cases with one or more NOD2 susceptibility alleles had increased proportions of abnormal Paneth cells compared with those without NOD2 susceptibility alleles. In this study, we also found an additive effect of NOD2 susceptibility alleles and ATG16L1 T300A on Paneth cell abnormalities. This finding is of interest because Paneth cell phenotypes have now been demonstrated to link 2 IBD susceptibility genes that have been suspected to act in a shared biochemical pathway. It has been proposed that IBD genes act in pathways, and our findings provide additional evidence that supports this theory.

Paneth Cell Phenotype Is Associated With Disease Prognosis

We next investigated if Paneth cell phenotypes were associated with the disease prognosis post resection. For this analysis, we used our expanded CD cohort (cases 1–119, Supplementary Tables 1 and 2), as well as an additional 59 CD cases that have not yet been genotyped, but had Paneth cell scoring and disease recurrence data (cases 120–178, Supplementary Table 3). In patients who received prophylactic therapy post resection (ie, suggestive of patients with a more aggressive clinical course before resection; n = 102), those with high proportions of abnormal Paneth cells (≥20%) had a significantly shorter time to disease recurrence compared with those with low proportions of abnormal Paneth cells (<20%) (P = .0200; Figure 5). A significant difference was not observed in patients who did not receive prophylaxis post resection (ie, patients with a less aggressive clinical course before resection) (Supplementary Figure 10). We conclude that Paneth cell phenotypes can identify clinically relevant subtypes of CD patients.
There is a pressing need to classify subtypes of CD based on underlying pathological mechanisms including genetics and not solely by clinical parameters. This need is highlighted by the fact that treatment with anti–tumor necrosis factor-α monoclonal antibodies, the most advanced biologic currently used, does not induce remission in the majority of CD cases, and the majority of initial responders do not maintain long-term remission.\(^3\)\(^,\)\(^4\) In addition, many potential therapeutics for CD have failed to induce or maintain remission of active disease.\(^2\)\(^,\)\(^5\) Although some of these “failed” therapeutics truly do lack efficacy, inappropriate end-point selection and patient heterogeneity have been cited as factors contributing to treatment inefficacy in clinical trials.\(^3\)\(^,\)\(^4\) It is becoming increasingly apparent that novel strategies to define and stratify CD patients that are based on genotype and other molecular parameters will be needed to progress toward improved diagnostics, prognostics, and therapeutics. Because the ability to test potential CD therapeutics in a particular subtype of patients might yield better outcomes, the abnormal Paneth cell phenotypes, as an objective biomarker that is both related to genetic susceptibility loci and disease pathogenesis mechanisms, should be explored as a method to identify a subtype of CD patients that has a similar disease pathogenesis mechanism. In this study, we observed a novel and important histological correlate to the Paneth cell phenotype, an inverse relationship between the presence of granuloma and the proportion of abnormal Paneth cells. This is, to our knowledge, the first report linking defects in a CD-relevant cell type and other, more classical histologic changes of CD. The standard practice for pathologic examination for CD includes extensive sampling of the specimen (at a minimum of 1 section/10 cm), and as there might be unsampled granulomas in patients where no granulomas were found, it is perhaps best to classify the 2 subsets of CD patients as “granuloma-rich” and “granuloma-poor.” We showed that the total proportion of abnormal Paneth cells, and in particular, excluded and diminished granules, were strong indicators of the granuloma-poor subtype. This is important because although we showed that \(NOD2\) status was associated with Paneth cell phenotype, we and others also showed that there was no significant correlation between \(NOD2\) status and granuloma,\(^3\)\(^6\)\(^–\)\(^3\)\(^8\) and that Paneth cell phenotype is the best predictor of granuloma. Our work supports the model that cell-specific readouts that can integrate the effects of both genetic and environmental factors are more informative and clinically relevant (Figure 6). There has been controversy over the biologic and prognostic implications of granulomas in CD,\(^3\)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\) with some recent studies indicating that granulomas might be linked to more aggressive clinical behavior\(^2\)\(^8\) and others that it is not.\(^3\)\(^7\)\(^,\)\(^8\) An immediate clinical implication of our finding is that stratification of CD based on Paneth cell phenotypes might be a more reliable approach for clinical management and clinical trial design, as features such as granuloma could be sparse and more likely to be undersampled, especially in biopsy specimens, and Paneth cell phenotypes are more easily analyzed within limited samples. Prospective studies using Paneth cell phenotypes as stratification for novel CD therapeutics might yield new insights.

In contrast to the success that has been achieved in treating single gene diseases (eg, chronic myelogenous leukemia and imatinib [Gleevec]),\(^3\)\(^9\)\(^–\)\(^4\)\(^1\) the development of effective therapeutic options for complex genetic diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and IBD has been difficult.\(^3\)\(^4\)\(^,\)\(^4\)\(^0\)\(^,\)\(^4\)\(^1\) Of the complex genetic diseases, CD studies are uniquely positioned to lead the way in the development of novel therapies. First, there is a clearer understanding of the genetic susceptibility loci that are associated with CD susceptibility compared to rheumatoid arthritis or systemic lupus erythematosus. Second, relevant tissue and cell types (not restricted to Paneth cells) are relatively easy to access. In contrast, for diseases such as type 1 diabetes, where there is a good understanding of the associated genetics, there is poor tissue availability.\(^5\) We believe that CD can be the prototype disease for moving forward the study of complex genetic diseases for both the development of new therapeutics and a more personalized approach to disease management.
Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.09.048.

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