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Polymorphisms in the TNF-α Promoter and Variability in the Granulomatous Response in Patients with Crohn's Disease

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ABSTRACT: Granulomas may be found in 30–70% of patients with Crohn's disease (CD). The etiology of granuloma formation in CD is presently unknown. Elevated levels of TNF-α are found within granuloma tissue, and are required to maintain granuloma formation in animal models. TNF-α production has been shown to influenced by TNF-α promoter polymorphisms. We hypothesized that heterogeneity for granulomas in CD might be influenced by the TNF-α promoter genotype. Patients with confirmed CD that had undergone full colonoscopy with multiple biopsies and/or surgical resection, served as the study group. One hundred healthy individuals served as a control population for genotyping. Patients and controls underwent genotyping for four TNF-α polymorphisms: 238G/A, 308 G/A,857 C/T, and 863 C/A. Inclusion and exclusion criteria were met in 155 patients (1–68 y). Polymorphisms in the TNF promoter were found in 16.6% (238G/A), 14.5% (308 G/A), 36.6% (857 C/T) and 30.7% (863G/A). No significant association was found for any of the individual polymorphisms with presence or absence of granulomas. In conclusion, we did not find an association between individual polymorphisms in the TNF-α promoter and presence of granulomas in CD. The reason for heterogeneity in granuloma formation in patients with CD remains elusive. **(*Pediatr Res* 59: 825–828, 2006)**

Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract (GIT) that can occur at any age. Upon histopathologic examination, it is characterized by a focal mononuclear inflammation along the GIT, and epithelioid granulomas (1,2).

Granuloma formation is a complex TH1 immune-mediated process caused by activated macrophages surrounding particulate sources of antigen or bacteria, producing nodules of inflammatory tissue. Activated macrophages responsible for this process, cause an inflammatory response through the secretion of cytokines such as TNF, Interleukin (IL)-1, IL-12 and chemokines. In response to the persistent signals, the activated macrophages develop increased cytoplasm and cytoplasmic organelles, which may resemble skin epithelial cells, the source of the designation "epithelioid cells." Activated macrophages may fuse to form multi-nucleated gianT-cells (3–5).

Although granulomas are a frequent and hallmark finding in other TH1 type diseases, significant heterogeneity exists regarding granuloma frequency and distribution in CD (6–10). The variability in frequency of granuloma detection, coupled with the hypothesis that Crohn's disease is not a response to single bacteria or pathogen, raise the following question; what causes phenotypic diversity in CD when it comes to granulomas?

One possible option is the type of pathogen. Several groups have postulated that CD may be caused by mycobacteria, however conflicting results from previous studies, along with the absence of evidence for infection in a significant subset of patients, raise doubts about this scenario (11–13). Recent studies have implicated intestinal flora including *Escherichia coli* in the pathogenesis of disease. Studies comparing the presence of strains of *E. coli* in CD and controls have demonstrated *E. coli* on the mucosa, in the intestinal epithelium, in mucosal macrophages, and in granulomas of patients with CD (14–17), but not in controls. This scenario is appealing, as it is consistent with an abnormal response to non-pathogenic intestinal flora. If this is the case, why do some patients develop granulomas, while others do not?

Another option is that the type of response, granulomatous or otherwise, may be dictated by variability in the underlying host response. Susceptibility to CD has been associated with defects in innate luminal immunity, and specifically with inherited defects in pattern recognition receptors of bacterial products. These include the NOD2/CARD15 gene, (an intracellular sensor for muramyl dipeptide) (18–21), and toll-like receptor 4 (TLR4), which recognizes LPS (24). At present, there is no evidence of an association between NOD2/CARD15 or TLR4 genotype, and presence of granulomas (22,23)

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Abbreviations: CD, Crohn's disease; **SNPs,** single nucleotide polymorphisms; **TLR,** toll-like receptors

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Granulomatous diseases and CD are characterized by TNF-α secretion. Elevated levels of TNF-α are found within granuloma tissue in CD and other granulomatous diseases, and are required for granuloma integrity in animal models and human diseases (14,25,26).

Polymorphisms that have been reported to increase (308G/A) or decrease circulating TNF-α (238G/A, 857 C/T, 863 C/A) (27–33), as well as playing a possible role in disease phenotype and susceptibility (27–39). The 857C/T and 863C/A polymorphisms appear to be located at transcription factor binding sites (32,34,35).

We examined these polymorphisms in an established cohort of CD patients, to evaluate if TNF-α genotype in CD is associated with the presence of granulomas.

MATERIALS AND METHODS

Study population. The study cohort consisted of patients with established CD recruited by pediatric or adult gastroenterology clinics, distributed throughout central and Northern Israel. One hundred DNA samples, ethnically matched for the Israeli population, were obtained from the National Laboratory for the Genetics of Israeli Populations at Tel Aviv University, and this group served as a control group for TNF-α polymorphisms. The study was authorized by an ethical review committee. Samples and data were obtained after informed consent was obtained.

Patients were eligible if CD was confirmed by established criteria based on clinical, radiologic, endoscopic and histopathological findings. Inclusion criteria included a full colonoscopy with multiple biopsies along the colon or the terminal ileum, or surgical resection. Patients with inflammatory bowel disease (IBD), like disease in the presence of a known immune deficiency state such as glycogen storage disease or chronic granulomatous disease, were excluded. All pathologic specimens were reviewed for presence of epithelioid granulomas by pathologists within participating centers. Patients included in this study were from a previously published cohort, in whom we established that the presence or absence of granulomas was unrelated to the number of biopsies sampled (mean number of biopsies 10.4 $\pm$ 6.2 with granulomas, *versus* 8.9 $\pm$ 5.6 biopsies without granulomas, NS), or to NOD2/CARD15 genotype (23).

Genetic analysis. The TNF-α polymorphisms examined included TNF 238G/A,308 G/A, 857 C/T, and 863 C/A. Genomic DNA was extracted from whole peripheral venous blood, using a commercially available kit (Gentra, Minneapolis, MN) in accordance with the manufacturer's instructions.

Mutations of the TNF-α promoter gene were analyzed by pyrosequencing technology (35). Polymerase chain reaction was performed in a 50 μL volume containing 10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTPs, 1 μM of each primer, 200 ng of genomic DNA and 1.25U of AmpiTaq Gold DNA polymerase (Perkin Elmer Applied Biosystem) with an initial denaturation step of 10min at 95°C to activate the polymerase followed by 35 cycles of 94°C; 15 s, 60°C; 45 s, 72°C; 45 s and final elongation of 10 min at 72°C. Predicted sizes were confirmed by agarose gel electrophoresis. PCR products were prepared for pyrosequencing analysis using the PSQ sample preparation kit according to the standard protocol from pyrosequencing AB and analyzed for the various SNPs on a PSQ 96MA pyrosequencer, using 0.2 μM sequencing primers and the SNP reagent kit according to standard protocols. The order of nucleotide dispensation was decided based on suggestions provided by PSQ HS 96 SNP software 1.0 (pyrosequencing AB) which was also used for automatic assay evaluation and genotype scoring.

Data analysis. Data analysis was carried out using SPSS 9.0 statistical analysis software (SPSS Inc., Chicago, IL, 1999). For continuous variables, such as age and disease duration, descriptive statistics are reported as mean $\pm$ SD. Categorical variables such as sex and the presence of granulomas and specific polymorphisms were described using frequency distributions. The χ^2 test was used to detect differences in categorical variables by granuloma prevalence. All tests are two-sided and considered significant at $p < 0.05$.

The study had 85% power to detect a 20% difference in granuloma frequency between groups for the least common polymorphism, and an 85% power to detect a 25% difference for the most common. Hardy Weinberg Equilibrium was not assumed during the analysis.

Table 1. *Entry data*

N = 155	Parameter
23.3 $\pm$ 12.8	Age of onset (years)
24.5 $\pm$ 13.6	Age biopsies (years)
56/44%	Gender m/f
40%	Site - ileal
40%	Site - ileocolonic
18%	Site - colon only
33.5%	Granulomas - final evaluation

RESULTS

One hundred ninety-nine patients were evaluated for this study. Complete pathologic, and TNF genotyping data, were available for 155 of these patients who met inclusion and exclusion criteria, all of whom had undergone colonoscopy with multiple biopsies or surgical resection. There age at disease ranged from 1 to 68 y (63 patients under age 17). Entry data are presented in Table 1. Approximately 40% of patients had ileal or ileal with upper intestinal disease, 18% had colitis only, 40% has ileocolonic disease, and the rest had disease confined to proximal gastro-intestinal tract above the ileum. Granulomas were present in 52 patients, and all but one patient had the granulomas detected by colonoscopy or surgery.

Data regarding the prevalence of TNF-α polymorphisms in our CD and a control population consisting of 100 healthy individuals are presented in Table 2. Allele frequencies in CD patients and controls respectively were 7.6% and 4.6% for 238G/A, 8.6% and 7% for 308 G/A, 21% and 20.6% for 857 C/T, and 17.3 and 22% for 863 C/A(all non-significant). TNF polymorphisms were not in Hardy-Weinberg equilibrium.

Data regarding the prevalence of granulomas according to TNF genotype are presented in Table 3. There was no apparent association between presence or absence of any of the polymorphisms in the whole cohort, or in a sub-analysis of surgical patients ($n = 46$). An additional sub-analysis that grouped polymorphisms by a purported common effect of the polymorphism on circulating TNF-α (TNF-238 G/A+TNF-863 C/A+TNF-857 C/T) *versus* absence of one or all of these polymorphisms, did not alter the conclusions from the initial analysis.

Data regarding prevalence of granulomas in pediatric onset *versus* adult-onset disease is not presented since data from this cohort has been previously published, and no significant difference in prevalence of granulomas was found (23).

Table 2. *Carriage of TNF-α polymorphisms in CD patients and healthy controls*

p-value	Controls with at least 1 allele (%)	Patients with at least 1 allele (%)	Polymorphism $n = 100$
NS	9.3	16.6	TNF 238 G/A
NS	14	14.5	TNF 308 G/A
NS	35	36.6	TNF 857 C/T
NS	40	30.7	TNF 863 C/A

Table 3. *Effect of TNF genotype on prevalence of granulomas*

p-value	Granuloma − $n = 103$ (%)	Granuloma + $n = 52$ (%)	Polymorphism
NS	11/102 (11)	11/50 (22)	TNF 238 G/A+
NS	17/102 (17)	8/51 (16)	TNF 308 G/A+
NS	39/102 (38)	17/51 (33)	TNF 857 C/T+
NS	29/102 (28)	18/52 (35)	TNF 863 C/A+

(%) rounded to the closest whole number.

DISCUSSION

The role of bacterial adhesion or invasion in CD has recently come to the forefront, and may be related to disease pathogenesis and chronicity. Defects in pattern recognition receptors for bacterial products are associated with the disease (18–21,24), and bacteria have been found to be abnormally adherent and to invade epithelial cells (14–18). If this is the case, presence of granulomas may be perceived to be a harmful but appropriate immune response to this constant exposure to bacteria bridging the integrity of the intestinal luminal-epithelial barrier. The ability to use this response to contain bacteria on the "wrong side of the fence" may be modulated by an individual's genetic makeup. The NOD2/CARD15 and TLR4 disease susceptibility genotypes have not been found to be associated with presence or absence of granulomas (22,23), raising the possibility that genes unrelated to disease susceptibility, such as those that may cause alterations in processes related to granuloma formation, may play a role. We explored the TNF-α promoter for a genotypic association, since TNF-α plays a pivotal role in both CD and in the granulomatous response, and functional polymorphisms that affect TNF-α are fairly common. Previous studies in animal and human models have shown that anti-TNF-α antibodies cause a reduction in granuloma size (36), and that TNF-α is critical for granuloma formation and integrity in TNF −/− mice and in human tuberculosis (25,37). In non-mycobacterial graulomatous diseases, such as Sarcoidosis, use of anti-TNF-α agents, such as thalidomide, pentoxyphylline and infliximab result in a clinical response (41).

We hypothesized that polymorphisms which may decrease TNF-α Transcription (238 G/A,857C/T,863C/A), might impede granuloma formation. We did not find an association between any of the individual polymorphisms, and presence or absence of granulomas. Since some of the polymorphisms are less frequent, we performed an additional analysis after grouping the polymorphisms into groups by perceived function. Even after including all patients with any polymorphism that may decrease circulating TNF, we did not find an association between TNF genotype and granulomas.

A significant bias involving our understanding of the granulomatous response is due the fact that much of our knowledge is extrapolated from animal and human models predominantly involving mycobacteria.

Several recently published studies suggest that the role of TNF-α is more complex than previously understood. Zganiacz *et al.* recently demonstrated that granulomas are formed, but disintegrate in TNF −/− mice exposed to *M. bovis* BCG. Granulomas remained preserved in these same animals if CD4 and CD8 T-cells were depleted, suggesting that TNF is not required for granuloma formation, but to inhibit and regulate an overwhelming T-cell response (38). Although one could hypothesize that a genotype with decreased TNF production might lead to more severe inflammation in CD because of a compromised ability to regulate a T-cell response, and decreased granuloma integrity, this is not consistent with what we know from clinical studies involving CD and granulomas. In fact, several studies have implied the opposite, that the presence of granulomas may be associated with more severe inflammation or disease (38–40).

Finally, there are inherent limitations to this study which can introduce bias. Sampling bias during colonoscopy, and evaluation of specimens from surgical resections, may influence the yield of granulomas in pathologic specimens. We did not find that the mean number of biopsies differed between patients with or without granulomas by colonoscopy. Surgical resections are not as common in pediatric disease, and use of surgical specimens may introduce selection bias regarding disease severity and duration. Additional statistical analysis of the findings in patients with surgical resection did not lead to a different conclusion. Although the study was underpowered to detect if the minor differences noted in granuloma frequency were due to the most frequent polymorphisms, the differences between the groups (5–7%) are of doubtful significance and unlikely to serve as an explanation for heterogeneity in granulomas observed. Similarly, since 238 G/A purported to reduce circulating TNF-α, was relatively infrequent in our population, our study would require greater numbers to determine a negative association with this polymorphism. To overcome this obstacle, we performed an additional analysis combining all polymorphisms likely to reduce TNF. This analysis did not find any association between TNF genotype and presence or absence of granulomas. Our cohort was not in Hardy-Weinberg equilibrium, possibly due to subpopulations derived from immigration over the last decades. This is unlikely to affect our results, since we did not assume Hardy Weinberg in the evaluation, and examined a phenotype within an uncommon disease cohort, and did not attempt to establish a disease association in comparison to the normal population.

In conclusion, we did not find evidence to suggest that the heterogeneity regarding presence of granulomas in CD is related to TNF-α promoter genotype. Since CD is multifactorial, and multiple genes may affect susceptibility and disease phenotype, the answer may be more complex and multifactorial as well. Since the activation of the TNF receptor complex initiates a downstream cascade of intermediate proteins, it is possible that alterations in these genes rather than the TNF-α promoter itself may harbor loss or gain of function, which may contribute to the variability in granuloma formation is some CD patients. Elucidation of all the susceptibility and phenotype associated genes, as well as environmental and microbiological factors affecting the disease, may allow us to answer this question not only for CD, but for other idiopathic granulomatous diseases as well.

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