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Innate Dysfunction Promotes Linear Growth Failure in Pediatric Crohn Disease and Growth Hormone Resistance in Murine Ileitis

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Abstract

Background—Growth failure remains a common complication of pediatric Crohn Disease (CD), and has been associated with small bowel involvement and need for surgery. We have reported that patients with elevated (≥ 1.6 mcg/mL) Granulocyte Macrophage Colony Stimulating Factor auto-antibodies (GM-CSF Ab) are more likely to experience complicated ileal disease requiring surgery. We hypothesized that concurrent GM-CSF Ab and *CARD15* risk allele carriage (*C15*⁺GMAb⁺) would be associated with growth failure in CD, and growth hormone (GH) resistance in murine ileitis.

Methods—We enrolled 229 pediatric CD patients at two sites and determined *CARD15* genotype, serum GM-CSF Ab and GH binding protein (GHBP), and height (HTz) and weight (WTz) z scores at diagnosis. Ileitis was induced in *card15* deficient mice by gm-csf neutralization and NSAID exposure. Hepatic GH receptor (Ghr) abundance and GH dependent Stat5 activation were determined by western blot, and *Igf-I* mRNA expression by real-time PCR.

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Results—Mean[95th CI] HTz at diagnosis was reduced to $-0.48[-4.2,2.3]$ in *C15*⁺GMAb⁺ patients, compared to $-0.07[-4.9,3.4]$ in disease controls, $p \leq 0.05$. Circulating GHBP, as a marker for tissue GHR abundance, was reduced in *C15*⁺GMAb⁺ patients. Hepatic Ghr abundance, GH induction of Stat5 tyrosine phosphorylation, and *Igf-I* mRNA expression were reduced in male *card15* deficient mice with ileitis due to gm-csf neutralization and NSAID exposure.

Conclusions—Innate dysfunction due to concurrent genetic variation in *CARD15* and neutralizing GM-CSF Ab is associated with linear growth failure in pediatric CD, and hepatic GH resistance in murine ileitis.

Keywords

granulocyte-macrophage colony stimulating factor; pediatric Crohn disease; linear growth; caspase recruitment domain family member 15; nucleotide-binding oligomerization domain containing 2

INTRODUCTION

Despite more frequent use of steroid-sparing therapies, linear growth impairment remains a common complication of pediatric Crohn disease (CD) (1). Growth velocity is often compromised both before and after the diagnosis, leading to a reduction in final adult height (2,3). Patients with extensive small bowel involvement are particularly at risk for growth impairment (3,4) and boys are typically more affected than girls (2,5). While recent efforts have explored mechanisms involved in growth impairment, many gaps exist in our knowledge of this important complication of CD.

While linear growth impairment had once been attributed to poor nutritional status and corticosteroid use, research over the last decade has suggested a role for genetic polymorphisms and pro-inflammatory cytokines. Our animal studies have characterized mechanisms by which endotoxin and TNF α induce growth hormone (GH) resistance via down-regulation of the tissue GH receptor (GHR) (6–8), and we have shown that suppression of circulating TNF α is associated with improved growth in CD (9). Two TNF α promoter polymorphisms which reduce circulating TNF α have been associated with increased linear growth in pediatric CD (10), while a polymorphism in the IL-6 promoter which increases IL-6 levels was associated with growth retardation. (11). However, pediatric patients with Ulcerative Colitis (UC) also experience increases in these cytokines, but typically grow normally (12). This has suggested that factors specifically associated with small bowel involvement and increased exposure to bacterial products including endotoxin might promote down regulation of the GHR and growth failure.

CARD15/NOD2 mutations are associated with small bowel involvement and reduced weight at diagnosis (13). However, when considered alone these have not been associated with reduced height (13,14). Our group has reported that neutralizing Granulocyte Macrophage Colony Stimulating Factor auto-antibodies (GM-CSF Ab) reduce neutrophil antimicrobial function and increase the likelihood of small bowel involvement and aggressive behavior requiring early surgery in CD (15). We have found that elevated GM-CSF Ab are associated with increased intestinal permeability and anti-endotoxin antibodies, in the absence of differences in systemic or intestinal inflammation (21). We developed an animal model of CD based upon these observations, and showed that on a background of *card15* deficiency, GM-CSF Ab administration followed by NSAID exposure led to a transmural ileitis (15). This murine model also demonstrated increased intestinal permeability and bacterial translocation (15). We therefore hypothesized that *CARD15* mutations may combine with

neutralizing GM-CSF Ab to promote small bowel location and growth retardation in children with CD, in association with down regulation of the GHR in this subset of patients, and in the corresponding animal model of ileitis. We found that pediatric CD patients with both a *CARD15* SNP and elevated GM-CSF Ab have reduced height at diagnosis, and in our complementary murine studies, we demonstrate decreased hepatic growth hormone signaling in male mice with ileitis.

MATERIALS AND METHODS

Human Subjects

A retrospective chart review was performed to determine age at diagnosis, gender, disease location and behavior based on the Montreal classification system (16), and height and weight at diagnosis, and for the group enrolled at the Cincinnati site, height one and two years after diagnosis, disease activity, and medication exposures. Height and weight were converted into standard deviation scores (z scores) using the Children's Hospital Boston Growth Calculator 2.01. Blood samples were drawn at the time of study enrollment and medication exposures were recorded.

CARD15 Genotyping

As previously described (15), DNA was isolated from whole blood and genotyped for the three common mutations in *CARD15* associated with increased risk for CD: NOD2R702W (SNP8), G908R (SNP12) and 1007fs (SNP13) in the Genetics Core Laboratories at CCHMC and MCH.

Serum GM-CSF Ab, GHBP, IGF-1, cytokine, and LBP measurements

GM-CSF Ab were quantified in serum by enzyme-linked immunosorbent assay (ELISA) (17). The following cytokine and growth factor measurements were obtained (9,18): serum tumor necrosis- α (TNF- α ; sensitivity: 0.05 pg/mL, inter-assay variation: 8.3%), and interleukin-6 (IL-6; sensitivity: 0.10 pg/mL, inter-assay variation: 7%) were measured using the Luminex platform and a custom human cytokine 2-plex high-sensitivity antibody bead kit (Millipore, Billerica, MA). Serum insulin-like growth factor-1 (IGF-1; sensitivity: 1.9 ng/mL, inter-assay variation: 7%; Immunodiagnostic Systems, Tyne & Wear, UK) and growth hormone binding protein (GHBP; sensitivity: 1.69 pmol/L, inter-assay variation: 6.3%; DSL, Webster, TX) were measured by enzyme-linked immunosorbent sandwich assay. Serum lipopolysaccharide binding protein (LBP; sensitivity: 4.4 ng/mL) was determined by solid-phase enzyme-linked immunosorbent sandwich assay (Hycult Biotechnology, Uden, The Netherlands).

Mouse model of ileitis

We induced ileitis in *Card15/Nod2* deficient (C15KO) mice (Jackson Laboratories) with anti-GM-CSF antibody administration (50 mcg, Pierce Biotechnology, Rockford, IL) at 4 weeks of age followed two weeks later by exposure to piroxicam (a NSAID) at 200 ppm in the chow for one week (Cincinnati Lab Supply, Cincinnati, OH) (15). C15KO mice provided with regular chow, or pre-treated with an isotype control antibody and then exposed to piroxicam, served as controls. Weights were monitored twice weekly. The mice were sacrificed at 7 weeks of age and whole blood and liver were harvested.

Immunoblot analysis of hepatic Ghr and pSTAT5 protein abundance

As we have previously published (7), nuclear and cytoplasmic proteins were prepared from livers using the NE-PER kit (Thermo Scientific, Rockford, IL). The nuclear (20 mcg) and cytoplasmic (100 mcg) proteins were loaded on to 4–12% graded Bis-Tris gel or 7.5% Tris

Acetate gel (Invitrogen, Carlsbad, CA) and underwent electrophoresis and electrotransfer to nitrocellulose membranes. The nitrocellulose membranes were then blocked at room temperature for 1 hour and incubated overnight at 4°C with antibodies specific for the GHR (AL-47), (7), pSTAT5 (Millipore, Temecula, CA), β -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) and SHPTP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:500–1:2000). Blots were washed and incubated with the appropriate horseradish-peroxidase-conjugate antibody (Santa Cruz Biotechnology). Chemiluminescence ECL (Perkins Elmer, Waltham, MA) was used to detect immune complexes and blots were developed using Blue Basic Autorad Film (ISC BioExpress, Kaysville, UT). Band signal intensity was measured by densitometry, with results for the target proteins (Ghr and pSTAT5) normalized to results for the loading control proteins (β -tubulin and SHPTP-1, respectively) (ImageQuant).

Real-time PCR Analysis of hepatic *Igf-1* and *Ghr* mRNA expression

As previously described (7), total liver RNA was extracted using TRIzol (Gibco Life Technologies). The RNA concentration was determined and complementary DNA was prepared using the AccuScript High Fidelity 1st Strand cDNA synthesis kit (Stratagene, Santa Clara, CA) per protocol. We used the same primers from our previous study for *mIgf-1* and total *mGhr* (7). The Brilliant SYBR Green real-time quantitative PCR assay was performed on a Stratagene Mx3000P machine, according to manufacturer's protocol (Stratagene, Santa Clara, CA). Standard curves were used to determine relative quantification of each target gene. Dissociation curves were implemented to confirm the absence of dimer-primers. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to normalize the quantification of target gene complementary DNA.

Murine serum LBP and Igf-1 Measurements

Serum LBP concentration was determined by a commercially available ELISA (Cell Sciences, Canton, MA) per manufacturer's protocol. Serum Igf-1 was determined by commercially available ELISA (Immunodiagnostic Systems, Fountain Hills, AZ) per manufacturer's protocol.

Statistical Analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc, Cary NC) and GraphPad PRISM version 4.01 (GraphPad Inc, San Diego, CA). Univariate analyses were conducted to identify any potential outliers and determine the normality of the data. Continuous variables were analyzed using 2-sample t-test and 1-way ANOVA with Newman-Keuls test for multiple comparisons, or their nonparametric alternatives, the Mann-Whitney test or Kruskal-Wallis with Dunns test for multiple comparisons, when the normality of the data was suspected. Odds ratio estimates were computed with 95% confidence intervals. Chi square test for trend was performed to determine the relationship between growth failure (HTz < -1) and GM-CSF Ab/*CARD15* status. Multivariate logistic regression analyses were conducted to observe the conditional relationships between height z-score, serum GM-CSF antibody concentration, and *CARD15* single nucleotide polymorphism (SNP) carriage, while controlling for age, gender and disease location. Logistic regression analyses were conducted to observe conditional relationships between ileal disease location and serum GM-CSF antibody concentration and *CARD15* single nucleotide polymorphism (SNP) carriage while controlling for age and gender. P value < 0.05 was considered significant.

ETHICAL CONSIDERATIONS

229 patients with Crohn's disease (CD) were recruited from two sites: Cincinnati Children's Hospital Medical Center (CCHMC) and Milwaukee Children's Hospital (MCH). Human studies were approved by the respective institutional review boards. Mice were maintained in conventional housing with free access to chow and water. Animal studies were approved by the CCHMC Institutional Animal Care and Use Committee.

RESULTS

Clinical and Demographic Characteristics

The clinical and demographic characteristics of the pediatric CD cohort, stratified by *CARD15* genotype and GM-CSF Ab status, are summarized in Table I. There were 45 patients with both a *CARD15* risk allele and elevated GM-CSF Ab (*C15*⁺GMAb⁺). The remaining 184 patients, those with one or neither factor, served as the disease controls. There was no difference in age at diagnosis, age at blood sample collection, gender, or frequency of moderate-to-severe disease activity at diagnosis between the groups. As shown in Table I and Supplemental Table I, there was a higher frequency of small bowel disease location in the *C15*⁺GMAb⁺ group (89% compared to 64% in disease controls, $p < 0.05$).

CARD15 risk allele carriage and GM-CSF Ab Status and Linear Growth at Diagnosis

Neither HTz nor WTz at diagnosis varied between CD sub-groups when considering *CARD15* risk allele carriage or GM-CSF Ab status alone. However, mean[95th CI] HTz at diagnosis was reduced to $-0.48[-4.2, 2.3]$ in the *C15*⁺GMAb⁺ group, compared to $-0.07[-4.9, 3.4]$ in disease controls, $p \leq 0.05$ (Table I). Consistent with this, there was a significantly higher frequency of patients with growth retardation ($HTz \leq -1$) and growth failure ($HTz \leq -1.8$) in the *C15*⁺GMAb⁺ group compared to disease controls (38% vs 18% and 16% vs 6%, respectively, $p \leq 0.05$). WTz did not vary between the groups, suggesting a specific effect upon linear growth. Chi square test for trend was performed to determine the relationship between growth retardation at diagnosis ($HTz \leq -1$) and GM-CSF Ab/*CARD15* risk allele status (see Table I). Patients with both elevated GM-CSF Ab and *CARD15* risk allele carriage exhibited the highest frequency of growth retardation compared to those with either elevated GM-CSF Ab or *CARD15* risk allele carriage, or neither factor ($p = 0.0202$). Data on medication exposures, disease activity, and height one and two years after diagnosis were available for the group enrolled at the Cincinnati site ($n = 109$). As shown in Supplemental Tables II and III, clinical disease activity during the first two years after diagnosis, and at the time of blood sampling, did not vary between the groups, with the exception of an increased frequency of moderate-to-severe disease activity in the disease control group between 18 and 24 months after diagnosis. Medication exposures during follow-up and at the time of blood sampling did not vary between the groups (Supplemental Table IV). Height z score within the four CD sub-groups did not change during the first two years after diagnosis (Supplemental Table II).

Clinical and Immuno-genetic Factors Associated with Height at Diagnosis

Univariate and multivariate logistic regression analyses were conducted to test for conditional relationships between HTz, serum GM-CSF Ab concentration, and *CARD15* risk allele carriage while controlling for age at diagnosis, gender, small bowel disease location, and WTz. In the univariate analysis, small bowel location and WTz were strongly associated with HTz (see Table II). We observed a trend towards an association between age ≥ 10 at diagnosis, elevated GM-CSF Ab, or *CARD15* risk allele carriage and reduced HTz at diagnosis. Male gender was not associated with HTz at diagnosis. A dichotomous variable of study participants who had both a *CARD15* risk allele and serum GM-CSF Ab ≥ 1.6 was

created and included in the analysis. An additive effect of the GM-CSF Ab and *CARD15* risk allele slope coefficients was observed for the dichotomous variable, which reached significance for association with reduced HTz (slope coefficient (SE) : -0.4117 (0.2), $p=0.04$). By comparison, the GM-CSF Ab/*CARD15* risk allele state was not associated with WTz (slope coefficient (SE) : -0.100 (0.1), $p=0.26$), suggesting an effect upon linear growth independent of variations in weight.

Consistent with prior reports, small bowel disease location was strongly associated with reduced HTz at diagnosis (slope coefficient (SE): -0.6425 (0.17), $p=0.0002$). Mean[95th CI] HTz at diagnosis was reduced to $-0.36[-0.53, -0.18]$ in patients with small bowel involvement, compared to $0.29[-0.03, 0.60]$ in those with colon-only involvement ($p=0.001$). The frequency of growth retardation ($HTz \leq -1$) was increased to 26% in patients with small bowel involvement, compared to 14% in those with colon-only involvement ($p=0.01$). However, the frequency of growth failure ($HTz \leq -1.8$) did not vary by disease location (8% with small bowel involvement compared to 7% with colon-only disease). In contrast to the results for the *CARD15*/GM-CSF Ab sub-groups, small bowel disease location was also strongly associated with reduced WTz at diagnosis (slope coefficient (SE): -0.6098 (0.20), $p=0.003$). Mean[95th CI] WTz at diagnosis was reduced to $-0.50[-0.70, -0.29]$ in patients with small bowel involvement, compared to $0.17[-0.24, 0.59]$ in those with colon-only involvement, $p \leq 0.001$.

Interactions found significant at the level of 0.15 in the univariate analysis were included in the multivariate logistic regression analysis. Multivariate logistic regression including age, gender, and the *C15*⁺GMAb⁺ state (Table II, part I) confirmed an association between HTz and the dichotomous *CARD15*/GM-CSF Ab variable. A model which included small bowel location (Table II, part II) reduced the effect of the *C15*⁺GMAb⁺ state upon HTz, suggesting that effects of the *C15*⁺GMAb⁺ were mediated in part by small bowel location. A final model which included WTz (Table II, part III) reduced the effect of small bowel location upon HTz, suggesting that the effect of small bowel location upon HTz was mediated in part by WTz.

Clinical and Immuno-genetic Factors Associated with Small Bowel Location and Weight at Diagnosis

We have recently reported that gm-csf neutralization interacts with *Card15* deficiency in mice to result in a transmural ileitis following NSAID exposure. We therefore examined the relationship between *CARD15* risk allele carriage, GM-CSF Ab status, and small bowel location. We confirmed that these factors were individually associated with small bowel location, with concurrent *CARD15* risk allele carriage and elevated GM-CSF Ab highly associated with small bowel location (see Supplemental Table V, slope coefficient (SE): 1.522 (0.50), $p=0.0023$). As expected, multivariate analysis confirmed that small bowel location was highly associated with WTz (see Supplemental Table VI, slope coefficient (SE): -0.64 (0.20), $p=0.002$).

Effect of the *C15*⁺GMAb⁺ state upon acute phase reactants and growth factors

As reduced growth in the *C15*⁺GMAb⁺ group was not accounted for by differences in weight, we asked whether systemic inflammation was increased or circulating growth factors reduced. The frequency of corticosteroid, immune modulator, or anti-TNF therapy did not differ between the groups at the time of blood sampling (Supplemental Table IV). Serum LBP is an acute phase reactant produced primarily in the liver. We have recently linked elevated LBP to reduced HTz at diagnosis in pediatric CD, and reduction in LBP with therapy to increase in HTz (9, 18). However, there was no difference in serum LBP concentration between the *C15*⁺GMAb⁺ group and disease controls (38 ± 5.2 mcg/mL

compared to 33.4 ± 2.1 mcg/mL, respectively). Consistent with this, neither the median(IQR) serum TNF α concentration (7(5,10) pg/mL compared to 10(5,12) pg/mL, respectively), nor the median(IQR) serum IL-6 concentration (7(4,16) pg/mL compared to 8(3,15) pg/mL, respectively) varied between the *C15*⁺GMAb⁺ group and disease controls. GH induction of liver IGF-1 production mediates some anabolic effects of GH, and we and others have shown that serum IGF-1 is reduced in children with CD (9). However, serum IGF-1 did not vary between the two groups (254 ± 21.8 ng/mL in the *C15*⁺GMAb⁺ group compared to 265 ± 15.6 ng/mL in disease controls).

Growth hormone binding protein (GHBP) is the extracellular domain of the growth hormone receptor (GHR), generated via cleavage of this domain with release into the circulation (8). As such, it may be used as a surrogate marker for tissue GHR abundance (19). In healthy children, serum GHBP is associated with body mass index (BMI) and linear growth (19). Serum GHBP was reduced in the *C15*⁺GMAb⁺ group in both males (601 ± 61.73 pmol/L vs 857 ± 59.78 pmol/L, $p < 0.05$) and females (829 ± 112.7 pmol/L vs 1201 ± 95.19 pmol/L, $p < 0.05$) compared to disease controls. (see Figures 1A and 1B). Importantly, this occurred in the absence of a difference in BMIz between the groups (data not shown). By comparison, serum GHBP did not differ between patients with small bowel involvement and those with colon-only disease (936 ± 59 vs 933 ± 82 , respectively). Collectively, these data showed that the *C15*⁺GMAb⁺ state was associated with a specific reduction in linear growth and serum GHBP.

Murine ileitis and increased markers of inflammation

We therefore asked whether an acute phase response (APR) would be induced, and the hepatic GH:Ghr:Igf-1 axis suppressed, in the corresponding animal model of ileitis. We have previously shown that gm-csf neutralization in C15KO mice, followed by NSAID exposure in the chow, results in a transmural ileitis (15). We measured serum LBP in C15KO mice with ileitis due to gm-csf neutralization and NSAID exposure (Ileitis group), C15KO mice fed regular chow (controls), and C15KO mice pre-treated with an isotype control antibody and then exposed to NSAID (NSAID group). We identified a significant increase in serum LBP in mice with both NSAID exposure alone and ileitis in both males (21.1 ± 1.75 mcg/mL in controls vs 64 ± 6 mcg/mL with NSAID exposure alone vs 65.11 ± 10.96 mcg/mL in mice with ileitis, $p < 0.001$) and females (12.47 ± 1.231 mcg/mL in controls vs 33 ± 12 mcg/mL with NSAID exposure alone vs 49.05 ± 4.992 mcg/mL in mice with ileitis, $p < 0.05$). Ileal histology scores did not differ between male and female mice (data not shown). The up-regulation of LBP confirmed activation of the hepatic APR with NSAID exposure alone, and in the murine model of ileitis, comparable to that observed in patients with CD.

Hepatic growth hormone receptor protein and mRNA expression is reduced in male mice with ileitis

We have previously reported that liver Ghr protein abundance and mRNA expression are reduced in IL-10 null mice with colitis (7). We therefore asked whether liver Ghr expression was also reduced in the murine model of ileitis, and whether this would vary by gender. Immunoblot analysis of hepatic Ghr abundance (see Figure 2A) demonstrated a significant reduction in males with ileitis compared to controls, from 2.05 ± 0.134 relative units to 1.479 ± 0.1858 relative units ($p < 0.05$). Ghr abundance did not change with NSAID exposure alone. Ghr abundance was preserved in females with ileitis compared to controls (1.170 ± 0.1364 relative units compared to 0.9121 ± 0.09927 relative units, respectively, $p = 0.16$). At the level of gene transcription, we observed a reduction in total *Ghr* transcripts in male mice with NSAID exposure alone or ileitis compared to controls (Figure 2C) from 98.1 ± 24.85 relative units to 36 ± 5 relative units and 47.6 ± 8.110 relative units, respectively, $p < 0.05$.

We did not observe a significant reduction in *Ghr* transcripts in female mice with NSAID exposure alone or ileitis compared to controls (2930 ± 1021 relative units and 1707 ± 817 relative units respectively, $p = 0.39$).

Growth hormone induced hepatic pSTAT5 abundance is reduced in male mice with ileitis

Anabolic effects of GH require activation of the STAT5b transcription factor. We have previously shown that the abundance of activated tyrosine phosphorylated STAT5 (pSTAT5) following GH administration was reduced in IL-10 null mice with colitis compared to controls (7). We therefore asked if GH activation of pSTAT5 (hGH, 3 mcg/gm IP 30 minutes prior to sacrifice) would be reduced in our murine model of ileitis. Immunoblots demonstrated a significant reduction in GH induced hepatic pSTAT5 in male mice with ileitis compared to controls, from 0.7016 ± 0.05049 relative units to 0.4406 ± 0.05297 relative units ($p < 0.001$) (Figure 3A). GH induced pSTAT5 abundance did not change with NSAID exposure alone. GH stimulated pSTAT5 abundance was preserved in females with ileitis compared to controls (0.5330 ± 0.04373 relative units and 0.4974 ± 0.1156 relative units, respectively, $p = 0.75$).

Hepatic *Igf-1* mRNA expression is reduced in male mice with ileitis

We have previously reported that serum Igf-1 concentration and total hepatic *Igf-1* mRNA expression were reduced in IL-10 null mice with colitis (7). Given this observation and the reduction of *Ghr* and pSTAT5 abundance in male mice with ileitis, we asked if *Igf-1* mRNA expression and serum Igf-1 concentration were reduced. Total *Igf-1* mRNA expression was reduced in male mice with ileitis compared to controls, from 1493 ± 428.2 relative units to 598.5 ± 14.82 relative units ($p < 0.01$). Total *Igf-1* mRNA expression did not change with NSAID exposure alone. There was not a significant reduction seen in the female mice with ileitis compared to controls (7721 ± 3895 relative units and 4085 ± 2310 relative units, respectively, $p = 0.44$). However, we did observe a significant reduction in serum Igf-1 in both male mice with ileitis or NSAID exposure alone compared to controls, from 488.1 ± 31.17 ng/mL in controls to 256 ± 42 ng/mL with NSAID exposure alone and 182.7 ± 49.63 ng/mL with ileitis ($p < 0.001$), and female mice with ileitis relative to controls, from 446.4 ± 152.5 ng/mL to 190.7 ± 38.73 ng/mL ($p < 0.05$). These differences in the hepatic GH:Ghr:Igf-1 axis were not associated with differences in weight gain over the three week course of the model. Male mice with ileitis gained $5.4(1)$ grams compared to $7(0.7)$ grams in male C15KO controls, while female mice with ileitis gained $3.7(0.6)$ grams compared to $3.8(0.9)$ grams in female C15KO controls.

Induction of the hepatic APR is associated with suppression of *Ghr* and *Igf-1* mRNA expression and circulating Igf-1

We then asked whether the degree of reduction in each of the components of the liver GH signaling axis would be associated with induction of the hepatic APR as measured by serum LBP. Consistent with prior studies from our group and others, we observed a significant association between increased serum LBP, and reduced liver *Ghr* and *Igf-1* mRNA expression, and circulating Igf-1 (see Figure 4). Interestingly however, neither liver *Ghr* protein abundance ($r = -.2962$, $p = 0.2327$) nor liver GH induced pSTAT5 nuclear abundance ($r = -.2742$, $p = 0.1579$) were significantly associated with serum LBP.

DISCUSSION

While several studies have examined the pathophysiology of growth failure in children with CD, many gaps in our knowledge remain. Previous studies have suggested a genetic predisposition to growth retardation in a subset. A longitudinal population-based study showed that children who underwent surgery were smaller throughout childhood, prior to

the clinical diagnosis of CD, compared to those who did not require surgery (12). A recent paper showed that the parents of patients with growth impairment had significantly decreased HTz compared to parents of children without growth impairment (20). Studies have consistently shown that small bowel disease location was associated with reduced linear growth (1,4). We have previously reported that elevated GM-CSF Ab in both pediatric and adult CD are strongly associated with ileal location and stricturing and penetrating disease requiring surgery (15). Our current studies have now shown that pediatric CD patients with both a *CARD15* SNP and elevated GM-CSF Ab have reduced HTz at diagnosis compared to children with CD with one or neither factor.

In our univariate analysis, we observed an additive effect of elevated GM-CSF Ab and a *CARD15* risk allele; the combination reached significance for association with reduced HTz. Small bowel location and WTz were also strongly associated with HTz. Consistent with our prior report, the *C15*⁺GMAb⁺ state was strongly associated with small bowel location. The *C15*⁺GMAb⁺ state was not associated with WTz, demonstrating an effect on linear growth independent of variation in weight. In our stepwise multivariate analysis, the inclusion of small bowel location reduced the effect of the *C15*⁺GMAb⁺ state upon HTz, while the inclusion of WTz reduced the effect of small bowel location upon HTz. We did not observe an effect of age or gender on HTz once they were included in the multivariate analysis. This differs from previous studies that found that age and male gender were associated with reduced linear growth (1,2). These differences may have been due to the inclusion of the specific genetic and immune factors in the current analysis, the pubertal status of the cohorts, or the definitions used to classify linear growth. Collectively, our analysis suggests that the *C15*⁺GMAb⁺ state suppresses linear growth in CD via a mechanism which involves small bowel location, independent of differences in weight.

Remarkably, we did not observe a difference in serum LBP, TNF α , or IL-6 between the *C15*⁺GMAb⁺ group and disease controls. This suggested that reduced growth was not due simply to global differences in inflammation. We have recently reported that children with CD and elevated GM-CSF Ab exhibit a specific increase in intestinal permeability and anti-endotoxin antibodies, in the absence of differences in systemic or intestinal inflammation (21). We have previously shown in animal models that endotoxin can directly reduce liver Ghr abundance, via both transcriptional and post-transcriptional mechanisms (6,8). Serum GHBP may be used as a surrogate marker for tissue GHR abundance and is associated with BMI and linear growth in healthy children (22,23). We identified a significant reduction in serum GHBP in *C15*⁺GMAb⁺ patients compared to disease controls. Our results suggest that the *C15*⁺GMAb⁺ state may lead to a specific reduction in tissue GHR as measured by serum GHBP, in the absence of a difference in overall weight or inflammatory status. Ongoing studies will determine whether this is due to increased permeability and chronic endotoxemia.

The patient-based studies could not establish a causal relationship between the *C15*⁺GMAb⁺ state and reduced tissue GHR abundance. We therefore asked whether induction of ileitis via gm-csf neutralization in the *Card15* deficient host would lead to down-regulation of the hepatic Ghr and GH resistance. We confirmed that the hepatic GH:Ghr:Igf-1 axis was profoundly suppressed in this setting, with an intriguing sexual dimorphism. We found that serum LBP concentration was elevated in the murine model of ileitis, with no gender differences, and that this was comparable to the degree of elevation observed in the patients. However, the hepatic Ghr and GH activation of STAT5 were significantly reduced only in male mice with ileitis. However, serum Igf-1 was reduced in both male and female mice with ileitis. We speculate that the gender differences we observed are secondary to differences in sensitivity to endotoxin dependent suppression of *Ghr* gene transcription and protein abundance. The reduction in serum Igf-1 observed in both sexes may be due to post-

Ghr effects of IL-6 upon Igf-1 stability (24). Collectively, the murine studies demonstrated that liver Ghr abundance and GH activation of the anabolic transcription factor Stat5 were suppressed in male mice with ileitis due to gm-csf neutralization and *Card15* deficiency, while circulating Igf-1 was reduced in both males and females. These changes occurred despite normal weight gain relative to controls, demonstrating an effect of the *C15*⁺GMAb⁺ state independent of variation in weight. The reduction in circulating Igf-1 was strongly associated with induction of the hepatic APR as measured by serum LBP, while the reduction in liver Ghr and pSTAT5 protein abundance were not. This was consistent with our observation in patients that suppression of circulating GHBP in the *C15*⁺GMAb⁺ group was not associated with a difference in systemic inflammation as measured by serum LBP or cytokines compared to disease controls.

Given the retrospective nature of our study, there are some limitations. The blood samples for measurement of GM-CSF auto-antibodies, cytokines, and growth factors were obtained on average three years after diagnosis (Table I). While our studies to date have shown that GM-CSF auto-antibodies are stable over time, it is possible that in some patients these may have been different at diagnosis (15). The results for the cytokines and growth factors are also limited by the cross-sectional nature of the analysis, and may not reflect differences that were present at diagnosis. We did not have access to Tanner staging of the patients, or bone age measurements, and so could not control for pubertal status. Mid-parental heights were not available, which would have been useful in estimating height deficits relative to genetic potential. We were not able to assess protein and calorie intake, or malabsorption, which may differ between CD patients with colon-only disease and those with small bowel involvement. We were not able to obtain a history for NSAID exposure, which may have influenced the serum cytokines or growth factors. Finally, we were not able to measure mucosal disease activity. An ongoing prospective study will address these potential weaknesses.

In summary, we have shown that defects in innate immunity due to GM-CSF auto-antibodies in a *CARD15* deficient host are associated with linear growth failure in children with CD, and induce liver GH resistance in a murine model of ileitis. Growth failure in the patients and GH resistance in the animal model occurred in the absence of differences in inflammation or weight, suggesting a specific effect of the *C15*⁺GMAb⁺ state. Based upon our recent study showing increased intestinal permeability and evidence of endotoxin exposure in children with CD and elevated GM-CSF Ab, we speculate that defective barrier function may directly induce GH resistance and growth failure in this setting (21). This would imply that therapies designed to augment barrier function might also restore GH action and growth in children with CD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APR	acute phase response
BMI	body mass index
CD	Crohn Disease
GHBP	growth hormone binding protein
GHR	growth hormone receptor
GM-CSF Ab	Granulocyte-Macrophage Colony Stimulating Factor auto-antibodies
IBD	Inflammatory Bowel Disease
IGF	insulin-like growth factor
IL	interleukin
LBP	lipopolysaccharide binding protein
NSAID	non-steroidal anti-inflammatory drug
PAP	Primary Alveolar Proteinosis
SNP	single nucleotide polymorphism
STAT	signals transducers and activators of transcription
TNF	tumor necrosis factor
UC	Ulcerative Colitis

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Figure 1. Serum Growth Hormone Binding Protein is Reduced in CD Patients with Combined *CARD15* SNP and Elevated GM-CSF Ab

Serum GHP concentration was determined by ELISA in (A) male *C15*⁺GMAb⁺ CD patients (n= 30) compared to male disease controls with one or neither of these factors (n=84), *p< 0.05 by Mann-Whitney test and (B) female *C15*⁺GMAb⁺ CD patients (n=20) compared to female disease controls with one or neither of these factors (n=60), *p< 0.05 by unpaired t-test. Data are shown as the mean $\pm$ SEM.

Figure 2. Hepatic Growth Hormone Receptor Expression is Reduced in Male Mice with Ileitis
 Ileitis was induced by NSAID exposure to *Card15*^{-/-} mice following Gm-csf neutralization. Liver protein and RNA were isolated from mice with ileitis, mice pre-treated with isotype control antibody and then exposed to NSAID (NSAID) and controls. (A and B) Ghr protein expression was determined by immunoblot and (C and D) total *Ghr* mRNA expression was determined by real-time PCR. Signal intensity was determined by densitometry in (A) male ileitis mice (n=6) compared to male NSAID (n=4) and controls (n=6) and (B) female ileitis mice (n=7) compared to female NSAID (n=5) and controls (n=6). β -tubulin was used to confirm uniform protein loading. Data are shown as the mean $\pm$ SEM; *p<0.05.

Figure 3. Growth Hormone Induced Hepatic pSTAT5 Activation is Reduced in Male Mice with Ileitis

Ileitis was induced by NSAID exposure to *Card15* $-/-$ mice following Gm-csf neutralization. Liver nuclear protein was isolated from mice with ileitis, mice pre-treated with isotype control antibody and then exposed to NSAID (NSAID) and controls 30 minutes after human GH injection (3mcg/gm, IP). Abundance of pSTAT5 was determined by immunoblot in (A) male ileitis mice (n=7), mice with NSAID exposure alone (n=5), and controls (n=6) and (B) female ileitis mice (n=5), mice with NSAID exposure alone (n=4), and controls (n=7). Signal intensity was determined by densitometry; SHPTP1 was used to confirm loading. Data are shown as the mean $\pm$ SEM; **p<0.01.

Figure 4. Circulating LBP and the Hepatic Ghr:Igf-1 Axis in Murine Ileitis

Ileitis was induced by NSAID exposure to *Card15*^{-/-} mice following Gm-csf neutralization (n=22). Serum LBP and Igf-I were determined by ELISA. Liver *Ghr* and *Igf-I* mRNA expression were determined by real-time PCR. Spearman correlations were determined as shown.

Linear Growth Stratified by *CARD15* Variant and GM-CSF Ab.

Table 1

Group	n	Age at Diagnosis	Age at Blood Sample Collection	Male	Small Bowel Location	WTz	HTz	HTz ≤ -1	HTz ≤ -1.8
<i>CARD15</i> -GMAb-	79	10.7 (1.9,18)	13.4 (2.67,21.1)	62%	57%	-0.15 (-2.5,4)	0.02 (-4.9,3.4)	19%	8%
<i>CARD15</i> +GMAb-	32	11.8 (4,17.8)	14.3 (9,20.8)	72%	63%	-0.42 (-2.9,5)	-0.11 (-1.8, 2.2)	25%	0%
<i>CARD15</i> -GMAb+	73	12.7 (2.5,18)	15.6 (8.7,22.5)	58%	71%	-0.29 (-4.4,6)	-0.16 (-3.6,2.2)	15%	7%
Disease Control	184	11.6 (11,12.2)	14.4 (2.67,22.5)	62%	64%	-0.25 (-4,5)	-0.07 (-4.9,3.4)	18%	6%
<i>CARD15</i> +GMAb+	45	11.8 (4.5,16)	14.0 (6.9,21.8)	62%	89%*	-0.43 (-4, 3.9)	-0.48* (-4.2,2.3)	38%*	16%*

CD patients were stratified into the group with both GM-CSF Ab ≥ 1.6 mcg/mL and a *CARD15* mutation (*CARD15*+GMAb+), the other three groups (*CARD15*+ or GMAb+), and these three groups combined (disease control). WTz: weight z score at diagnosis, HTz: height z score at diagnosis. Data are shown as mean (range) or relative frequency.

* p≤0.05 vs. the disease control group.

Table II

Clinical, Genetic, and Immune Determinants of Height Z Score at Diagnosis

Variable	Univariate Analysis β (SE), p-value	Multivariate Analysis (I) β (SE), p- value	Multivariate Analysis (II) β (SE), p-value	Multivariate Analysis (III) β (SE), p-value
Age at diagnosis ≥ 10	-0.26(0.2), 0.14	-0.31(0.2), 0.09	-0.20(0.2), 0.26	-0.02(0.2), 0.86
Male gender	-0.05(0.2), 0.76	-0.01(0.2), 0.1	-0.06 (0.2), 0.72	-0.12(0.1), 0.35
GM-CSF Ab ≥ 1.6 mcg/mL	-0.27(0.2), 0.10			
<i>CARD15</i> mutation	-0.27(0.2), 0.12			
<i>C15</i> ⁺ GMAb ⁺	-0.41(0.2), 0.04	-0.42(0.2), 0.04	-0.26(0.2), 0.19	-0.25(0.1), 0.08
Small bowel location	-0.64(0.2), 0.0002		-0.58(0.2), 0.0008	-0.22(0.13), 0.08
Weight z score	0.59(0.2), 0.0001			0.56(0.04), 0.0001

C15⁺GMAb⁺:CD group with both a *CARD15* mutation and GM-CSF Ab ≥ 1.6 mcg/mL.