Analysis of *IL12B* Gene Variants in Inflammatory Bowel Disease

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Abstract

**Background:** *IL12B* encodes the p40 subunit of IL-12, which is also part of IL-23. Recent genome-wide association studies identified *IL12B* and *IL23R* as susceptibility genes for inflammatory bowel disease (IBD). However, the phenotypic effects and potential gene-gene interactions of *IL12B* variants are largely unknown.

**Methodology/Principal Findings:** We analyzed *IL12B* gene variants regarding association with Crohn’s disease (CD) and ulcerative colitis (UC). Genomic DNA from 2196 individuals including 913 CD patients, 318 UC patients and 965 healthy, unrelated controls was analyzed for four SNPs in the *IL12B* gene region (rs3212227, rs17860508, rs10045431, rs6887695). Our analysis revealed an association of the *IL12B* SNP rs6887695 with susceptibility to IBD (p = 0.035; OR 1.15 [95% CI 1.01–1.31]) including a trend for rs6887695 for association with CD (OR 1.41; [0.99–1.31], p = 0.066) and UC (OR 1.18 [0.97–1.43], p = 0.092). CD patients, who were homozygous C/C carriers of this SNP, had significantly more often non-stricturing, non-penetrating disease than carriers of the G allele (p = 6.8×10⁻⁵; OR = 2.84, 95% CI 1.66–4.84), while C/C homozygous UC patients had less often extensive colitis than G allele carriers (p = 0.029; OR = 0.36, 95% CI 0.14–0.92). In *silico* analysis predicted stronger binding of the minor C allele of rs6887695 to the transcription factor RORγt which is involved in Th17 differentiation. Differences regarding the binding to the major and minor allele sequence of rs6887695 were also predicted for the transcription factors HSF1, HSF2, MZF1 and Oct-1. Epistasis analysis revealed weak epistasis of the *IL12B* SNP rs6887695 with several SNPs (rs11889341, rs7574865, rs7568275, rs8179673, rs10181656, rs7582694) in the *STAT4* gene which encodes the major IL-12 downstream transcription factor STAT4 (p<0.05) but there was no epistasis between *IL23R* and *IL12B* variants.

**Conclusions/Significance:** The *IL12B* SNP rs6887695 modulates the susceptibility and the phenotype of IBD, although the effect on IBD susceptibility is less pronounced than that of *IL23R* gene variants.

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Introduction

The identification of the IL-23/Th17 pathway as a key regulator of intestinal immune homeostasis and proinflammatory responses in defense to microbial infection has elucidated new potential therapeutic targets in inflammatory bowel diseases (IBD) [1,2,3,4]. Genome-wide association studies (GWAS) and large cohort studies demonstrated that *IL23R* [3,5,6,7] and additional genes involved in Th17 differentiation (e.g., *IL12B*, *JAK2*, *TIF2*, *STAT3*, *CCR6*, *IL2/IL21* and *TNFSF15*) are associated with the susceptibility to Crohn’s disease (CD) and partly also to ulcerative colitis (UC) [5,8,9,10,11]. Moreover, a pathway analysis using data from the Wellcome Trust Case Control Consortium (WTCCC) uncovered significant associations of CD and IL-12/IL-23 pathway components, harbouring 20 genes such as *IL12B*, *JAK2*, *STAT3* and *CCR6* [12]. Functionally, the proinflammatory cytokines IL-12 and IL-23 play critical and unique roles in bridging the innate and adaptive immune systems in IBD [4,13].
and are produced primarily by activated dendritic cells (DCs) and macrophages in response to microbial stimulation. IL-12 promotes the differentiation of naive CD4+ T cells into mature interferon-γ (IFN-γ)-producing Th1 effector cells and is a potent stimulus of natural killer and CD8+ T cells [14,15]. In contrast, IL-23, a heterodimeric cytokine composed of a p19 subunit and a p40 subunit of which the latter is shared with IL-12, is required for the generation of memory T cells and drives differentiation of Th17 cells [16,17]. Th17 cytokines such as IL-17A, IL-17F, IL-21, IL-22, IL-26 and the Th17 chemokine CCL20 have been particularly implicated as key cytokines involved in the intestinal inflammation of CD [1,18,19,20,21,22,23].

On the genetic level, IL12B encodes the IL-12 p40 subunit shared by IL-12 and IL-23 cytokines while IL23R encodes one of the two subunits of the IL-23 receptor [24]. Antibody therapy directed against the IL-12/IL-23 p40 subunit demonstrated some clinical efficacy in CD patients [25]. Since the first identification of IL23R as susceptibility gene in IBD by Duerr et al. [3], various GWAS and cohort studies have confirmed IL23R not only as a major susceptibility gene in IBD but also in the pathogenesis of other autoimmune diseases such as psoriasis [26,27] and ankylosing spondylitis [28], implicating common proinflammatory pathways. Genetic variants in the IL12B region have also been associated with the susceptibility to psoriasis, ankylosing spondylitis, and infectious diseases such as leprosy and tuberculosis [29,30,31,32,33]. In contrast, data on IL12B variants and their role in asthma [34,35], rheumatoid arthritis [36] or multiple sclerosis [37] remain controversial. While recent GWAS meta-analyses by Barrett et al. [5], Franke et al. [9], and Anderson et al. [10] established IL12B as IBD susceptibility gene, smaller studies showed inconsistent results [38,39]. Cohort studies in the Spanish [38] and Japanese [39] population demonstrated an association of IL12B SNPs with the susceptibility to IBD; however with different results, reporting associations with CD susceptibility in the Japanese cohort (rs6887695) [39] and to UC (rs6887695) but not to CD susceptibility in the Spanish cohort [38]. Moreover, the phenotypic effects of IL12B and potential gene-gene interactions contributing to IBD susceptibility are largely unknown.

Since the exact role of IL12B for IBD susceptibility in the German population and IBD phenotype behaviour remains unclear, we aimed to perform a detailed genotype-phenotype analysis in a large IBD cohort and an analysis for potential epistatic interactions with other gene variants involved in IL-12 and IL-23 signaling and implicated in IBD susceptibility such as IL23R and STAT4 [3,40] which encodes the major IL-12-induced downstream transcription factor STAT4.

Methods

Ethics statement

The study was approved by the Ethics committee of the Medical Faculty of Ludwig-Maximilians-University Munich and written, informed consent was obtained from all patients prior to the study. Study protocols adhered to the ethical principles for medical research involving human subjects of the Helsinki Declaration (as detailed under: http://www.wma.net/en/30publications/10policies/b3/index.html).

Study population and IBD phenotype assessment

Overall, the study population (n = 2196) consisted of 1231 IBD patients including 913 patients with CD, 318 patients with UC, and 963 healthy, unrelated controls, all of Caucasian origin. The demographic and clinical data (behaviour and location of IBD, disease-related complications, immunosuppressive therapy and history of previous surgeries) of the patients were recorded by analysis of patient charts and a detailed questionnaire including an interview at time of enrolment. The diagnosis of CD or UC was determined according to established guidelines based on endoscopic, radiological, and histopathological criteria. Patients with CD were assessed following the Montreal classification based on the age at diagnosis (A), location (L), and behaviour (B) of the disease [41]. In patients with UC, anatomic location was also based on the Montreal classification using the criteria ulcerative proctitis (E1), left-sided UC (distal UC; E2), and extensive UC (pancolitis; E3). Patients with indeterminate colitis were excluded from the study. The demographic characteristics of the IBD study population were collected blind to the results of the genotype analyses and are summarized in Table 1.

DNA extraction and genotyping of the IL12B variants

From all study participants, blood samples were taken and genomic DNA was isolated from peripheral blood leukocytes using the DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer’s guidelines. Four IL12B SNPs (rs3212227, rs17860508, rs10045431 and rs6887695) were genotyped by PCR and melting curve analysis using a pair of fluorescence resonance energy transfer (FRET) probes in a LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany) as described in previous studies [6,40,42,43]. These IL12B SNPs were selected based on previous studies showing associations with CD or other autoimmune diseases such as psoriasis. Specifically, the SNPs rs3212227 and rs6887695 were selected from the study of Cargill and co-workers [29], while rs6887695 was also investigated in the study of Parkes et al. [8]. The SNP rs10045431 was selected from the study of Barrett and co-workers [5]. Additionally, the SNP rs17860508 located within the promoter of IL12B was included because of its potential functional relevance regarding gene expression [44]. The total

Table 1. Demographic characteristics of the IBD study population.

|                         | Crohn’s disease n = 913 | Ulcerative colitis n = 318 | Controls n = 965 |
|-------------------------|-------------------------|-----------------------------|------------------|
| **Gender**              |                         |                             |                  |
| Male (%)                | 48.9                    | 52.2                        | 63.5             |
| Female (%)              | 51.1                    | 47.8                        | 36.5             |
| **Age (yrs)**           |                         |                             |                  |
| Mean ± SD               | 40.9±13.2               | 44.2±14.8                   | 46.0±10.3        |
| Range                   | 15–83                   | 17–88                       | 19–68            |
| **Body mass index**     |                         |                             |                  |
| Mean ± SD               | 23.0±4.2                | 23.9±4.5                    |                  |
| Range                   | 13–41                   | 15–54                       |                  |
| **Age at diagnosis (yrs)** |                       |                             |                  |
| Mean ± SD               | 26.1±12.3               | 28.9±14.5                   |                  |
| Range                   | 1–78                    | 2–81                        |                  |
| **Disease duration (yrs)** |                       |                             |                  |
| Mean ± SD               | 13.4±8.9                | 12.2±8.3                    |                  |
| Range                   | 0–47                    | 1–50                        |                  |
| **Positive family history of IBD (%)** |           |                             |                  |
|                         | 16.8                    | 17.4                        |                  |

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were included in the analysis. The minor alleles including the flanking sequences 15 bp upstream of the tagging SNPs were genotyped by PCR and melting curve analysis using a pair of FRET probes in a LightCycler fluorescence resonance energy transfer (FRET) assay. The PCR comprised an initial denaturation step (95°C for 10 min) and 45 cycles (95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec). The melting curve analysis comprised an initial denaturation step (95°C for 1 min), a step rapidly lowering the temperature to 40°C and holding for 2 min, and a heating step slowly (1 acquisition/°C) increasing the temperature up to 95°C and continuously measuring the fluorescence intensity. The results of the melting curve analysis were confirmed by analyzing two patient samples for each possible genotype using sequence analysis. For sequencing, the total volume of the PCR was 100 µl containing 250 ng of genomic DNA, 1 × PCR-buffer (Qiagen, Hilden, Germany), a final MgCl2 concentration of 2 mM, 0.5 mM of a dNTP mix (Sigma, Steinheim, Germany), 2.5 units of HotStar Plus Taq™ DNA polymerase (Qiagen, Hilden, Germany), a final MgCl2 concentration of 2 mM, and 0.25 pmol of each primer (TIB MOLBIOL). The PCR comprised an initial denaturation step (95°C for 5 min), 35 cycles (denaturation at 94°C for 30 sec, primer annealing at 65°C for 30 sec, extension at 72°C for 30 sec) and a final extension step (72°C for 10 min). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Vaterstetten, Germany). All sequences of primers and FRET probes and primer annealing temperatures used for genotyping and for sequence analysis, respectively, are given in Supplementary Tables S1 and S2.

In silico analysis of transcription factor binding sites
SNPs rs3212227, rs17860508, rs10045431 and rs6887695 were analyzed for potential transcription factor binding sites applying the online tool TFSEARCH (http://www.cbrp.jp/research/db/TFSEARCH.html). It is based on the TRANSFAC database developed at GFB Braunschweig, Germany [45]. The threshold score for binding sites was set to 75.0 (score = 100.0 * (weight of sum−min)/(max−min); max. score = 100). For each SNP, major and minor alleles including the flanking sequences 15 bp upstream and downstream were analyzed. Only human transcription factors were included in the analysis.

Statistical analyses
Power calculation was performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/plink/). Each genetic marker was tested for Hardy-Weinberg equilibrium in the control population. For data evaluation, we used PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) and R-2.13.1. (http://cran.r-project.org). Haplotypes tests were performed in a sliding-window approach including up to 4 markers. Epistasis between different SNPs was tested using the –epistasis option on PLINK. Genotype-phenotype associations were assessed using logistic regression.

Results

The IL12B polymorphisms rs6887695 is associated with increased IBD susceptibility
In all three subgroups (CD, UC, and controls), the allele frequencies of the IL12B SNPs (rs3212227, rs17860508, rs10045431, rs6887695) were in accordance with the predicted Hardy-Weinberg equilibrium (Table 2). The IL12B SNP rs6887695 showed an association with increased IBD susceptibility (p = 0.035; OR 1.15 [95% CI 1.01–1.31]). In addition, there was a trend for rs6887695 for association with CD (OR 1.41 [95% CI 0.99–1.31], p = 0.066) and UC (OR 1.18 [95% CI 0.97–1.43], p = 0.092) and a trend for association of rs10045431 with UC (OR 1.41 [95% CI 0.96–1.98], p = 0.083; Table 2).

IL12B haplotype analysis
We next performed a detailed haplotype analysis in our IBD cohort. As shown in Tables 3 and 4, we could not demonstrate significant associations of IL12B haplotypes with CD and UC susceptibility. A haplotype of all four investigated SNPs (rs3212227-rs17860508-rs10045431-rs6887695) showed a trend for association with CD (p = 0.053); however, this potential association signal did not reach statistical significance and was uncorrected for multiple testing.

Genotype-phenotype analysis: C/C homozygosity for the SNP rs6887695 is associated with non-stricturing, non-penetrating Crohn’s disease
The majority of previous GWAS meta-analyses showing an association with IL12B were performed in CD patients with predominant ileal involvement [5,9]. To exclude that only a certain IBD subphenotype such as ileal CD is associated with IL12B, we further investigated potential associations of IL12B SNPs with the anatomic location in IBD patients Genotype-phenotype analysis showed a weak association of rs3212227 with colonic CD (p = 0.04) but not with ileal involvement. In addition, we analyzed the IL12B SNP rs6887695, for which we found a trend for association with CD and UC, regarding potential phenotypic consequences in CD and UC. CD patients, who were homozygous C/C carriers of this SNP, had significantly more often a non-stricturing, non-penetrating disease phenotype than carriers of the G allele (= combined group of heterozygous C/G and wildtype G/G carriers; p = 6.8 × 10−5 (Table 3). This strong association remained significant following Bonferroni correction for multiple testing. C/C homozygous carriers of the IL12B SNP rs6887695 had also less stenoses than G carriers (p = 0.038) and there was a trend towards less penetrating disease (p = 0.062), less fistulas (p = 0.055), and less surgery (p = 0.063; Table 5). In UC, homozygous C/C carriers of the IL12B SNP rs6887695 had significantly more often left-sided UC (p = 0.006) but less often extensive UC (p = 0.029) than the combined group of heterozygous C/G and wildtype G/G carriers (Table 6).
Analysis for epistasis of IL12B with IL23R and STAT4 gene variants regarding susceptibility to Crohn’s disease

Next, we analyzed potential evidence for epistasis of IL12B variants with other CD susceptibility genes involved in IL-12 and Th17 signaling, including CD-associated variants in the IL23R and STAT4 gene [3,6,40]. Analysis for gene-gene interaction revealed weak epistasis of IL12B SNP rs6887695 with 7 out of the 8 analyzed STAT4 gene variants (p<0.05) regarding CD susceptibility (Table 7). However, following Bonferroni correction, none of these associations remained significant. In addition, there was no evidence for epistasis between IL23R and IL12B variants (Table 7).

In silico analysis of IL12B SNPs identifies potential differences in transcription factor binding to major and minor alleles

To analyze if SNPs in the IL12B region modify the binding of transcription factors to DNA and thereby modulating gene expression, we analyzed SNIPs rs3212227-rs17860508-rs10045431-rs6887695 including the surrounding sequences for potential binding sites as described in the methods section. The results are shown in Table 8. Interestingly, the highest differences in binding scores were found for the major and the minor allele of rs6887695, the only SNP that was associated with overall IBD susceptibility in our study cohort. Moreover, we observed significant associations with specific CD and UC phenotypes. While the transcription factors HSF1, HSF2, MZF1 and Oct-1 were predicted to bind with very high probability to the sequence comprising the major G allele, predicted Table 3.

Haplotypes of IL12B SNPs in the CD case-control sample and omnibus p-values for association with CD susceptibility.

| SNP combination | Omnibus p-value |
|-----------------|----------------|
| rs3212227-rs17860508 | 0.957 |
| rs17860508-rs10045431 | 0.333 |
| rs10045431-rs6887695 | 0.053 |
| rs3212227-rs17860508-rs10045431-rs6887695 | 0.035 |

The category “Inflammatory bowel disease” represents the combined CD and UC cohort. Minor allele frequencies (MAF), allelic test P-values, and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control cohorts. Significant associations (p<0.05) are highlighted in bold fonts, suggestive associations (p<0.10) are depicted in italic fonts.

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Table 4.

Haplotypes of IL12B SNPs in the UC case-control sample and omnibus p-values for association with UC susceptibility.

| SNP combination | Omnibus p-value |
|-----------------|----------------|
| rs3212227-rs17860508 | 0.614 |
| rs17860508-rs10045431 | 0.199 |
| rs10045431-rs6887695 | 0.126 |
| rs3212227-rs17860508-rs10045431 | 0.288 |
| rs17860508-rs10045431-rs6887695 | 0.206 |
| rs3212227-rs17860508-rs10045431-rs6887695 | 0.035 |

The category “Inflammatory bowel disease” represents the combined CD and UC cohort. Minor allele frequencies (MAF), allelic test P-values, and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control cohorts. Significant associations (p<0.05) are highlighted in bold fonts, suggestive associations (p<0.10) are depicted in italic fonts.

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Table 2.

Associations of IL12B gene markers in CD and UC case-control association studies.

| SNP | Minor allele | Crohn’s disease | Ulcerative colitis | Inflammatory bowel disease | Controls |
|-----|--------------|----------------|-------------------|---------------------------|----------|
|     |              | n = 913         | n = 318           | n = 1231                   | n = 965  |
| MAF | p value | OR [95% CI] | MAF | p value | OR [95% CI] | MAF | p value | OR [95% CI] | MAF | p value | OR [95% CI] |
| rs3212227 | G       | 0.209 | 0.684 | 0.97 [0.82–1.13] | 0.214 | 0.777 | 1.03 [0.83–1.29] | 0.206 | 0.820 | 0.98 [0.85–1.14] |
| rs17860508 | TTAGAG | 0.814 | 0.843 | 1.02 [0.88–1.14] | 0.489 | 0.854 | 1.02 [0.85–1.22] | 0.486 | 0.903 | 1.00 [0.89–1.14] | 0.484 |
| rs10045431 | A     | 0.271 | 0.258 | 0.83 [0.70–1.00] | 0.252 | 0.803 | 0.83 [0.68–1.02] | 0.266 | 0.109 | 0.89 [0.78–1.02] | 0.288 |
| rs6887695 | C     | 0.329 | 0.966 | 1.41 [0.99–1.31] | 0.393 | 0.922 | 1.10 [0.97–1.43] | 0.330 | 0.035 | 1.15 [1.01–1.31] | 0.300 |

The category “Inflammatory bowel disease” represents the combined CD and UC cohort. Minor allele frequencies (MAF), allelic test P-values, and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control cohorts. Significant associations (p<0.05) are highlighted in bold fonts, suggestive associations (p<0.10) are depicted in italic fonts.

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binding to the minor C allele was substantially lower (Table 8). On the other hand, the binding score for the transcription factor RORα was higher for the minor C allele (Table 8). Therefore, differential DNA binding of transcription factors in this genomic region followed by differential gene transcription might be one reason for the disease-modifying abilities of this SNP that we observed in our genotype-phenotype analysis.

**Discussion**

This study presents a detailed genotype-phenotype analysis investigating IL12B SNPs as potential susceptibility gene variants in a large Caucasian IBD cohort. A major focus of this study was the analysis of potential epistatic interactions with key gene variants of the IL-12 and IL-23/Th17 pathway such as IL23R and STAT4. We demonstrated that the IL12B variant rs6887695 is weakly associated with overall IBD susceptibility (p = 0.035), with trends for association with both CD and UC susceptibility. Moreover, a haplotype block formed by the four investigated IL12B SNPs showed a trend for association with CD (p = 0.05). Considering our findings and the strong association signal for this gene in the recent GWAS [9,10], IL12B can be regarded as established IBD susceptibility gene. In contrast to the very large IBD GWAS, our sample size (n = 2196) was smaller, resulting in limited power, particularly for the UC cohort. For example, a power calculation, which assumed an OR of 1.2 and an allele frequency of 0.20 for the rarest IL12B variant rs3212227, demonstrated that our study had 63.1% power to detect a nominal significant finding (alpha = 0.05) in the CD cohort but had only a power of 38.2% in the UC cohort. However, based on the results of this study, the strength of a potential association of IL12B with IBD is several log-fold weaker than that shown for

**Table 5. Genotype-phenotype-analysis of SNP rs6887695 in patients with Crohn’s disease (CD).**

| IL12B   | (1) CC | (2) CG | (3) GG | P_CC | OR_CC |
|---------|--------|--------|--------|------|-------|
| rs6887695 | n = 100 | n = 394 | n = 409 |      |       |
| Location |
| Terminal ileum | n = 80 | n = 331 | n = 357 | 1.000 | 0.92  |
| (L1) (13.8%) | (16.6%) | (12.9%) |         | [0.45–1.88] |
| Colon | 6 | 43 | 49 | 0.158 | 0.53 |
| (L2) (7.5%) | (13.0%) | (13.7%) |         | [0.20–1.30] |
| Ileocolon | 60 | 230 | 258 | 0.514 | 1.23 |
| (L3) (75.0%) | (69.5%) | (72.3%) |         | [0.70–2.17] |
| Upper GI | 3 | 3 | 4 | 0.076 | 3.79 |
| (L4) (3.8%) | (0.9%) | (1.1%) |         | [0.76–16.69] |
| Behaviour¹ |
| Non-stricturing & Non-penetrating | 31 | 65 | 75 | 6.8 × 10⁻⁸* | 2.84 |
| (B1) (43.1%) | (22.2%) | (23.1%) |         | [1.66–4.84] |
| Stricturing | 14 | 87 | 85 | 0.160 | 0.63 |
| (B2) (19.4%) | (29.7%) | (26.2%) |         | [0.32–1.19] |
| Penetrating | 27 | 141 | 165 | 0.062 | 0.61 |
| (B3) (37.5%) | (48.1%) | (50.8%) |         | [0.36–1.04] |
| Use of immuno-suppressive agents² | 50 | 202 | 225 | 0.604 | 0.84 |
| (n = 477/583) | (79.7%) | (82.1%) | (82.1%) | [0.42–1.69] |
| Surgery because of CD³ | 35 | 188 | 200 | 0.063 | 0.63 |
| (n = 423/808) | (42.2%) | (52.9%) | (54.2%) | [0.39–1.027] |
| Fistulas | 33 | 172 | 193 | 0.085 | 0.67 |
| (n = 398/825) | (39.3%) | (47.8%) | (50.7%) | [0.41–1.08] |
| Stenosis | 42 | 221 | 225 | 0.038 | 0.62 |
| (n = 488/827) | (48.3%) | (61.1%) | (59.5%) | [0.39–0.98] |

P_CC: P-value for testing for differences between homozygous carriers of the C allele (C/C) and heterozygous and non-carriers of the C allele. OR_CC: corresponding odds ratios and 95% confidence intervals (95% CI). Significant P-values (<0.05) are depicted in bold. P-values showing a trend towards significance are depicted in italic fonts. P-values marked with an asterisk * remained significant after Bonferroni correction.

¹Disease behaviour was defined according to the Montreal classification. A stricturing disease phenotype was defined as presence of stenosis without penetrating disease. The diagnosis of stenosis was made surgically, endoscopically, or radiologically (using MRI enteroclysis).

²Immunosuppressive agents included azathioprine, 6-mercaptopurine, 6-thioguanin, methotrexate, infliximab and/or adalimumab.

³Only surgery related to CD-specific problems (e.g. fistulectomy, colectomy, ileostomy) was included.

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IL23R in our cohort [6] and other major CD susceptibility genes such as NOD2 and ATG16L1 (Supplemental Table S3), suggesting a more important role for IL-23R than IL-12/23 p40 in the genetic susceptibility to IBD. This is in agreement with the recent IBD meta-analyses [9,10], which showed much stronger IBD association signals for \(\text{IL23R}\) than for \(\text{IL12B}\). Similarly, smaller studies failed to show an association of \(\text{IL12B}\) SNPs with IBD or showed only weak associations with CD or UC [46,47,48] (see Supplemental Table S4 for an overview on published studies on \(\text{IL12B}\) in IBD). Even large studies such as the study by Festen et al., which included 1,455 UC patients and 1,902 controls, was unable to show an association of \(\text{IL12B}\) with UC [49], further demonstrating that very large patient cohorts are necessary to show convincing associations with this gene locus. Therefore, \(\text{IL23R}\) is a more important genetic modifier of IBD susceptibility than \(\text{IL12B}\), suggesting a more important pathogenic role of Th17 cells, which express IL-23R, than Th1 cells, which develop under the control of IL-12.

Currently, there are limited data on how \(\text{IL12B}\) may functionally influence IBD susceptibility in humans. A very recent study in mice demonstrated that a polymorphism in the coding region of murine \(\text{Il12b}\) promotes IL-12p70 and IL-23 heterodimer formation [50]. The authors hypothesized that the high synthesis rate of IL-12/23 cytokines resulting from efficient binding may lead to rapid proinflammatory skewing of immune responses and disturbance of the homeostatic balance resulting in higher susceptibility for IBD [50]. Other gene variants may also modulate IL-12 expression as recently demonstrated by our group showing increased basal levels of IL-12p40 in CD patients with two mutated \(\text{NOD2}\) alleles [51]. Currently, it is unclear which \(\text{IL12B}\) SNP is the “true” disease-causing variant. We therefore performed a detailed in \textit{silico} analysis of \(\text{IL12B}\) regarding potential transcription factor binding sites and demonstrated for

### Table 6. Genotype-phenotype-analysis of SNP rs6887695 in patients with ulcerative colitis (UC) for which detailed phenotypic data based on the Montreal classification was available.

| \(\text{IL12B}\) | (1) CC | (2) CG | (3) GG | \(P_{\text{CC}}\) | \(OR_{\text{CC}}\) |
|----------------|-------|-------|-------|----------------|----------------|
| rs6887695 | \(n = 34\) | \(n = 145\) | \(n = 138\) |  |  |
| Location | \(n = 193\) | \(n = 25\) | \(n = 94\) | \(n = 74\) |  |  |
| Proctitis (E1) | 3 | 10 | 11 | 1.000 | 0.96 |
| (12.0%) | (10.6%) | (14.9%) | \([0.21–3.78]\) |  |  |
| Left-sided UC (E2) | 9 | 10 | 11 | \(0.006^*\) | 3.94 |
| (36.0%) | (10.6%) | (14.9%) | \([1.07–7.03]\) |  |  |
| Extensive UC (E3) | 13 | 74 | 52 | 0.029 | 0.36 |
| (52.0%) | (78.7%) | (70.3%) | \([0.14–0.92]\) |  |  |
| Extra-intestinal manifestations | 3 | 26 | 24 | 0.747 | 0.66 |
| (27.3%) | (35.1%) | (37.5%) | \([0.13–2.91]\) |  |  |
| Use of immunosuppressive agents | 22 | 97 | 82 | 0.818 | 1.23 |
| (78.6%) | (77.6%) | (71.9%) | \([0.45–3.57]\) |  |  |
| Abscesses | 2 | 6 | 4 | 0.365 | 1.77 |
| (12/240) | (7.7%) | (5.4%) | (3.9%) | \([0.25–9.41]\) |  |  |

For each variable, the number of patients included is given. \(P_{\text{CC}}\) P-value for testing for differences between homozygous carriers of the C allele and heterozygous/non-carriers of the C allele. \(OR_{\text{CC}}\): corresponding odds ratios and 95% confidence intervals (95% CI). Significant \(P\)-values (<0.05) are depicted in \textbf{bold fonts}. \(P\)-values marked with an asterisk remained significant following Bonferroni correction.

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### Table 7. Analysis for gene-gene interaction of \(\text{IL12B}\) with \(\text{IL23R}\) and \(\text{STAT4}\) variants, respectively, regarding susceptibility to Crohn’s disease (CD).

| \(\text{IL12B}\) SNPs | rs3212227 | rs17860508 | rs10045431 | rs6887695 |
|----------------|-------|-------|-------|-------|
| IL23R SNPs | \(\text{rs1004819}\) | 0.408 | 0.731 | 0.474 | 0.450 |
| \(\text{rs7517847}\) | 0.902 | 0.895 | 0.743 | 0.956 |
| \(\text{rs10489629}\) | 0.577 | 0.284 | 0.757 | 0.561 |
| \(\text{rs2201841}\) | 0.497 | 0.428 | 0.080 | 0.189 |
| \(\text{rs11455804}\) | 0.793 | 0.852 | 0.819 | 0.110 |
| \(\text{rs11209026}=p\text{Arg381Gln}\) | 0.280 | 0.752 | 0.929 | 0.181 |
| \(\text{rs1343151}\) | 0.970 | 0.745 | 0.874 | 0.971 |
| \(\text{rs10889677}\) | 0.485 | 0.688 | 0.108 | 0.120 |
| \(\text{rs11209032}\) | 0.311 | 0.473 | 0.890 | 0.427 |
| \(\text{rs1459565}\) | 0.641 | 0.640 | 0.518 | 0.624 |
| STAT4 SNPs | \(\text{rs11889341}\) | 0.732 | 0.092 | 0.160 | 0.025 |
| \(\text{rs7574865}\) | 0.845 | 0.069 | 0.223 | 0.031 |
| \(\text{rs7568275}\) | 0.725 | 0.115 | 0.229 | 0.045 |
| \(\text{rs8179673}\) | 0.659 | 0.120 | 0.160 | 0.035 |
| \(\text{rs10181656}\) | 0.618 | 0.115 | 0.230 | 0.040 |
| \(\text{rs7582694}\) | 0.818 | 0.085 | 0.162 | 0.021 |
| \(\text{rs10174238}\) | 0.354 | 0.200 | 0.625 | 0.247 |

Significant associations are highlighted in \textbf{bold fonts}. None of the associations remained significant following Bonferroni correction.

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| **IL12B SNP** | **Factor** | **Consensus sequence** | position relative to SNP (5’ to 3’) | Binding score major allele | Binding score minor allele |
|----------------|----------------------------|---------------------|-------------------------------------|--------------------------|--------------------------|
| rs6887695 (approx. 65 kb upstream of IL12B): | HSF2 | NGAANWTCCK | −1 to +8 | 85.9 | 68.6 |
| | AML-1a | TGCGGT | −6 to −1 | 85.4 | 85.4 |
| | MZF1 | NGNGGGGA | −6 to +1 | 84.3 | 67.0 |
| | Oct-1 | NNGAATKCANNNN | −5 to +9 | 82.5 | 70.6 |
| | AML-1a | TGCGGT | −14 to −9 | 81.4 | 81.4 |
| | AML-1a | TGCGGT | −9 to −4 | 81.4 | 81.4 |
| | Th1/IFN | NNNGATCTCNGMWT | −1 to +14 | 80.4 | 80.8 |
| | HSF1 | RGAANRTTCN | −1 to +8 | 79.0 | 62.7 |
| | ROR | NWAWNNAGGTCAN | −10 to +2 | 64.4 | 75.9 |
| rs10045431 (approx. 57 kb upstream of IL12B): | Oct-1 | CWNAWTKWSATRYN | −2 to +11 | 79.6 | 75.5 |
| | SRY |AACAWAM | +4 to +10 | 77.3 | 77.3 |
| | Pbx-1 | ANCAATCAY | −2 to +6 | 75.5 | 69.6 |
| | Sp1 | GRGGCGRGGGW | −11 to −2 | 75.3 | 75.3 |
| | Tst-1/Oct-6 | NNGAATKCANNNN | −4 to +10 | 70.8 | 81.2 |
| rs17860508* (approx. 2.7 kb upstream of IL12B): | GATA-3⁶ | NNGATARGN | 0 to +9 | 82.5 | 74.1 |
| | AML-1a | TGCGGT | −6 to −1 | 83.7 | 83.7 |
| | MZF1 | NGNGGGGA | −13 to −8 | 82.7 | 82.7 |
| | Oct-1¹ | NNRTATNANNN | −13 to −6 | 80.9 | 80.9 |
| | Sp1¹ | GRGGCGRGGGW | −6 to +1 | 79.3 | 77.5 |
| | Tst-1/Oct-6 | NNGAATKCANNNN | −7 to +3 | 75.3 | 61.6 |
| rs3212227 (3’-UTR of IL12B): | C/EBP | NNTKTGGWNANN | −13 to −1 | 87.7 | 87.7 |
| | Brm-2/Oct-3 | NNCATNSRWAATNNR | −1 to +16 | 85.1 | 83.2 |
| | TATA | NCTATAAAAAR | −11 to −2 | 84.7 | 84.7 |
| | SRY | AACAWAM | −10 to −4 | 80.9 | 80.9 |
| | Oct-1 | TNGATGNTAAT | −1 to +10 | 80.2 | 79.6 |
| | Tst-1/Oct-6 | NNGAATKCANNNN | 0 to +14 | 79.2 | 77.1 |
| | CDP CR | NAYTAGATSSS | −3 to +6 | 78.5 | 73.1 |
| | Oct-1 | CWNAWTKWSATRYN | −7 to +6 | 77.6 | 83.7 |
| | SRY | AACAWAM | −15 to −9 | 77.3 | 77.3 |
| | C/EBP | NNTKTGGWNANN | −1 to +10 | 76.2 | 76.9 |
| | CDP CR | NAYTAGATSSS | −5 to +4 | 75.3 | 73.1 |
| | SRY | NWAAACAWANN | −9 to +2 | 71.8 | 76.2 |

Binding scores differing more than 5 points between major and minor alleles are depicted in **bold**. Scores differing more than 10 points are depicted in **bold italic**. The binding score threshold for each allele was set to 75.0.

¹Different consensus sequences for the same transcription factor are caused by the deduction of the sequences from different matrices in the TRANSFAC database [45].

*For rs17860508, more than two alleles exist. For the major allele, the score comprises the highest score from all four non-minor alleles (-, G, GC, TTAGA). &= score for the GC allele; # = score for the (-) allele; $= score for the TTAGA allele.

UTR = untranslated region;

Nucleotide codes: K = G or T, M = A or C, R = A or G, S = C or G, W = A or T, N = A, G, C or T.

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rs6887695 (out of the four analyzed IL12B SNPs) the highest likelihood of changes in transcription factor binding. This is consistent with the results of our genotype analysis in which only rs6887695 was associated with overall IBD susceptibility and potential epistasis with STAT4 SNPs.

For rs6887695, the in silico analysis predicted changes in the binding of the transcription factors HSF1, HSF2, MZF1, Oct-1 and RORγ. HSF1 plays a role in protecting against DSS-induced colitis [52]. MZF1 and Oct-1 are transcriptional regulators of SERPINA3 [53,54] which has been implicated as one of the epithelium-derived genes involved in the antibacterial defense [55]. In addition, our in silico analysis predicted for the transcription factor RORγ stronger binding to the minor C allele. Interestingly, RORγ plays together with RORγ1 a key role in the development of Th17 cells which are involved in the pathogenesis of CD and UC [1] and may explain the association of rs6887695 with increased IBD susceptibility found in our study. Although Th17 cells are recognized as proinflammatory T cell population, their major effector cytokines IL-17A and IL-22 may have also protective functions under certain circumstances [19,56,57,58,59]. Similarly, studies indicated for RORγ not only proinflammatory effects but also a role as negative regulator of inflammatory responses [60]. For example, RORγ inhibits TNF-α-induced IL-6, IL-8 and COX-2 expression in human primary smooth-muscle cells by negatively interfering with the NF-κB signaling pathway and reducing p65 translocation [60]. This may correlate with our genotype-phenotype analysis which demonstrated a less severe disease phenotype for homozygous C/C carriers of the IL12B SNP rs6887695. CD patients, who were homozygous C/C carriers of this SNP, had significantly more often non-stricturing, non-penetrating disease than carriers of the G allele (p = 6.8 x 10^-5), while C/C homozygous UC patients had less often extensive colitis than G allele carriers (p = 0.029).

IL12B is another example for a common susceptibility gene for CD and UC which is supported by the results of our study showing a trend for rs6887695 for association with both CD and UC. Out of 99 currently known IBD susceptibility gene loci (71 in CD and 47 in UC), at least 28 susceptibility loci are shared between CD and UC [9,10]. Remarkably, the strongest cluster of common CD and UC susceptibility genes is formed by genes related to the IL-23/Th17 pathway including IL12B [61]. Similar to IL23R, IL12B is also a shared susceptibility gene with other IBD-associated diseases such as psoriasis [29] and ankylosing spondylitis [28], providing an explanation of the increased incidence of these extraintestinal manifestations in IBD patients.

The initial GWAS focused on CD patients with ileal CD. To exclude that the associations found in recent GWAS meta-analysis are based only on certain IBD subphenotypes, we performed a detailed genotype-phenotype analysis focusing also on the anatomic disease localization. Interestingly, genotype-phenotype analysis showed an association of IL12B rs3212227 with colonic CD but not with ileal CD. It is likely that many of the susceptibility loci, which are shared by CD and UC, may predispose to a common phenotype such as colonic (and not ileal) IBD. This is supported by the association of the IL12B SNP rs3212227 with colonic CD and the trend for an association signal of rs6887695 with both CD and UC susceptibility found in our study. Similarly, in another study from New Zealand, carriers of the minor C allele of the IL12B SNP rs6887695 had a decreased risk of ileal disease [48]. A recent study analyzing the same SNP (rs6887695) suggested additional environmental triggers regarding the risk of this IL12B SNP on CD susceptibility. Analyzing differences in associated genes between smoking and non-smoking CD patients, it implicated a complex gene-environment interaction demonstrating an association of IL12B SNP rs6887695 in non-smoking, but not in smoking patients [62].

In addition, our analysis of potential gene-gene interactions revealed weak epistasis of IL12B SNP rs6887695 with 7 out of 8 analyzed STAT4 gene variants (p < 0.05). This is highly interesting, given that STAT4 is the major downstream transcription factor of IL-12. However, considering our limited sample size and the borderline significance of this interaction, which was lost after Bonferroni correction, this potential gene-gene interaction needs further analysis in very large cohorts or GWAS meta-analyses. However, in contrast to our hypothesis-driven study, which analyzed SNPs in the IL12B gene encoding IL-12 p40 and the gene STAT4 encoding the major IL-12 downstream transcription factor STAT4, GWAS are limited by the problem of multiple testing to a much greater extent than our study. Given the large number of SNPs analyzed in GWAS, the correction factor (to correct for multiple testing) is much higher than in our study, which may result in the elimination of potentially true gene-gene interactions. Moreover, in the study by Anderson et al. no formal testing for epistasis has been performed [10]. In addition, in the manuscript by Franke et al. [9], only epistasis between the 71 significantly associated CD loci, which did not include STAT4, was analyzed.

In summary, in contrast to Th17 cell-modifying and strongly IBD-associated IL23R gene variants, IL12B variants have a lesser role in the susceptibility to CD and UC in the German population, suggesting a more important role of IL-23R expressing Th17 cells than Th1 cells in the CD pathogenesis. This observation is supported by similar results of large GWAS [5,9,10], in which IL23R showed stronger associations with IBD susceptibility than IL12B, although these GWAS clearly established IL12B as IBD susceptibility gene. Our IL12B in silico analysis and the results of our genotype analysis suggest rs6887695 as likely disease-causing IL12B variant. Homozygous C/C carriers of this SNP were protected against stricturing and penetrating CD and showed less often extensive UC which may be related to the alteration of the binding of certain transcription factors such as RORγ as predicted by our in silico analysis. However, there is a need for further investigation potential epistasis between the main IBD susceptibility genes as major pathomechanism in the disease pathogenesis. Further in-depth mapping and functional studies are required to clarify the “true” disease-causing IL12B variant and its pathogenic role in IBD susceptibility.

Supporting Information

Table S1 Primer sequences (F: forward primer, R: reverse Primer), FRET probe sequences, and primer annealing temperatures used for genotyping of IL12B variants. Note: FL: Fluorescin, LC610: LightCycler-Red 610; LC640: LightCycler-Red 640; LC670: LightCycler-Red 670. The polymorphic position within the sensor probe is underlined. A phosphate is linked to the 5’-end of the acceptor probe to prevent elongation by the DNA polymerase in the PCR given based on a median split. (DOC)
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**Table S2** Primer sequences used for the sequence analysis of the IL12B variants. (DOC)

**Table S3** Comparison of the association signals of IL12B with the association signals of the three most strongly CD-associated genes NOD2, IL23R and ATG16L1 in the Munich IBD case-control cohort. Minor allele frequencies (MAF), allelic test P-values, and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control cohorts. Details on the analyses of NOD2, IL23R and ATG16L1 gene variants were published in previous studies. (DOC)

**Table S4** Overview of published studies on IL12B in patients with inflammatory bowel diseases. CD: Crohn’s disease; UC: ulcerative colitis. (DOC)

**Author Contributions**

Conceived and designed the experiments: SB JG. Performed the experiments: JW MW MF CJ JD. Analyzed the data: DC JG SB. Contributed reagents/materials/analysis tools: JG SB CS J. Stallhofer BG DC T. Otscheknu T. Olszak. Wrote the paper: J. Seiderer SB JG JD. Recruited, interviewed and treated the participating patients: CT BF J. Seiderer J. Stallhofer T. Otscheknu T. Olszak SB. Provided funding for the study: SB JG J. Seiderer BG.

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