Experimental colitis and translation to human inflammatory bowel disease

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CHAPTER 3

A TAQI POLYMORPHISM IN THE 3'UTR OF THE IL-12 P40 GENE CORRELATES WITH INCREASED IL-12 SECRETION

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Abstract

Interleukin-12 (IL-12) is a key cytokine for the induction of Th1 immune responses. We evaluated whether a TaqI polymorphism in the 3'UTR of the IL-12 p40 gene affects secretion of IL-12 in vitro, and whether this polymorphism is associated with susceptibility to Crohn's disease (CD). IL-12 p40 and p70 secretion by monocytes in relation to genotype was determined in 63 healthy donors. Genotype and allele frequencies of the TaqI polymorphism in 150 CD patients were compared with 145 ethnically matched healthy controls (HC). No significant association was found between genotype and IL-12 p40 secretion after stimulation of monocytes with SAC+IFNg. In contrast, increasing IL-12 p70 secretion was found across the categories of noncarriers, heterozygotes and homozygotes for the variant allele (median values ± SEM: 147 ± 27, 282 ± 51 and 482 ± 34 pg/ ml, respectively; $P<0.005$). The allele and genotype frequencies of this polymorphism in patients with CD did not differ statistically significantly from HC. The presence of a TaqI polymorphism in the IL12 p40 3'UTR correlates with increased in vitro IL-12 p70, but not p40 secretion. While this polymorphism does not appear to be correlated with susceptibility to CD in the limited population of patients tested here, it could influence the occurrence of the disease in certain subsets of patients.

Introduction

Interleukin-12 (IL-12) is a 75 kDa heterodimeric cytokine that is composed of two disulfide-bonded polypeptide chains of 35 and 40 kDa [1]. Whereas the p35 chain is constitutively expressed in many cell types, expression of IL-12 p40, and therefore the p70 heterodimer, occurs mainly in dendritic cells, macrophages and monocytes after one of several stimuli [2–4].
IL-12 plays a key role in the modulation of the immune response by providing the stimulus for CD4+ T cells and NK cells to differentiate towards Th1, IFNγ secreting cells, which in general is associated with cell-mediated immunity. In this capacity, IL-12 plays a key role in the defense against intracellular microorganisms. In addition, excessive IL-12 production has been found in several organ-specific autoimmune diseases, including rheumatoid arthritis, [5] type I diabetes mellitus, [6] multiple sclerosis (MS) [7] and Crohn's disease (CD)[8]. The latter are associated with a strong genetic component in disease susceptibility, which renders the possibility that genetic alterations in the genes affecting the height of the IL-12 response are involved in the susceptibility to autoimmune as well as infectious diseases. Indeed, it has been shown that mutations in the IL-12 p40 gene or the IL-12 receptor lead to reduced IL-12 signaling, which in turn is associated with serious impairment of immunity to intracellular bacteria [9,10].

CD is a chronic granulomatous disease of the intestine that has been shown to be associated with a Th1 T-cellmediated inflammation and high IL-12/IFNg production at histologically affected sites [8]. In addition, studies in animal models of intestinal inflammation have shown that IL-12 is a key cytokine for susceptibility to colitis. Thus, antibodies to IL-12 can prevent or abrogate established disease in a mouse model of colitis induced by the intrarectal administration of the haptenating agent trinitro benzene sulfonic acid (TNBS-colitis) [11]. Interestingly, in a recent study we observed that one of the genetic loci mediating susceptibility to TNBS-colitis maps to a region on chromosome 11 which includes the IL-12 p40 gene. In addition, we were able to demonstrate that the same locus also controls the IL-12 response to LPS [12]. This raises the possibility that a genetic abnormality in mounting IL-12 responses to bacterial products is involved in the susceptibility to intestinal inflammation.

In humans, the gene for the IL-12 p35 subunit resides on chromosome 3 at 3p12–3q13.2, whereas the IL-12 p40 subunit is located at an independent locus on chromosome 5 at 5q31–33 [13]. Recently, the genomic sequence of the IL-12 p40 subunit was determined [14]. Whereas no polymorphisms were found in the coding region of this gene, several intronic polymorphisms

and a TaqI poly- morphism in the 3'UTR at position 1188 were identified.

The latter may have functional relevance, since the 3'UTR region is able to influence the amount of translated protein by several mechanisms, including effects on mRNA stability as well as on transcriptional activity [15], [16]. The TaqI polymorphism was recently found to be associated with susceptibility to both IDDM and MS, but the biological relevance of this polymorphism to these diseases has not been identified [17,18]. In the present study, we attempted to rectify this situation by investigating whether this polymorphism is associated with altered IL-12 secretion by monocytes. Also, given the established role of IL-12 in CD, we investigated whether this polymorphism is associated with susceptibility to this disease.

Materials and methods

Patients and controls

A total of 63 healthy anonymous blood donors attending the Blood Bank of the National Institutes of Health were included to study the relation between the IL-12 p40 TaqI polymorphism and IL-12 secretion. Except for two females, all subjects were males in the age range of 24–67 years (mean: 39 years).

To study the role of the TaqI polymorphism in susceptibility to CD, a total of 150 patients with CD (53 males and 97 females) attending the outdoor patient clinic of the Vrije Universiteit Medical Center were included. All patients had established disease, as defined by clinical, radiological, endoscopic and histological criteria. A group of 145 age, sex and ethnically matched healthy blood donors from the Netherlands was used as the control group. This group of healthy controls and the frequency distribution of the TaqI IL-12 p40 gene polymorphism have been described previously [17].

Media and reagents

HBSS (Biofluids, Rockville, MD, USA) was used as wash medium. Complete culture media consisted of RPMI 1640 (Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated FCS (Invitrogen, Rockville, MD, USA), 100 U/ml penicillin, 100 mg/ml streptomycin (Biofluids), 2mm l-glutamine (Biofluids), 5% (v/v) NCTC (Sigma-Aldrich Inc., St Louis, MO, USA), 50 mg/ ml gentamycin sulfate (BioWhittaker, Walkersville, MD, USA).

Lipopolysaccharide (LPS) from Salmonella enteritidis (L-6011) was obtained from Sigma, recombinant human IFN-g from R&D (R&D systems, Minneapolis, MN, USA), and Staphylococcus aureus strain Cowan (SAC) from Calbiochem (Calbiochem, La Jolla, CA, USA). PElabeled antibodies to human CD14 as well as the human IL-12 p70 OptEIAt ELISA and the human IL-12 p40 BD OptEIAt ELISA were obtained from PharMingen (BD Pharmingen, San Diego, CA, USA).

Isolation of monocytes and cell culture

Monocytes were obtained from normal healthy donors at the NIH blood bank and isolated by aphaeresis and gradient densitometry. Immediately after isolation of monocytes, cells were washed three times in HBSS and counted. The viability of cells, as determined by trypan blue

staining, was 495% for each sample. The percentage of CD14 positive cells was determined with a CD14- specific antibody by FACS analysis using a BD FACS Calibur. The percentage of CD14 positive cells was always between 75% and 80%.

Cells were cultured in triplicate in 24-well plates (Corning Incorporated, Corning, NY, USA) at $2x10^6$ cells/well in a total volume of 1ml complete culture media. Cultures were incubated in the presence or absence of LPS alone, LPS and interferon-gamma (IFN-g), or SAC and IFN-g. After 20 h, supernatants were collected, aliquoted and stored at -80°C until cytokine measurements, and thawed only once. The cell pellet was stored at -80°C and used for DNA isolation using a commercially available DNA isolation kit (Gentra Systems).

Cytokine measurement

Measurement of IL-12 p40 and p70 was assessed by ELISA according to the manufacturer's recommendations. The lower limits of detection of IL-12 p70 and IL-12 p40 were 7.8 and 31.3 pg/ml, respectively. All cytokine measurements were done in triplicate and repeated twice, with an intra- and inter-assay variability of <10%.

TaqI RFLP analysis

Genotyping for the TaqI polymorphism in the 3'UTR of the IL-12 p40 gene was performed as previously described [14]. In short, 50 ng of genomic DNA was PCR amplified using primers that cover the TaqI restriction site. After PCR amplification, the product was cut with the TaqI restriction enzyme for 16 h at 65°C. After restriction enzyme digestion, products were visualized on an ethidium bromide stained 2% agarose gel.

Results

In vitro secretion of IL-12 p40 and IL-12 p70 by monocytes from healthy volunteers

Monocytes from healthy volunteers did not exhibit detectable spontaneous secretion of IL-12 p40 or p70, nor did they exhibit detectable secretion of the IL-12 components after stimulation with LPS alone. However, stimulation of cells with LPS+IFNγ or SAC+IFNγ (Figures 1a and 1b) elicited in general robust induction of IL-12 secretion, although the level of secretion varied

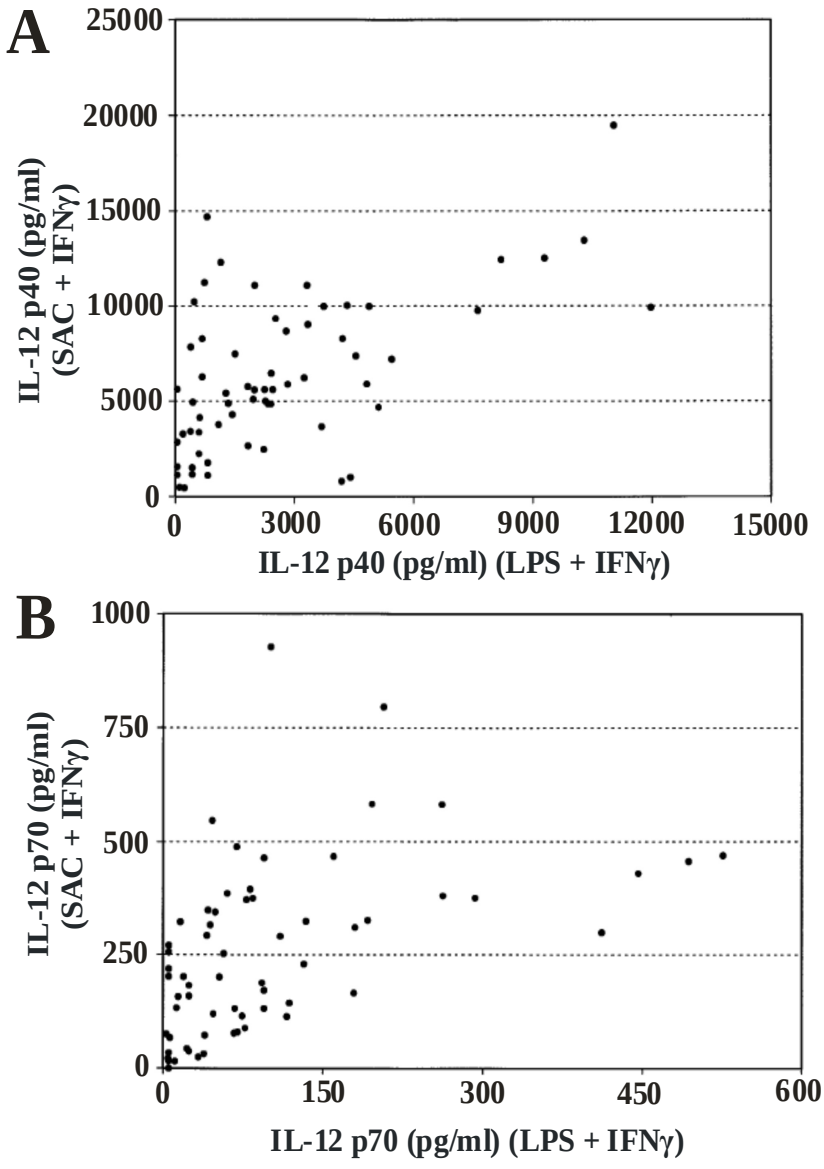

Figure 1. Secretion of IL-12 by human monocytes after stimulation of cells with either LPS+IFNγ or SAC+IFNγ: **A** secretion of IL-12 p40; **B** secretion of IL-12 p70. A significant correlation was found between stimulation of cells with either LPS+IFNγ or SAC+IFNγ for both p40 ($P<0.0001$) and p70 ($P<0.0001$).

widely. The median secretion of IL-12 p40 after stimulation of monocytes with LPS+IFNγ and SAC+IFNγ was 1976 pg/ml (range: 102–12040) and 5623 pg/ml (range: 469–19460), respectively. The median secretion of IL-12 p70 after stimulation of monocytes with LPS+IFNγ was 46 pg/ml (range: <50–545) and 218 pg/ml (range: <50– 928) after stimulation with SAC+IFNγ.

A highly significant correlation was found between IL- 12 p40 secretion after stimulation of cells with either LPS+IFNγ or SAC+IFNγ ($P<0.0001$; Figure 1a). A similarly significant correlation was found for IL-12 p70 secretion (Figure 1b). The results from both stimulation regimens were essentially similar; thus, we will only present the data for stimulation with SAC+IFNγ.

Since only two donors were female, a separate analysis after stratification for gender could not be made. However, the two females secreted values that fall within the mean ± SD of the overall group. Among the 61 males, 34 were of African-American descent, whereas 27 were white Caucasians. No statistically significant differences in IL-12 p40 or IL-12 p70 secretion were found between these two groups (results not shown).

In vitro secretion of IL-12 p40 and IL-12 p70 by monocytes in relation to the TaqI gene polymorphism

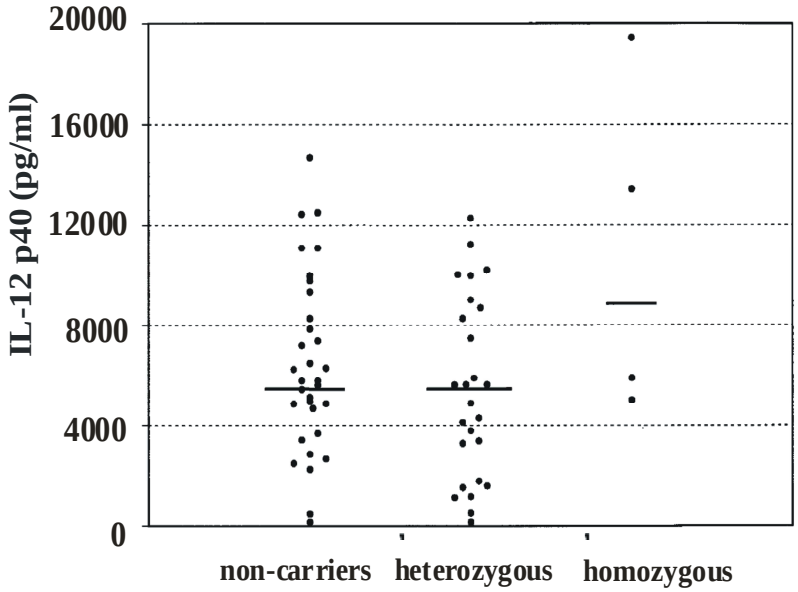

Figure 2. Secretion of IL-12 p40 in relation to a TaqI restriction fragment length polymorphism at position 1188 in the 3'UTR of the IL-12 p40 gene after stimulation of monocytes with SAC+IFNγ. Non-carriers, heterozygous and homozygous indicate absence, carrier ship and homozygosity for the TaqI cutting site.

Of the 63 healthy blood donors tested, a total of 35 did not carry the TaqI cutting site in the 3'UTR of the IL-12 p40 gene, whereas 24 were heterozygous for this polymorphism, and four homozygous. The allele frequency of the common allele (74%) was somewhat lower than that previously reported in a Caucasian population where the allele frequency was found to be ~ 80% [14]. This difference may be due to the relatively low number of individuals in this study or to the fact that this study included a large number of individuals from African- American descent, in which the frequency of this allele has been shown to be lower [14].

As shown in Figure 2, no significant correlation was observed between secretion of IL-12 p40 after stimulation of cells with SAC+IFNγ and the TaqI polymorphic site across the categories of non-carriers, heterozygous and homozygous individuals (P=0.3). In contrast, as shown in Figure 3, a highly significant correlation was found between IL-12 p70 secretion and this polymorphism; thus, increasingly higher values were found among the categories of non-

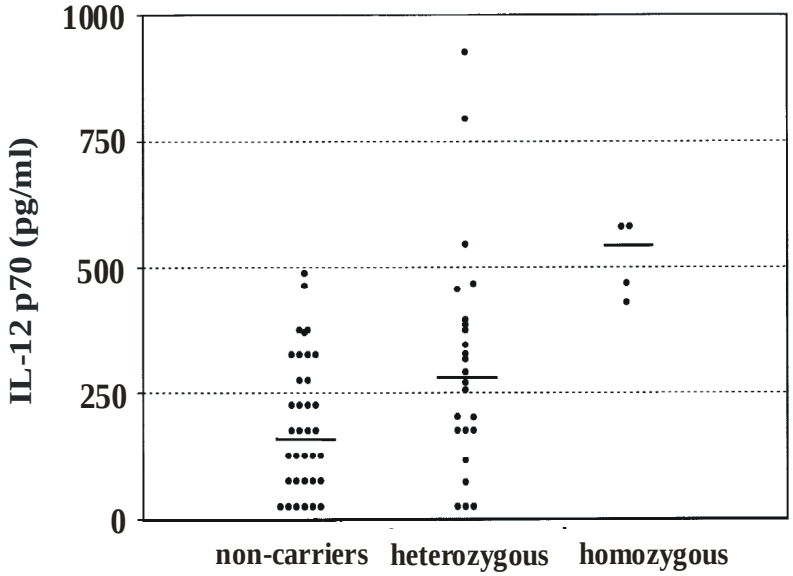

Figure 3. Secretion of IL-12 p70 in relation to a TaqI restriction fragment length polymorphism at position 1188 in the 3'UTR of the IL-12 p40 gene after stimulation of monocytes with SAC+IFNγ. Increasing IL-12 p70 secretion was found across the categories noncarriers, carriers and individuals homozygous for this polymorphism (P<0.005).

carriers, heterozygous and homozygous individuals (P<0.005).

Since all four homozygous individuals were unrelated African-American males, we subsequently analyzed the data in this group of individuals separately. Again, no association was

found with IL-12 p40 secretion, whereas a highly significant association was found with IL-12 p70 secretion, similar to the overall group (data not shown).

Frequency of the TaqI gene polymorphism in patients with Crohn's disease and healthy controls

Subsequently, we determined the genotype and allele frequencies of the TaqI polymorphism in a group of 150 patients with CD and 145 controls. As can be seen from Table 1, no significant differences in allele and genotype frequencies were found between patients with CD and controls.

Table 1. Allele and genotype frequencies of the TaqI restriction fragment length polymorphism in the 3'UTR of the IL-12 p40 gene in patients with CD and healthy controls

	Genotype frequencies n (%)			Allele frequencies %	
	AA	AB	BB	A	B
Patients (n = 150)	92 (61	51 (34)	7 (5)	78	22
Controls (n = 145)	98 (68)	38 (26)	9 (6)	81	19

A indicates the wild-type allele. B indicates the presence of the TaqI polymorphic site.

Discussion

Given their central role in the immune response, the IL- 12 genes are potential candidate genes for infectious as well as Th1-mediated autoimmune diseases. Indeed, increased expression of IL-12 is found in several immune-mediated diseases, whereas mutations in the IL-12 genes and its receptors are associated with increased susceptibility to Th1-mediated infectious diseases. Sequencing of the IL-12 p40 gene revealed no polymorphism in the coding sequence, indicating a high level of conservation in humans. Several intronic polymorphisms however were identified, as well as a TaqI RFLP at position 1188 in the 3'UTR [14]. The latter is of particular interest, since it has been shown to be associated with susceptibility to at least two diseases: multiple sclerosis [17] and type I diabetes mellitus [18].

To elucidate the biological significance of the 3'UTR polymorphism in the etiology or course of a disease, it is mandatory to study the relation between genotype and phenotype. For this reason, we studied in vitro IL-12 secretion by stimulated human monocytes in relation to this polymorphism. We found that the TaqI polymorphism was associated with increased secretion of IL-12 p70 by stimulated human monocytes. Individuals homozygous for the polymorphism were high producers of IL-12 p70 in vitro, non-carriers were low producers, and individuals that were heterozygous were intermediate producers. In contrast, we did not find an association between genotype and the ability of monocytes to produce IL-12 p40.

The fact that the TaqI polymorphism in the p40 gene has an effect on IL-12 p70 secretion, but not on IL-12 p40 secretion, is of considerable interest and, at first glance, may appear counterintuitive. One possibility is that the polymorphism affects p40 homodimer formation, which, in turn, influences IL-12 expression, as previously demonstrated [19]. Another possibility is that the TaqI polymorphism affects p70 heterodimer formation because it is in linkage disequilibrium with a polymorphism in the coding region that affects heterodimer formation. It is now known that amino acid substitutions in the IL-12 p40 and p35 gene dramatically affect p70 heterodimer formation, [20] and while in a study by Huang et al [14] no polymorphisms in the coding region of the human IL-12 p40 gene were found, the presence of such polymorphism cannot be excluded. Indeed, sequence variations in the coding region of the p40 gene between different inbred strains of mice have recently been identified [12], [21]. Finally, it is unlikely that the observed differences in p70 secretion are mediated through differential p35 expression, since the IL-12 p35 gene is located on another chromosome than the p40 gene.

Our findings are somewhat contrary to the results obtained by Morahan and colleagues, who demonstrated that the TaqI polymorphic site was associated with decreased IL-12 p40 mRNA expression by EBV transformed cell lines [18]. It must be noted however that this study was done in cell lines, and focused on p40 mRNA expression rather than on p40 or p70 protein secretion, and investigated basal expression instead of stimulated expression. Interestingly, our findings in humans are in general in accordance with data we have generated in the mouse. In particular, we have found dramatic differences in serum IL-12 p70 secretion, but not p40 secretion, between different inbred strains of mice after in vivo LPS challenge [12]. We were able to map these differences to a region on mouse chromosome 11, which contains the IL-12 p40 gene. Thus, similar to the present observation in humans, the region containing the IL-12 p40 gene in the mouse seems to affect IL-12 p70 rather than p40 formation. We are currently further investigating the genetic and molecular basis for these findings.

The IL-12 genes are excellent candidate genes for CD; a well-established Th1-mediated inflammatory disorder of the gastrointestinal tract. Animal studies as well as studies in patients have revealed a central role of IL-12 in disease pathogenesis, and the clinical effect of anti-IL12 monoclonal antibodies is under current investigation. In addition, the recent identification of CARD15, a gene involved in NFkB activation as a susceptibility gene for CD, emphasizes the important role of the innate immune system in the pathogenesis of CD [22, 23]. Although the data from the present study did not reveal a significant association with the IL-12 p40 TaqI polymorphism, this does not necessarily exclude a role for the IL-12 gene in the susceptibility to CD. The relatively small sample size in combination with the rare frequency of the variant allele may not have been powerful enough to detect such an association. As was recently shown for IDDM, [18], [24] large-scale family-based linkage studies are necessary to establish the role of the IL-12 genes in the susceptibility to CD. These efforts are currently underway.

In conclusion, we have demonstrated that a polymorphism that is associated with susceptibility to at least two Th1-mediated diseases affects protein expression of IL-12p70, providing a rational base to further explore this genetic variation in the etiology of IL-12-mediated diseases.

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