CHAPTER 2

A POLYMORPHISM IN THE CODING REGION OF IL12B PROMOTES IL-12P70 HETERODIMER FORMATION IN COLITIS SENSITIVE SJL/J MICE.

A. Zwiers¹,², D. Seegers³, T. Konijn², J.J. Garcia-Vallejo², J. Samsom⁴, I. Fuss³, W Strober³, G. Kraal¹ and G. Bouma²

Dept. of Gastroenterology¹ and Molecular Cell Biology and Immunology², Vrije Universiteit Medical Center, Amsterdam, the Netherlands; The Mucosal Immunity Section, National Institutes of Health, Bethesda, MD, USA³; Dept. of Pediatric Gastroenterology and Nutrition, Erasmus University Medical Centre–Sophia Children's Hospital, Rotterdam, The Netherlands⁴
Abstract

Interleukin-12 (IL-12) and interleukin-23 (IL-23) are key cytokines involved in the induction of Th1 and Th17 immune responses. Both cytokines are heterodimers, sharing a common subunit (IL-12p40) coded for by the *IL12b* gene. Genome wide association studies have recently implicated *IL12b* as a susceptibility gene for Crohn’s disease and Ulcerative Colitis. In a previous study in mice we identified the locus harboring this gene as a susceptibility region for trinitrobenzene sulfonic acid (TNBS) induced colitis. Here we investigated whether polymorphisms in the *IL12b* gene influence the synthesis of IL-12 in the highly susceptible SJL/J mouse strain.

By generating two sets of constructs, in which p35 was linked to either the polymorphic SJL/J IL-12p40 or the wild type IL-12p40 from C57Bl/6, it was found in transfection studies that constructs with the SJL/J derived variant synthesized significantly more IL-12p70 relative to IL-12p40 compared to the wild type C57BL/6 variant. This could not be attributed to differences in synthesis rate or secretion implicating a higher affinity of SJL/J derived IL-12p40 for its IL-12p35 subunit. This higher affinity is not confined to IL-12 synthesis but is also implicated in IL-23 synthesis. Conformational changes in the p40 molecule may underlie more efficient binding to p35, resulting in enhanced synthesis of the mature dimeric cytokine. It is concluded that the higher susceptibility found in the SJL/J strain for TNBS induced colitis is based on an aberrant high synthesis rate of the IL-12p70 cytokine, leading to a rapid pro-inflammatory skewing of the immune response and distortion of the homeostatic balance.
Introduction

Crohn’s disease (CD) and Ulcerative Colitis (UC) are chronic inflammatory disorders of the gastrointestinal tract, which together form the two main entities of inflammatory bowel diseases (IBD).

Although the exact pathogenesis of IBD still remains unknown it is apparent that in its etiology, genetic factors as well as environmental components are involved [1-3]. Epidemiological, family and twin studies revealed that this genetic component is not attributable to the action of one or of a few genes with a large effect but that it rather is the cumulative effect of small contributions of many genes [4]. In addition, disease displays a marked clinical heterogeneity. Until the introduction of genome wide association studies (GWAS) the identification of the genes involved has proven to be difficult and only a few were unequivocally associated with IBD. These include the gene encoding NOD2 - also known as CARD15-, associated with CD [5-7], the TNFSF15 gene (in the Japanese population) [8], the IL-23 receptor gene [9] and the ATG16L1 gene [10]. Now a recent genome-wide meta-analysis on previous CD scans confirmed 11 loci that were found in previous genetic studies [11] and it additionally reported significant evidence for another 21 loci. Nevertheless it should be kept in mind that mutations in these genes only contribute to a minority of the overall risk.

Genetic analysis of animal models for a particular disease may circumvent part of the problems encountered in dissecting complex genetic traits in human disease. Apart from the advantage of genetic and clinical homogeneity in such a model, animal models also offer the possibility of systematic analysis of gene function once a gene has been identified. In one such murine model for intestinal inflammation, the trinitrobenzene sulfonic acid (TNBS) inducible colitis model, we have recently shown that differences in susceptibility to colitis in susceptible SJL/J mice and resistant C57Bl/6 mice are regulated by two loci on the mouse genome, Tnbs1 on chromosome 9 and Tnbs2 on chromosome 11 [12].

In a further search for logical candidate genes within Tnbs2 it was found that this region harbors the gene encoding the interleukin-12 p40 (IL-12p40) chain. IL-12p40 can covalently heterodimerize with a 35-kDa light chain (known as p35 or IL-12α) to form the biologically active pro-inflammatory cytokine IL-12p70 [13]. Furthermore, it can heterodimerize with another light chain, p19, to form IL-23 [14], a cytokine that can drive chronic inflammation via the Th17 T-cell subset by production of the proinflammatory cytokine IL-17 [15]. In this re-
A polymorphism in the coding region of *il12b* promotes IL-12p70 heterodimer formation in colitis sensitive SJL/J mice.

spect it is noteworthy that recently mutations in the receptor for IL-23 have been identified as a risk factor for IBD [9].

That the gene encoding IL12p40 is a very likely candidate gene can be inferred from the key observation that development of colitis in this model can be prevented or even reversed by using antibodies against the interleukin 12p40 subunit [16]. Additional support for the thesis that the gene coding for the IL-12p40 subunit might be responsible for the linkage with *Tnbs1* came from the observation that challenging mice intraperitoneally with sublethal doses of LPS led to profound IL-12p70 responses in the colitis susceptible SJL/J strain, which were substantially higher than in the colitis resistant C57Bl/6 strain. In a subsequent genetic analysis we demonstrated that these differences were mediated by the same chromosomal region on chromosome 11 [12]. Taken together, these findings strongly indicate that SJL/J mice have a genetic preponderance to mount high IL-12 responses when challenged with the appropriate bacterial stimuli.

In the current study we intended to further unravel the molecular mechanism underlying these strain differences and found that not only IL-12p70 but also IL-23 responses are different in both strains and that this could not be attributed to differential expression of the IL-12 p40 chain. We hypothesize that polymorphisms found in the coding region of *IL12b* lead to more efficient heterodimer formation and larger quantities of functional IL-12p70 protein, by changed affinity of the p40 chain for the p35 chain.
Materials and Methods

Animals
Specific pathogen-free, 5–6-week-old male SJL and C57Bl/6 mice were obtained from the National Cancer Institute.

Mice were maintained in the National Institute of Allergy and Infectious Diseases animal holding facilities. Animal use adhered to National Institutes of Health Laboratory Animal Care Guidelines.

DO11.10 mice (OVA-specific TCR transgenic mice) were bred at the Vrije Universiteit University Medical Center. Experiments were approved by the Animal Experiments Committee of the Vrije Universiteit University Medical Center.

Induction of TNBS Colitis
For the induction of colitis, mice were first lightly anesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). To induce colitis, 3.15 mg TNBS (Sigma Chemical Co., St. Louis, MO) was mixed and dissolved with an equal amount of 100% ethanol. A total volume of 150 µL of the TNBS-ethanol mixture was slowly administered per rectum via a 3.5F catheter equipped with a 1-mL syringe. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 30 seconds after the injection. The mice were killed 4 days after induction of colitis.

IL-12 IL-23 and IFN-γ induction
From each strain, 3 male mice were intraperitoneally injected with a sublethal dose of 300 µg LPS. Six hours after administration, mice were bled and IL-12 p70, IL-12 p40 and IL-23 were measured in the serum. Preparation of spleen cell suspensions for OVA activated IFN-γ induction by IL12p70 was performed as described elsewhere [17]. In short, spleens from 8-12 weeks old DO11.10 mice were strained through a 100-µm gauze. Erythrocytes in the splenocyte suspension were lysed by incubation with lysis buffer (150 mM NH4Cl, 1 mM NaHCO3, pH 7.4) for 5 min on ice. Cells were diluted to 5x10^5 cells/ml and incubated with 100nM OVA 323–339 peptide and the appropriate amount of IL-12p70 and/or IL-12p40. After 48 hrs IFN-γ production was measured.
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**Reagents**

Lipopolysaccharide (LPS) from *Salmonella enteritidis* was obtained from Sigma (Sigma-Aldrich, St. Louis, MO). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside and kifunensine were obtained from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). OVA\(_{323-339}\) peptide was purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). IL-12 p40, IL-12 p70 and IL-23, were determined using enzyme-linked immunosorbent assay kits. The IL-12p40 and IL-12p70 kits were obtained from PharMingen (PharMingen, Alphen a/d Rijn, The Netherlands), the IL-23 kit was purchased from eBioscience (ITK diagnostics, Uithoorn, The Netherlands). IFN-γ production was measured with the Cytometric Bead Array (CBA) -Mouse Th1/Th2 Cytokine- Kit supplied by PharMingen (PharMingen, Alphen a/d Rijn, The Netherlands). Restriction enzymes were supplied by New England Biolabs (Westburg, Leiden, The Netherlands).

**Plasmids and site-directed mutagenesis**

Plasmid DNAs encoding the C57BL/6 IL 12p40 and IL 12p35 genes were obtained from ATTC (http://www.atcc.org). The retroviral vector pBMN-I-GFP was purchased from the Nolan Lab (http://www.stanford.edu/group/nolan/). The SJL/J variant of IL 12p40 was generated from the C57BL/6 DNA using the QuickChange II XL site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The retroviral constructs, B6n and SJn, were generated by subcloning the murine IL-12p35 gene in the XhoI/NotI site which is located proximal of the Internal Ribosomal Entry Site (IRES) and the C57BL/6 or SJL/J IL 12p40 gene at the NcoI/SalI located distal of the IRES.

Constructs were verified by sequencing. This revealed that in one of our clones the first base of the ATG start codon, i.e. the adenosine, and the last nine bases, including the TGA stop codon, of the IL 12p35 gene were deleted. This gave rise to a shift in the reading frame of the IL 12p35 gene, as a consequence of which this clone was only able to produce IL 12p40 and no IL-12p70. This clone was used to make constructs synthesizing only p40 from C57BL/6 or SJL/J origin, constructs B6p40 and SJp40 respectively.
Transfection, cell culture and inhibition of glycosylation.

Retroviral constructs were transfected in the Phoenix packaging cell line obtained from the ATCC (http://www.atcc.org) as previously described [18]. 48 – 72 hours after transfection viral supernatants were collected and filtered. Subsequently BW5147 thymoma cells were infected using a centrifugation-facilitated protocol. Cells were grown in RPMI 1640 (HEPES buffered 10mM) containing 10% (v/v) fetal calf serum and 1 % pen/strep. Supernatants were collected by centrifugation and either used for direct analysis or frozen at -20 C for later analysis. To block glycosylation cells were incubated during 5 days in the presence of the N-glycosylation inhibitor kifunensine (Kitasatospora kifusinense, 2 µg/ml; Calbiochem, USA) or the O-glycosylation inhibitor Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (Benzyl-α-GalNAc, 4 mM, Sigma, The Netherlands). Efficiency of inhibition was assessed by flow cytometry using the biotinylated lectins Con A (Concanavalin A, Vector Laboratories, USA) and HPA (Helix pomatia agglutinin, Sigma, The Netherlands). Incubation with kifunensine or Benzyl-α-GalNAc results in an increase in immature N-glycans, highly reactive with Con A and/or Tn Ag, recognized by HPA.

Electrophoresis and Western blotting.

Non-denaturing SDS-PAGE was performed on 4-12% NuPage Bis-Tris gradient gels (Novex; Invitrogen, Breda, The Netherlands) with MOPS running buffer (Novex; Invitrogen, Breda, The Netherlands) in a X cell II mini gel/blot module (Novex; Invitrogen, Breda, The Netherlands) according to the manufacturers protocol. After electrophoresis the separated bands were transferred onto a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands) in the NuPageTransfer Buffer (Novex; Invitrogen, Breda, The Netherlands) containing 10% methanol per gel. Unoccupied places on the membrane were blocked with 5% skimmed milk in phosphate buffered saline (PBS) containing 0.05% Tween 20 (blocking buffer) for 1 hour at room temperature. Incubation with the primary antibody, rat anti-mouse IL-12 Mab - clone C17-8 - (Endogen/Perbio, Etten-Leur, The Netherlands), was performed over night at room temperature in blocking buffer. After washing with PBS and Tween 20 (0.05%) incubation with peroxidase-labeled goat anti-rat antibody (Brunschwig; Amsterdam, The Netherlands) followed. Antibody binding was detected using a chemiluminescence detection system (ECL; Amersham Biosciences, Roosendaal, The Netherlands) according to the
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manufacturer’s instructions. Molecular weights were estimated by simultaneous electrophoresis and transfer of fluorescence labeled molecular weight markers (MagicMark Western Standard; Invitrogen, Breda, The Netherlands).

\textit{Glycoprotein-lectin Immunosorbent Assay (GLIA)}

The glycosyl status of both forms of IL-12p40 was determined with the procedure described by Hampel et al. \cite{19}. In short, the capturing antibody from the mouse IL-12p40 kit (PharMingen, Alphen a/d Rijn, The Netherlands) was coupled to polystyrene microtiter ELISA plates. After blocking of unoccupied sites with 1% BSA in Trisbuffer incubation with equimolar amounts of either SJL/J type or C57BL/6 type p40 followed. The presence of specific glycan moieties was analyzed with a panel of lectins with different specificities (see Table 1). The IL-12p40-lectin complex was quantified with ExtraAvidin peroxidase conjugate. Background absorbance was measured by incubating with buffer instead of IL-12p40. Samples were analyzed in pentuplicate.

\begin{table}[h]
\centering
\begin{tabular}{ll}
\hline
\textbf{Lectin} & \textbf{Carbohydrate Binding Specificity} \\
\hline
\textit{Canavalia ensiformis} agglutinin (ConA) & TerminalαMan, Manα3[Manα6]Man \\
\textit{Galanthus nivalis} (GNA) & TerminalαMan \\
\textit{Helix pomatia} agglutinin (HPA) & αGalNac(terminal) \\
\textit{Maackia amurensis} agglutinin (MAA) & Neu5Acα(2,3)Gal \\
\textit{Sambucus nigra} agglutinin (SNA) & Neu5Acα2,6Gal or α2,6GalNAc \\
\textit{Ricinus communis} agglutinin (RCA), (GlcNAc, N-acetylglucosamine; Neu5A, sialic acid; Gal, galactose; GalNac, N-acetylgalactos-amine; Man, mannose.) & TerminalβGal \\
\hline
\end{tabular}
\caption{Lectins used for identification of Oligosaccharide content of mutant and WT IL-12p40.}
\end{table}
Results

IL12 p70 responses in colitis susceptible versus colitis resistant mice.

When SJL/J and C57Bl/6 mice were injected with a sublethal dose of LPS and serum IL12 responses were determined after 6 hours SJL/J mice produced significantly higher amounts of IL12 p70 as compared to C57BL/6 mice (mean 1721 ± 367 pg/ml versus 102 ± 95 pg/ml; P<0.001; figure 1), confirming previous observations [12]. This vigorous IL12 response in SJL/J mice could be limited to a great extent by co-injection of rmIL10. As can be seen from figure 1, injection of 4 µg rmIL10 resulted in an 80% reduction in IL-12 responses in the SJL/J strain indicating that the measured responses are biologically relevant and not due to the mode of investigation.

Importantly, whereas the expression of IL-12 p70 was significantly different between strains, the p40 levels did not significantly differ between the two strains as previously described and confirmed in this study (results not shown).

Figure 1. Serologic cytokine responses to administration of LPS in SJL/J and C57BL/6 mice. Mice were injected intraperitoneally with a sublethal (300 µg) dose of S. Enteritidis dissolved in phosphate-buffered saline, or with a same dose of S. Enteritidis LPS concomitant with 4 µg rmIL10. Bars represent mean ± SD of one representative experiment out of 3, each involving 5 mice per group. (■ C57BL/6 mice, □ SJL/J mice.)
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**Figure 2.** Different susceptibility to TNBS-induced colitis in SJL/J and C57BL/6 mice. Distribution of weight changes (mean ± SD compared with baseline weight) 4 days after the induction of TNBS colitis in SJL/J and C57BL/6 mice. One experiment representative of 3 independent experiments, each including 5 mice in each group, is shown (■ C57BL/6 mice, □ SJL/J mice).

**Figure 3.** Serologic cytokine responses to intrarectal administration of TNBS in SJL/J and C57BL/6 mice. Mice were instilled with TNBS dissolved in ethanol. Four days after the induction of colitis, mice were sacrificed and the serum IL12 responses determined. Bars represent mean ± SD of one representative experiment out of 3, each involving 5 mice per group (■ C57BL/6 mice, □ SJL/J mice).

As previously described, SJL/J mice reared under the specific pathogen–free conditions in our
animal facility were highly susceptible to TNBS-induced colitis. Thus, when given TNBS, all mice in this mouse strain quickly developed diarrhea accompanied by severe weight loss (average, 18% ± 8% of body weight 4 days after induction of colitis) (figure 2). In addition, all affected mice showed severe macroscopic and microscopic disease at this time. In contrast, C57BL/6 mice reared under the same conditions were highly resistant to TNBS-induced colitis. Thus, although mice of this strain also developed weight loss when given TNBS (typically 10%–15%) during the first 24 hours after administration of TNBS, they quickly recovered; by day 4, 90% of mice regained their original weight (figure 2). In addition, by this time, their colons looked normal both macroscopically and histologically.

In view of the important role of IL-12 in colitis and the differential expression of this cytokine in the resistant C57BL/6 and susceptible SJL/J strain we determined the serum IL-12 responses in mice after induction of TNBS colitis. Albeit there was a wide variation, IL-12 responses were significantly higher in SJL/J mice as compared with C57BL/6 mice, 4 days after the induction of TNBS colitis (P<0.05; figure 3), clearly showing a relationship with the production of IL-12 and the susceptibility for TNBS colitis.

**Polymorphisms in the coding region of il12b promote formation of IL12-P70 heterodimers.**

The above and previous data indicate that susceptibility to colitis is accompanied by increased serum IL12 p70 responses and that colitis susceptible mice have highly increased IL12p70 responses when challenged with LPS. The genetic region that regulates the susceptibility is linked to the same region on chromosome 11 harboring the IL-12 p40 gene. The fact that SJL/J mice produce more IL12 could not be attributed to higher secretion of IL12p40, since serum responses after LPS challenge of the p40 protein were comparable between strains. IL-12 is a heterodimeric protein consisting of a p40 subunit (encoded by il12b on chromosome 11) and a p35 subunit (encoded by il12a on chromosome 3). An explanation for the observed serological differences in IL12p70 but not p40 after LPS challenge might come from the observation that certain amino acid residues in IL12p40 are critical for the affinity for the p35 chain, which is important for the dimerization and secretion of the combined molecule. Indeed, it was previously shown for human IL-12 that a single amino acid change in the IL-12 p40 subunit gave rise to dramatic differences in the formation of the mature heterodimer [20]. To address the possibility that a similar mechanism underlies our observed differences in p70
but not p40 secretion, we sequenced the p40 coding region from SJL/J and C57BL/6 mice. In agreement with published data, we found that SJL/J and C57BL/6 IL-12p40 differ in their genomic sequence at two sites giving rise to two amino acid substitutions: (Met (C57BL/6) -> Thr (SJL/J) at position 169 and Phe (C57BL/6) -> Leu (SJL/J) at position 294 relative to the Methionine startcodon.

Subsequently, we investigated whether these sequence differences influenced heterodimer formation. We generated bicistronic retroviral vectors harboring the IL12p40 from either strain in conjunction with the same p35 chain. In bicistronic expression plasmids the gene under control of the IRES is usually much less efficiently translated than the gene at the cap-dependent first cistron. Therefore, to mimic as closely as possible the physiological situation, the p35 gene was placed under the control of first cistron, since p35 is constitutively expressed whereas p40 is only upregulated after stimulation of the cell. Subsequently, we infected murine BW5147 thymoma cells with the packaged retroviral constructs. Cells containing the retroviral construct were selected by limiting dilution and positive clones were cultured at a concentration of $1.10^5$ for 4 days, whereafter the concentrations of p40 and p70 were determined. If the SJL/J derived p40 has a higher affinity for the p35 chain than

![Figure 4](image-url)
C57BL/6 derived p40, this would result in more efficient generation of p70 and consequently a lower concentration of free p40 in the supernatant. To address this hypothesis, we determined the ratio of p70 over free p40 as a measure for the affinity of the p40 chain for the p35 chain. We used ratios rather than absolute values of p70 and p40 since ratios are independent from inter-assay variation and copy number of the integrated gene. As can be seen from figure 4, the ratio p70/p40 in clones transfected with SJL/J derived p40 was significantly higher than in clones transfected with C57BL/6 derived p40 (P<0.0001), indicating that the SJL/J derived polymorphic variant of p40 indeed promotes the formation of the p70 heterodimers.

An alternative explanation for the differences in p70/p40 ratios in this in vitro system could be different excretion of the polymorphic variant of the p40 chain. However, this possibility was excluded by determining the concentration of p40 from both strains intracellularly and in the supernatant. Thus, as shown in figure 5, an excellent correlation was found between the amount of p40 intracellularly and in the supernatant.

**Figure 5.** Intracellular and extracellular concentrations of IL12 p40. IL-12p40 concentrations were determined with ELISA in supernatants and cell lysates from transfected cells containing plasmids only expressing expressing SJL/J type IL-12p40 or C57BL/6 type IL-12p40. Intracellular amounts of p40 are given relative to the excreted amount of IL-12p40. Bars represent mean ± SD (■ extracellular IL-12p40, □ intracellular IL12-p40).
A polymorphism in the coding region of *il12b* promotes IL-12p70 heterodimer formation in colitis sensitive SJL/J mice.

**Biological consequences of the mutant form of p40**

In addition to exert an effect on the formation of the amount of biologically p70 produced, the polymorphic variant might also affect the three-dimensional configuration of the protein and thereby the affinity for the IL-12 receptor. To address this question, we determined the *in vitro* IFN-γ responses in OVA primed DO11.10 cells, carrying the transgenic TCR for OVA, after culturing them in the presence of IL-12p70 (at 8 ng/ml) derived from the respective constructs. As can be seen from figure 6, equal amounts of IL-12 p70 from either strain resulted in identical induction of IFN-γ. In addition, adding increasing amounts of either of the two p40 variants resulted in a similar mild, dose-related inhibition of IFN-γ production. Thus, the polymorphic variant of p40 does not have an effect on the affinity of p70 for its receptor or the ability to induce IFN-γ, nor has free p40 differential effects on inhibition of the IFN-γ response.

**Figure 6.** Effect of the polymorphic p40 variants on IFN-gamma secretion. Splenocytes from DO11.10 mice were incubated with OVA peptide in the presence of IL-12p70 and increasing amounts of IL-12p40. After 48 hr IFNγ responses were measured in the supernatants. Bars represent mean ± SEM. (■ = induction by C57BL/6 type IL-12p70 together with C57BL/6 derived IL-12p40 amounts as indicated, □ = induction by mutant SJL/J type IL-12p70 together with SJL/J derived IL-12p40 amounts as indicated).

The polymorphic variant of *IL12* results in differential glycosylation

Western Blot analysis showed that SJL-derived p40 has a higher molecular weight as compared to C57BL/6 derived p40, suggestive for increased glycan synthesis figure 7. This
can either be due to enhanced N-glycosylation or to an additional O-glycosylation event as a consequence of the Met to Thr change in the SJL/J type of IL-12p40 as Thr can be subjected to O-glycosylation.

In a next series of experiments we investigated these possibilities and their possible involvement in the observed higher affinity of the SJL/J type of IL-12p40 for IL-12p35.

First, we investigated differences in glycosylation status between both strains using a glycoprotein-lectin binding assay. Here, a panel of lectins with different specificities was used (Table 1). Most lectins did not show a signal above the background level probably due to binding of the lectins to glycans of the capturing antibody (results not shown). With SNA however the SJL/J type IL-12p40 showed a significantly higher degree of staining than did
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**Figure 8.** Differences in sialic acid content of both IL-12p40 variants. Equimolar amounts of either SJL/J type or C57BL/6 type IL-12p40 were subjected to glycoprotein-lectin immunosorbent assay analysis with a panel of different lectins. Only the results with SNA are shown as the other lectins did not generate a signal above background staining. Results are given as the relative absorbance at 450 nm. Samples were run in pentuplicate, bars represent mean ± SD. (□: SJL/J type IL-12p40, ■: C57Bl/6 type IL-12p40).

**Figure 9.** Differences in P70/p40 ratios obtained after incubation with or without glycosylation inhibitors. IL-12p40 and IL-12p70 ratios were determined in the supernatants of cells transfected with either the SJL/J or C57Bl/6 derived constructs which were cultured in the presence of BGN an inhibitor of O-glycosylation, the solvent for BGN (methanol) or with kifunensine an inhibitor of N-glycosylation. Bars represent mean ± SD. (■ = ratio’s from C57Bl/6 derived constructs, □ = ratio’s from SJL/J type IL-12p40 derived constructs KIF = kifunensine).

the C57BL/6 type (figure 8). This stronger signal is indicative for the presence of more sialic
acid units and thus for increased glycosylation as already suggested by the Western Blot experiments, but as these sialic residues can be added to N-linked as well as to O-linked sugars in the Golgi apparatus this assay was not discriminative of the primary type of glycosylation.

In a following series of experiments we investigated the consequences of inhibiting the glycosylation process. We cultured cells transfected with either the SJL/J or C57Bl/6 derived constructs in the presence of 4 mM Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BGN), an inhibitor of O-glycosylation or with the inhibitor of N-glycosylation kifunensin. After an incubation period of 36 hours the p70/p40 ratios were determined. As can be seen from figure 9, culturing in the presence of Kifusenin resulted in a clear decline in p70/p40 ratios from the SJL/J derived clones. Interfering with O-glycosylation however did not result in decreased ratios, implicating that if this type of glycosylation is present, it does not interfere with preferred heterodimerization as seen with SJL/J derived clones.

The SJL/J derived IL-12p40 subunit promotes IL-23 formation

To investigate whether the enhanced affinity of IL-12p40 also applies for its other counterpart, p19 with which it heterodimerizes to IL-23, we challenged mice with LPS and measured IL-
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12p40 and IL-23 synthesis. As can be seen from figure 10, the SJL/J strain responded with a significantly higher IL-23 serum level compared with that of C57Bl/6 while at the same time the IL12p40 level between both strains did not differ. This indicates that the SJL/J derived IL-12p40 subunit also displays a higher affinity for the p19 subunit.
Discussion

From studies in both human and murine IBD it is clear that IL-12 plays a crucial role in the pathogenesis of Crohn’s disease (CD). Human mucosal antigen presenting cells from CD patients secrete increased amounts of IL-12p70 [21] and in the murine TNBS induced colitis model, IL-12 is overexpressed by Dendritic cells [22].

Genetic analysis has revealed that one of the two loci governing susceptibility to TNBS colitis in the susceptible SJL/J strain located on chromosome 11 harbors the gene for the IL-12p40 subunit of Interleukin–12 [12] and that this SJL/J locus is associated with higher IL-12 responses than found in C57BL/6 mice carrying the resistant genotype. Surprisingly this higher response was not accompanied with a concomitant increase in IL-12p40 synthesis, also no differences were observed in the regulatory regions between the two strains upon sequencing. However the two strains did differ at two sites in the structural part of the gene. As it is known for human IL-12 that amino acid changes can dramatically influence formation of the mature heterodimer we investigated whether such is the case here [20].

To test this hypothesis we generated vectors containing both subunits of IL-12 and found that supernatants of cells containing constructs with the SJL/J derived Il-12p40 synthesized significantly more IL-12p70 relative to also secreted IL-12p40 than did cells containing the C57BL/6 derived form of p40. Due to the use of constructs the transcriptional and translational regime for both forms of IL-12p70 is the same implying that any difference in synthesis must be attributed to posttranslational events leading to an increased affinity of both subunits for each other. An alternative explanation for the results however could be that the amino acid changes would negatively influence the secretion potential of the IL-12p40 subunit. This would imply in our case that the differences in ratios are the consequence of retarded secretion of the SJL/J free IL-12p40 chain. That mutations in the IL-12p40 chain can influence secretion was shown by Ha et al [23]. This possibility was excluded by determining the concentration of p40 from both strains intracellularly and in the supernatant which revealed an excellent correlation between the amount of p40 intracellularly and in the supernatant.

It is conceivable that the mutation which influences the affinity of both subunits for each other could also have consequences for binding to the IL-12 receptor resulting in an impaired signaling of IL-12. If such were the case this should be reflected in the synthesis of
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IFN-γ the main effector molecule of IL-12 signaling. We did not find such differences ruling out an asymmetry between both forms in this aspect.

Subsequently we investigated whether altered glycosylation could have an influence on this altered affinity. We found in accordance with Carra et al [24] that interfering with N-glycosylation did indeed reduce the affinity but that interfering with O-glycosylation had no effect, ruling out a possible involvement of O-glycosylation on heterodimer formation.

Interestingly, the incubation with kifunensin did not completely resolve the preferred heterodimer formation seen with the SJL/J type IL-12p40 (figure 9). This implies that besides the known effect of N-glycosylation, in the SJL/J type an other mechanism is at play, these could very well be altered electrostatic interactions between the both subunits as a consequence of the amino acid changes. The higher MW of the SJL/J derived IL-12 p40 as seen on Western blotting could still be a consequence of O-glycosylation but on the other hand also be a consequence of an altered structure in the SJL/J derived chain which makes the N-glycosilated residues more accessible to secondary glycosylation in the Golgi apparatus. This mechanism is involved in the formation of complex-type N-glycans as opposed to the high-mannose type N-glycans which are thought to be a consequence of inaccessibility of the sugar residues to secondary glycosylation due to protein folding for a review see [25]. This structural difference then has to be a consequence of the amino acid changes in the SJL/J strain and it is very conceivable that these are the same changes that would promote the enhanced heterodimer formation of IL-12p70 seen with the SJL/J’s.

As p40 can not only heterodimerize with p35 to form IL-12p70 but also with p19 resulting in the related pro-inflammatory cytokine IL-23 it is conceivable that the mechanism which facilitates the formation of IL-12p70 could also facilitate the formation of IL-23. Indeed the higher synthesis rate of IL-23 in SJL/J as compared to C57BL/6 without a concomitant higher synthesis of IL-12p40 is indicative that in SJL/J its IL-12p40 has also a higher affinity for p19.

Mucosal homeostasis is a dynamic process depending on the balance between pro- and anti-inflammatory signals. With a genetic susceptible individual, or strain, a defective regulation of immunologic responses could be the cause of a pathogenic response to bacterial, dietary and self-antigens. In the SJL/J the same initiating event would inherently give rise to a higher synthesis rate of pro-inflammatory cytokines, skewing the balance of pro- and anti-inflammatory signals to a more pro-inflammatory tendency than in C57BL/6 animals. Thus
given a big enough challenge it is wholly conceivable that that with SJL/J the balance would tip over to a pro-inflammatory route, whereas with C57BL/6 the process would stay within the bandwidth of homeostasis. In this context it is noteworthy that treatment with bacterial LPS, a strong inducer of IL-12 synthesis, renders genetically resistant C57BL/6 mice as susceptible as SJL/J mice to Theiler’s Virus-Induced Demyelinating Disease [26].

It should be kept in mind that to develop colitis this “defect” on its own is not enough. Genomic screening revealed that besides the locus harboring the gene for IL-12p40, another locus on chromosome 9 is also involved in rendering susceptibility or resistance to TNBS colitis. In a parallel study (results to be published elsewhere) we found strong evidence that this locus harbors a gene involved in epithelial barrier integrity. Thus in the SJL/J mice the enhanced entry of the luminal content as a consequence of impaired mucosal integrity would meet a more pro-inflammatory prone environment and this subsequently would lead to an irreversible shift of the balance to inflammation and ultimately colitis.

In conclusion: we have found ample evidence that due to polymorphisms in the SJL/J derived IL-12p40 chain the IL-12p40 subunit of IL-12 and IL-23 has a higher affinity for its p35 subunit or p19 subunit respectively. This leads to an inherently higher synthesis rate of the mature cytokine which contributes, by distorting the homeostatic balance, to the higher susceptibility found in the SJL/J strain for TNBS colitis.
A polymorphism in the coding region of \textit{il12b} promotes IL-12p70 heterodimer formation in colitis sensitive SJL/J mice.

References


