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A Polymorphism in the Coding Region of Il12b Promotes IL-12p70 and IL-23 Heterodimer Formation

Antonie Zwiers,*‡ IVan J. Fuss,‡ Diana Seegers,* Tanja Konijn,‡ Juan J. Garcia-Vallejo,‡ Janneke N. Samsom,§ Warren Strober,‡ Georg Kraal,‡ and Gerd Bouma*†

IL-12 and IL-23 are heterodimeric cytokines involved in the induction of Th1 and Th17 immune responses. Previous work indicated that a region on chromosome 11 encoding the IL-12p40 subunit regulates strain differences in susceptibility to murine trinitrobenzene sulfonic acid-induced colitis. In addition, this region determines strain differences in LPS-induced IL-12 responses. In this study, we investigated how polymorphisms in the coding region of murine Il12b influence IL-12 and IL-23 heterodimer formation. Transfection studies using constructs containing IL-12p35 linked to IL-12p40 from the colitis-resistant C57BL/6 strain or to the polymorphic p40 variant from the colitis-susceptible SJL/J strain demonstrated that SJL/J-derived p40 constructs synthesized significantly more IL-12p70 than did constructs harboring the C57BL/6-p40 variant. This could not be attributed to differences in synthesis rate or secretion, implicating a greater affinity of SJL/J-derived IL-12p40 for its IL-12p35 subunit. This greater affinity is also associated with increased IL-23 synthesis. In addition, C57BL/6 mice transgenic for the SJL/J p40 variant synthesized significantly more IL-12p70 upon LPS challenge and were more prone to develop colonic inflammation than did C57BL/6 mice transgenic for the C57BL/6-p40 variant. The more efficient binding of the polymorphic Il12b variant to p35 and p19 is most likely due to conformational changes following differential glycosylation as a consequence of the polymorphism. The high synthesis rate of the mature cytokines resulting from this efficient binding can lead to rapid proinflammatory skewing of immune responses and distortion of the homeostatic balance underlying the greater susceptibility for colitis. The Journal of Immunology, 2011, 186: 3572–3580.

C rohn’s disease (CD) and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract, which are the two main entities making up the inflammatory bowel diseases (IBDs). There is general consensus that these diseases have their basis in a disturbed mucosal immune response that, in turn, results from the underlying presence of multiple genetic and environmental factors affecting one or both forms of the disease (1–4).

Recent genome-wide association studies (GWAS) established that ≥30 genetic loci are associated with IBD susceptibility (4, 5). These findings confirm the complex genetic basis of IBD already evident from earlier epidemiological, family, and twin studies. One of the strongest gene associations observed involves the gene encoding IL-23R, part of the heterodimeric membrane receptor for the proinflammatory cytokine IL-23. The latter drives inflammation via its ability to sustain IL-17 production and is composed of a p40 chain, common to IL-23 and IL-12, and a p19 chain unique to IL-23 (6). Of particular interest to the current study, the gene encoding the common p40 subunit, IL12B, was also shown to be genetically linked to IBD susceptibility (7). In addition, a recent pathway analysis of pooled GWAS data confirmed these findings, as well as demonstrated that other genes involved in the IL-12–IL-23 pathway (containing 19 other genes) are involved in CD susceptibility (8).

The genetic information described above nicely complements previous immunological findings demonstrating that the IL-12–IL-23 signaling pathway has a key role in mediating the inflammation present in CD (9). Indeed, mAbs against IL-12 may induce clinical responses and remissions in patients with active CD. This treatment is associated with decreases in Th1-mediated inflammatory cytokines at the site of disease (10). Thus, it is reasonable to postulate that the genetic variation in the genes encoding these cytokines and cytokine receptors acts by causing an exaggeration of the inflammation driven by these factors. However, studies to substantiate this possibility have not been reported.

We previously found that differences in susceptibility to experimental trinitrobenzene sulfonic acid (TNBS)-induced colitis between mouse strains map to at least two regions on the mouse genome: one on chromosome 11 harboring the gene encoding the p40 subunit and another on chromosome 9 (11). In addition, we demonstrated that the colitis-sensitive SJL/J strain exhibits high IL-12p70 responses following i.p. challenge with LPS, whereas colitis-resistant C57BL/6 mice exhibit low responses; importantly, these differences are under the control of a gene that is present in the same region of chromosome 11 that was identified as a susceptibility factor for TNBS colitis (11). Taken together, these findings strongly suggested that SJL/J mice have a genetically determined predisposition to mount high IL-12 responses when challenged with the appropriate bacterial stimulus and that this high IL-12 responsiveness is at the basis of colitis susceptibility. It is unlikely that this had to be attributed to differences in

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transcription, because sequencing of the first kilobase of the promoter region of Il12b (i.e., the region that harbors key binding sites for transcription factors) (12) in both strains of mice failed to reveal distinguishing polymorphisms in any known regulatory region (data not shown).

A possible clue to understanding the differences in responses to LPS between SJL/J and BL/6 mice was that the increased SJL/J IL-12p70 response was not accompanied by increases in the IL-12p40 response. This suggested that sequence variation in the coding region of Il12b of SJL/J mice leads to more efficient heterodimer formation and, thus, larger quantities of IL-12p70, because of changed affinities of the p40 chain for the p35 chain. We explored this possibility with studies of SJL/J IL-12p40 function under in vitro and in vivo conditions.

Materials and Methods

Animals

Specific pathogen-free, 5–6-week-old male SJL/J and C57BL/6 mice were obtained from the National Cancer Institute.

Mice were maintained in the National Institute of Allergy and Infectious Diseases animal housing 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 Medical Center. Experiments involving these mice were approved by the Animal Experiment Committee of the Vrije University Medical Center.

Reagents

LPS from Salmonella enteritidis was obtained from Sigma-Aldrich (St. Louis, MO). Benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside (BGN), kifunensine, and OVA232-339 peptide were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). IL-12p40, IL-12p70, and IL-23 were determined using ELISA kits: the IL-12p40 and IL-12p70 kits were obtained from Pharmingen (PharMingen, Alphen a/d Rijn, The Netherlands), and the IL-23 kit was purchased from eBioscience (ITK diagnostics, Uithoorn, The Netherlands).

IFN-γ, TNF-α, IL-6, and MCP-1 production was measured with the Cytometric Bead Array. The Mouse Th1/Th2 Cytokine Kit was purchased from PharMingen (PharMingen, Alphen a/d Rijn, The Netherlands), and the Mouse Inflammation Kit was used for TNF-α, IL-6, and MCP-1; both were supplied by Pharmingen. Restriction enzymes were supplied by New England Biolabs (Westburg, Leiden, The Netherlands).

Induction of TNBS colitis

For the induction of colitis, mice were lightly anesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). To induce colitis, 3.15 mg TNBS (Sigma Chemical, St. Louis, MO) was mixed and dissolved with an equal amount of 100% ethanol. A total volume of 150 μl 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 s after the injection. The mice were killed 4 d after the induction of colitis.

IL-12, IL-23, and IFN-γ induction

Five male mice from each strain were injected i.p. with a sublethal dose of 300 μg LPS. Six hours after administration, mice were bled, and IL-12p70, IL-12p40, and IL-23 were measured in the serum. Preparation of spleen-cell suspensions for OVA-activated IFN-γ induction by IL-12p70 was performed as described elsewhere (13). In short, spleens from 8–12-week-old DO11.10 mice were strained through 100-μm mesh. The suspension was washed once with phosphate-buffered saline, and 2×10^7 cells/mouse were injected into each mouse. Cells were stimulated with 10 μg/ml OVA232-339 peptide and 2 μg/ml BGN (4 mM, Sigma). After 48 h, supernatants were collected and analyzed for IL-12 and IFN-γ.

Generation of IL-12–transgenic mice

An artificial EcoRV restriction site was generated just upstream of the start codon of the murine Il12b. At the 3′ end, a BamHI site was generated surrounding the stop codon. The products were loaded on a 1% agarose gel and purified with Qiagen column purification (Qiagen, Valencia, CA) expression vector after Sal I-Not I double digestion and electroporation, followed by Qiagen column purification (Qiagen, Valencia, CA) and ethanol precipitation.

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME); they were bred and maintained under approved protocols at the National Institute of Allergy and Infectious Diseases Transgenic/Knockout Mouse Facility (Frederick, MD). One-cell mouse embryos were flushed from female oviducts by standard methods (15). Male pronuclei were microinjected with the isolated DNA construct, and the embryos were implanted into pseudopregnant females.

Retroviral constructs

The retroviral vector pBMN-I-GFP was purchased from the Nolan Lab (http://www.stanford.edu/group/nolan/). 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-deriviled IL-12p40 construct at the Ncol-Sall located distal of the IRES. Constructs were verified by sequencing. In one of our clones, the first base of the IRES start codon (i.e., the adenosine), as well as the last nine bases, including the TG2 stop codon, of the IL-12p55 gene were deleted. This gave rise to a shift in the reading frame of the IL-12p35 gene; consequently, this clone was able to produce IL-12p40 but 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 American Type Culture Collection (http://www.atcc.org), as previously described (14). Viral supernatants were collected and filtered 48–72 h after transfection. Subsequently, BWS147 thymoma cells were infected using a centrifugation-facilitated protocol. Cells were grown in RPMI 1640 (HEPES buffered, 10 mM) containing 10% (v/v) FCS and 1% penicillin/streptomycin. Single-cell clones were generated by limiting dilution. Supernatants were collected by centrifugation, and positive clones were identified by ELISA.

To block glycosylation, cells were incubated for 4 h in the presence of the N-glycosylation inhibitor kifunensine (Kitasatosporia kifusinense, 2 μg/ml; Calbiochem) or the O-glycosylation inhibitor BGN (4 mM, Sigma). Efficiency of inhibition was assessed by flow cytometry using the biotinylated lectins Con A, peanut agglutinin (Vector Laboratories), Helix pomatia agglutinin (HPA), and Galanthus nivalis agglutinin (GNA) (Sigma). Incubation with kifunensine results in an increase in immature N-glycans, which is highly reactive with Con A and GNA, whereas incubation with BGN results in an increase in immature O-glycans, which is highly reactive with HPA and peanut agglutinin.

Generation of IL-12 transgenic mice

An artificial EcoRV restriction site was generated just upstream of the start codon of the murine Il12b. At the 3′ end, a BamHI site was generated surrounding the stop codon. The products were loaded on a 1% agarose gel at 150 V for 2 h, and isolated from the gel with a gel-purification kit (Qiagen). The constructs were subsequently cloned into a pBluescriptII KS(−) expression vector (Stratagene) containing a 2.5-kb stretch of a human Growth Hormone gene (kindly provided by Dr. Frank Scheiffele, Department of Gastroenterology, Infectious Diseases, and Rheumatology, Charité, Berlin, Germany) that was cloned into the BamHI and NotI restriction sites of the vector. Ligation of the vector and insert was performed with a Rapid DNA Ligation Kit (Boehringer Mannheim, Indianapolis, IN). Colonies were tested for the insert with restriction digestion with EcoRV and BamHI and confirmed by sequencing.

A stretch of 1040 bp of the p40 promoter (from position −974 relative to the start site of transcription to position +57, including the first noncoding exon, bp 1–12) (12) was amplified by PCR using primers designed from a C57BL/6 mouse and a SJL/J mouse. Direct sequencing did not reveal genetic variation between the two strains in any regulatory part of the promoter; therefore, the C57BL/6-derived construct was used for both transgenic lines. A SalI restriction site was added to the 5′ region, whereas an EcoRV site was added to the 3′ end and cloned into the pBluescript vector. The 4.5-kb transgene constructs were isolated from the pBluescriptII KS(−) expression vector after Sal I-Not I double digestion and electroporation, followed by Qiagen column purification (Qiagen, Valencia, CA) and ethanol precipitation.

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME); they were bred and maintained under approved protocols at the National Institute of Allergy and Infectious Diseases Transgenic/Knockout Mouse Facility (Frederick, MD). One-cell mouse embryos were flushed from female oviducts by standard methods (15). Male pronuclei were microinjected with the isolated DNA construct, and the embryos were implanted into pseudopregnant females.
Transgenic founders were identified by screening tail DNA by PCR amplification, using the following primers: forward, 5'-CAC ATC TGC TGC TCC ACA-3'; reverse, 5'-TGA TGA TGT CCC TGA A-3'.

Electrophoresis and Western blotting
Nondenaturing SDS-PAGE was performed on 4–12% NuPage Bis-Tris gradient gels with MOPS running buffer in an X cell II mini gel/ blot module (all from Novex; Invitrogen, Breda, The Netherlands), according to the manufacturer’s protocol. After electrophoresis, the separated bands were transferred onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell, Hertogenbosch, The Netherlands) in NuPage Transfer Buffer (Novex; Invitrogen) containing 10% methanol per gel. Unoccupied places on the membrane were blocked with 5% skimmed milk in PBS containing 0.05% Tween 20 (blocking buffer) for 1 h at room temperature. Incubation with the primary Ab, rat anti-mouse IL-12 MAb (clone C17-8; Endogen/Perbio, Etten-Leur, The Netherlands), was performed overnight at room temperature in blocking buffer. Washing with PBS and Tween 20 (0.05%) was followed by incubation with peroxidase-labeled goat anti-rat Ab (Brunschwig; Amsterdam, The Netherlands). Ab binding was detected using a chemiluminescence detection system (ECL; Amersham Biosciences, Rosendaal, The Netherlands), according to the manufacturer’s instructions. Molecular masses were estimated by simultaneous electrophoresis and transfer of fluorescently labelled molecular mass markers (MagicMark Western Standard; Invitrogen).

Glycoprotein-lectin immunosorbent assay
The glycosyl status of both forms of IL-12p40 was determined with the procedure described by Hampel et al. (16). In short, the capturing Ab from the mouse IL-12p40 kit (PharMingen) was coupled to poly styrene microtiter ELISA plates. Blocking of unoccupied sites with 1% BSA in Tris buffer was followed by incubation with equimolar amounts of SJL/J- or C57BL/6-derived p40. The presence of specific glycan moieties was analyzed with a panel of lectins with different specificities (Table I). 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.

Histologic assessment of tissues
Colonic and cecal tissue specimens obtained 4 d after induction of colitis were fixed in 10% buffered formalin phosphate (Sigma-Aldrich, St. Louis, MO). The specimens were embedded in paraffin, cut into sections, and stained with H&E. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 as described previously (17): 0, no evidence of inflammation; 1, low level of infiltration; 2, marked degree of lymphocyte infiltration, with infiltration seen in 10–25% of high-power fields, crypt fields and no structural changes observed; 3, moderate lymphocyte infiltration, with infiltration seen in >25% of high-power fields, large bowel wall thickening, with ulceration; and 4, marked degree of lymphocyte infiltration, with infiltration seen in ≥50% of high-power fields, high vascular density, crypt elongation with distortion, and transmural bowel wall thickening with ulceration.

Table I. Lectins used for identification of oligosaccharide content of mutant and wild-type IL-12p40

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Carbohydrate-Binding Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>TerminalαMan, Manα3[Manα6]Man</td>
</tr>
<tr>
<td>GNA</td>
<td>TerminalαMan</td>
</tr>
<tr>
<td>HPA</td>
<td>αGalNac (terminal)</td>
</tr>
<tr>
<td>M. amurensis agglutinin</td>
<td>Neu5Acα(2,3)Gal</td>
</tr>
<tr>
<td>SNA</td>
<td>Neu5Acα2,6Gal or α2,6GalNac</td>
</tr>
<tr>
<td>R. communis agglutinin</td>
<td>TerminalβGal</td>
</tr>
</tbody>
</table>

Gal, galactose; GαNac, N-acetylgalactosamine; Man, mannosae; Neu5A, sialic acid.

Statistics
The Mann–Whitney U test was used to evaluate whether the medians of the collected data were statistically significantly different from each other. This was performed using GraphPad version 4.0 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com).

Results
Polymorphisms in the coding region of Il12b promote formation of IL12-p70 heterodimers
As alluded to above, we hypothesized that the increased IL-12p70 secretion in SJL/J mice is the consequence of more efficient heterodimer formation resulting from genetic polymorphism in Il12b. In agreement with published data, we found that the genomic p40 sequences of SJL/J and C57BL/6 mice differ at two sites and that these differences give 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 start codon. The Met → Thr substitution is situated opposite from the plane where p40 and p35 interact (amino acid positions 195–203, 261–268, and 315–322).

Next, to determine whether these structural differences in IL-12p40 from the two strains led to differences in their capacities to form heterodimers, we generated bicistronic retroviral vectors harboring the IL-12p40 from each strain in conjunction with their common p35 chain and determined the capacity of these vectors to produce IL-12p70. In constructing these vectors, we took note of the fact that the IL-12p40 chain is produced in excess in IL-12p70–expressing cells (18); therefore, to mimic the physiologic condition as closely as possible, we placed the p40 chain in the higher-producing cap citron and the p35 chain in the lower-producing IRES citron. In the studies performed, murine BWS147 thymoma cells were transduced with the packaged retroviral constructs; cells containing the constructs were selected by limiting dilution and cultured for 4 d, after which the concentrations of secreted IL-12p40 and IL-12p70 were measured and used to determine the ratio of p70/free p40 as a measure of the affinity of the p40 chain for the p35 chain. Ratios, rather than absolute values, of p70 and p40 were used because ratios were independent of interassay variation and copy number of the integrated gene. As shown in Fig. 1A, the ratio of p70/p40 in clones transfected with SJL/J-derived p40 was consistently higher than in clones transfected with C57BL/6-derived p40 (p < 0.001), regardless of the amount of DNA transfected. This indicated that the SJL/J-derived polymorphic variant of p40 more readily forms p70 heterodimers than does the C57BL/6-derived p40 variant. The above-mentioned measurements could also be explained when a difference exists between the two transfected cell lines in the secretion of the IL-12p70 or IL-12p40 product. Because the constructs used differ only in their IL-12p40 region, this difference must be a consequence of the polymorphism. To exclude an effect of differential secretion, the IL-12p70 ratio over free p40 was measured intracellularly and extracellularly. Indeed, as shown in Fig. 1B, the ratios in both the intracellular as well as in the extracellular compartment were significantly higher for the SJL/J derived construct as compared to the C57BL/6 derived construct (p < 0.001).

This showed that the observed differences cannot be ascribed to differential secretion of the IL-12p40 or IL-12p70 molecule but are a consequence of preferential heterodimerization with the SJL/J-derived–type IL-12p40.

Taken together, these in vitro findings provide a molecular basis for our previous in vivo observations demonstrating significantly higher serum IL-12p70 responses in SJL/J mice compared with C57BL/6 mice upon LPS challenge.
The SJL/J-derived IL-12p40 subunit also promotes IL-23 formation

In parallel studies, we investigated whether the polymorphism in the p40 subunit also affects affinity for the p19 subunit and, thus, the amount of IL-23 produced by SJL/J and C57BL/6 cells. Mice of both strains were challenged with LPS and serum IL-12p40, and IL-23 levels were measured by ELISA. As can be seen in Fig. 2, the SJL/J strain responded with a significantly higher IL-23 serum level compared with that of the C57BL/6 strain. In accordance with our previous findings, the IL-12p40 level in the two strains did not differ (SJL/J versus C57BL/6: 73,358 pg/ml ± 11,560 pg/ml and 66,864 pg/ml ± 15,455 pg/ml, respectively; p = NS). This indicated that the SJL/J-derived IL-12p40 subunit also displays a greater affinity for the p19 subunit. Although it cannot be excluded that strain-specific differences in production of the p19 subunit of IL-23 might be responsible for these results, this is less likely because sequencing of the first kilobase of the promoter region, as well as the coding sequence, of SJL/J IL-23A did not reveal sequence variation compared with C57BL/6 IL-23A (data not shown). In addition, the genetic analysis of both strains (11) was not indicative for linkage with the IL23A subunit on chromosome 10.

Biological consequences of the SJL/J polymorphic form of IL-12p40

In addition to an effect on heterodimer formation as shown in the previous experiments, the polymorphic variant might also affect the three-dimensional configuration of the protein and, thereby, its affinity for the IL-12R. 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 seen in Fig. 3, equal amounts of IL-12p70 from either strain resulted in identical induction of IFN-γ release by DO11.10 splenocytes.

In previous studies, it was reported that p40, either as a monomer or as a dimer (p40)2, can act as an inhibitor of IFN-γ synthesis by competing with IL-12p70 for its receptor (19). Thus, in additional studies, we determined whether constructs derived from the two strains expressing different polymorphisms produced p40 with different inhibitory capacities. However, as shown in the Western blot studies depicted in Fig. 4, we were limited to the study of p40 monomers because the constructs did not produce dimers. Therefore, we could only investigate a possible influence of the polymorphisms on the antagonizing effect with the p40 monomer.

FIGURE 1. A, IL-12p70 synthesis relative to IL-12p40 synthesis. IL-12p70 and IL-12p40 concentrations were measured by ELISA in the supernatants of cells transfected with plasmids expressing SJL/J-type IL-12p70 (○) or C57BL/6-type IL-12p70 (■). The results are given as the ratio of IL-12p70 over IL-12p40. Shown are data from individual clones of one representative transfection of three. From each transfection, multiple clones of each construct were used per measurement. B, Intracellular and extracellular IL-12p70/IL-12p40 ratios. IL-12p70 and p40 concentrations were determined with ELISA in supernatants and cell lysates from transfected cells containing plasmids expressing SJL/J-type (○) IL-12p70 and p40 or C57BL/6-type (■) IL-12p70 and p40. The results are given as the ratio of IL-12p70 over free IL-12p40. Bars represent mean ± SD of one representative experiment of two. Each measurement was performed in duplicate.

FIGURE 2. Serologic cytokine IL-23 response to administration of LPS in SJL/J (○) and C57BL/6 (■) mice. Mice were challenged i.p. with a sublethal dose of 300 μg LPS. After 6 h, mice were bled, and IL-23 was measured in the serum by ELISA. Bars represent mean ± SD of a representative experiment of three, each involving five mice per group.

FIGURE 3. Effect of the polymorphic p40 variants on IFN-γ 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 h, IFN-γ responses were measured in the supernatants. Bars represent mean ± SEM; each measurement was performed in quadruplicate. □, Induction by C57BL/6-type IL-12p70 together with C57BL/6-derived IL-12p40; ○, induction by mutant SJL/J-type IL-12p70 together with SJL/J-derived IL-12p40.
Polymorphic variants of IL-12p40 associated with differential glycosylation

As shown in Fig. 4, Western blot analysis revealed that SJL/J-derived p40 has a greater molecular mass compared with C57BL/6-derived p40, suggesting that the SJL/J-derived variant is associated with increased glycosylation. This can be due to enhanced N-glycosylation or, alternatively, it can be the consequence of an additional O-glycosylation event, because the Met to Thr change in the SJL/J-type of IL-12p40 variant is subjected to O-glycosylation.

To investigate these possibilities, we first determined differences in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin-binding assay. To this end, we determined the ability in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin–binding assay. To this end, we determined the ability in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin–binding assay. To this end, we determined the ability in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin–binding assay. To this end, we determined the ability in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin–binding assay. To this end, we determined the ability in glycosylation of IL-12p40 from both strains using a glycoprotein-lectin–binding assay.

Adding increasing amounts of either of the two p40 variants resulted in a similar mild, dose-related inhibition of IFN-γ production in the in vitro assay system described above (Fig. 3). Thus, the polymorphic variation of p40 does not have an effect on the affinity of p70 for its receptor or the ability to induce IFN-γ; in addition, free p40 monomers exhibiting different polymorphisms do not have differential effects on inhibition of the IFN-γ response.

Increased production of IL-12p70 in transgenic C57BL/6 mice harboring the SJL/J-derived IL-12p40 variant

In additional studies, we determined to what extent the above in vitro studies of cells transduced with retroviruses expressing different p40 variants reflected the in vivo expression of IL-12p70 in mice with these variants. Therefore, we generated transgenic C57BL/6 mice that carried transgenes expressing the SJL/J variant or the C57BL/6 variant of the p40 subunit under the IL-12 p40 promoter. From the several founders initially present for each construct, we randomly chose two founders from each transgenic line for our experiments. We challenged these transgenics with LPS i.p. and determined their ability to produce IL-12p70 (as measured in the serum). As shown in Fig. 6, mice bearing a transgene expressing glycoprotein-binding specificities (Table I). As shown in Fig. 5A, the SJL/J-derived–type IL-12p40 showed a significantly higher degree of staining with the Sambucus nigra agglutinin (SNA) lectin than did the C57BL/6-derived IL-12p40. In contrast, the other lectins showed no difference between the two types of IL-12p40 (data not shown). Increased binding of the SJL/J-derived variant to SNA is indicative of the presence of more sialic acid units and, thus, of increased glycosylation, as suggested by the Western blot experiments. Sialic residues can be added to N-linked and O-linked sugars in the Golgi apparatus; therefore, this assay did not discriminate between these types of glycosylation.

We next investigated the consequences of inhibiting the glycosylation on p40 to p35 binding. In this study, we cultured cells transfected with the SJL/J- or C57BL/6-derived constructs in the presence of 4 mM BGN, an inhibitor of O-glycosylation, or with kifunensin, an inhibitor of N-glycosylation; following incubation of the cells for 48 h (at which time inhibition of glycosylation was evident, Supplemental Fig. 2), we determined the p70/p40 ratios of secreted IL-12. As seen in Fig. 5B, culturing in the presence of kifunensin resulted in a clear decline in the p70/p40 ratio in IL-12 secreted from cells expressing the SJL/J-derived constructs, albeit not to the level seen with C57BL/6 constructs. In contrast, culturing in the presence of BGN resulted in only slightly decreased ratios, and this decrease could be totally ascribed to the effect of methanol in which BGN, but not kifunensin, is dissolved. This implies that if O-glycosylation is present, it does not participate in the increased heterodimerization seen with SJL/J-derived constructs.

A. Differences in sialic acid content of both IL-12p40 variants. Equimolar amounts of 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; 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. B. 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 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 kifunensin (an inhibitor of N-glycosylation). Bars represent mean ± SD of one representative experiment of two; each measurement was performed in duplicate. ■, Ratios from C57BL/6-derived constructs; □, ratios from SJL/J-type IL-12p40-derived constructs.

FIGURE 4. Western blot analysis of the two polymorphic variants of IL-12 p40. Supernatants from transfected cell lines expressing only the SJL/J-derived IL-12p40 or the C57BL/6-derived IL-12p40 were run on 4–12% gradient gels under nondenaturing conditions. After transfer to 0.45 μm nitrocellulose, the blotted bands were immunodetected with a specific rat anti-mouse IL-12p40 mAb and were visualized with peroxidase-labeled rabbit anti-rat IgG Abs. A molecular mass standard is displayed in the left column. Individual lanes are shown for esthetic reasons. The original blot is shown as Supplemental Fig. 1.

FIGURE 5. A. Differences in sialic acid content of both IL-12p40 variants. Equimolar amounts of 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; 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. B. 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 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 kifunensin (an inhibitor of N-glycosylation). Bars represent mean ± SD of one representative experiment of two; each measurement was performed in duplicate. ■, Ratios from C57BL/6-derived constructs; □, ratios from SJL/J-type IL-12p40-derived constructs.
the SJL/J variant exhibited a significantly higher level of IL-12p70 synthesis than did mice bearing the C57BL/6 variant, whereas there was no difference in the IL-12p40 synthesis at the translational level (Fig. 6) or at the transcriptional level (Supplemental Fig. 3).

No interlineage difference between founder lines was observed. Because all parameters at the transcriptional and translational levels are exactly the same in these mice, the differences observed are very likely due to enhanced IL-12p70 synthesis resulting from preferential heterodimerization of IL12-p35 with the SJL/J IL12-p40 variant.

Role of the Il12b polymorphism in colitis susceptibility

The above-mentioned experiments clearly indicated that the strain differences in IL-12p70 formation can be related to polymorphisms in the coding region of Il12b. This difference in response is likely to be relevant to the difference in TNBS colitis susceptibility in the two strains, because it was shown that TNBS colitis is driven by IL-12–mediated responses. In a next series of experiments, we sought to further establish this relationship. We first determined serum IL-12p70 responses in colitis-resistant C57BL/6 mice and colitis-susceptible SJL/J mice 4 d after induction of TNBS colitis. As shown in Fig. 7, IL-12 responses were significantly higher in SJL/J mice compared with C57BL/6 mice (p < 0.05). This cannot be accounted for by an initially higher basal level in the SJL/J mice, because there was no difference between the strains at day 0 (results not shown). Again, similar to the previous LPS challenge studies, the IL-12p40 responses were not different between strains (data not shown).

In a final series of experiments, we determined whether C57BL/6 mice, which are normally highly resistant to the induction of TNBS colitis, are rendered susceptible to such induction if they are bearing a transgene expressing the SJL/J-derived p40 variant. To this end, intrarectal TNBS was administered to transgenic C57BL/6 mice carrying the SJL/J-derived p40 variant transgene and to transgenic C57BL/6 mice carrying the C57BL/6-derived variant transgene; in addition, intrarectal TNBS was administered to control SJL/J mice and to nontransgenic C57BL/6 littermates. Three days after the TNBS challenge, mice were sacrificed and evaluated for the presence of colitis. It should be noted that the differences in susceptibility to TNBS colitis are under polygenic control and that the region on chromosome 11 was only one of several genetic risk loci (11). Thus, in the absence of other genetic risk factors derived from the SJL/J strain, it was to be expected that the transgene would have only limited effect on the trait in the C57BL/6 strain. As can be seen in Fig. 8A, 8 of 10 SJL/J mice developed severe weight loss, whereas in all other groups, weights had normalized at day 3. Remarkably, in the transgenic mice bearing the SJL/J variant, histological signs of mild to moderate inflammation were seen in 8 of 10 mice (Fig. 8D); however, this did not reach statistical significance. In contrast, such abnormalities were seen in only a small proportion of mice bearing the transgene expressing the C57BL/6-derived p40 variant, whereas control SJL/J and C57BL/6 mice were positive and negative, respectively, for histological signs of inflammation (Fig. 8B).

Representative histological slides are shown in Fig. 8C (a larger magnification is provided in Supplemental Fig. 4). Thus, transgenic mice carrying the SJL/J-derived p40 variant are more prone to develop an inflammatory reaction in the colonic mucosa upon TNBS challenge, although, as predicted, the reaction was too mild to result in frank clinical signs of colitis.

Discussion

Studies of CD and murine models of this disease provide clear evidence that IL-12p40 plays a crucial and nonredundant role in disease pathogenesis. Human mucosal APCs from CD patients secrete increased amounts of IL-12p70 (20), and treatment of patients with this disease with anti–IL-12p40 results in marked amelioration of disease. In addition, IL-12p40 is overexpressed by dendritic cells in the two common murine models of CD, TNBS colitis (21) and cell-transfer colitis (22); treatment with anti–IL-12p40 is a very effective agent for preventing or treating the inflammation (17).

Although some controversy persists as to whether the main downstream effector cytokine in Crohn’s-like inflammation is IFN-γ or IL-17, the role of IL-12p40 as a component of IL-12p70 or IL-23, the “master” cytokines supporting the production of these downstream cytokines, is unequivocal. More recently, an etiological role for the IL-12/IL-23 axis in CD has been supported by the fact that polymorphisms in the gene encoding IL-12 p40 (IL12B) and the
gene encoding the IL-23 receptor (IL23R) are genetically linked to disease susceptibility; however, the mechanism by which these polymorphisms contribute to disease susceptibility or resistance remains to be determined.

Genetic analysis of experimental colitis revealed that one of the two loci governing susceptibility to TNBS colitis in the susceptible SJL/J strain is located on chromosome 11 and harbors the gene for the IL-12p40 subunit of IL-12 (11). Moreover, it was established that this SJL/J locus is associated with greater IL-12 responses than found in C57BL/6 mice carrying the resistant genotype. Surprisingly, this higher response was not accompanied by a concomitant increase in IL-12p40 synthesis, and no difference was observed in the sequence of the regulatory regions of the genes in the two strains. However, the two strains differed at two sites in the structural part of the gene. Because previous in vitro mutagenesis experiments showed that amino acid changes in human IL-12 could dramatically influence the formation of the mature heterodimer (23), we hypothesized that these structural differences

FIGURE 8. A, Weight loss after induction of colitis by rectal instillation of TNBS at day 0 (mean ± SD relative to day 0, which was set as 100%; each group involved 10 mice). □, C57BL/6 mice carrying the SJL/J-derived p40 variant; ▲, C57BL/6 mice carrying the C57BL/6-derived p40 variant; ■, nontransgenic littermates; ◇, SJL/J wild-type control. B, Histological scoring of inflammation was done on H&E-stained slides. Results are grouped according to their genetic background: SJL/J, nontransgenic SJL/J wild-type mice, SJL Tg, C57BL/6 mice carrying the SJL/J-derived p40 variant; BL6 Tg, C57BL/6 mice carrying the C57BL/6-derived p40 variant; littersmates, nontransgenic littersmates. Within a bar, each section represents the percentage of mice that showed no signs of inflammation (white); mild signs of inflammation (light grey); moderate signs of inflammation (dark grey); or severe signs of inflammation (black). C, Histologic analysis of the colon of transgenic mice, nontransgenic littersmates, and SJL/J wild-type mice after TNBS colitis induction. Representative H&E-stained cross-sections of colon specimens are shown. A, Normal-appearing colon from resistant mice predominantly found in mice carrying the C57BL/6-derived p40 variant and nontransgenic littersmates. Examples of mild (B) to moderate (C) forms of colitis predominantly found in mice carrying the SJL/J-derived p40 variant. D, Severe colitis found in the colitis-susceptible SJL/J wild-type control group. D, Distribution of histological scoring of the transgenic mouse strains. Scoring was done on H&E-stained slides. SJL Tg, C57BL/6 mice carrying the SJL/J-derived p40 variant; BL6 Tg, C57BL/6 mice carrying the C57BL/6-derived p40 variant. 0, no evidence of inflammation; 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation. The average score of each group is indicated by the horizontal line.
also lead to effects on the formation of the heterodimer and, thus, contribute to the mechanism of differences in colitis susceptibility of the SJL/J and C57BL/6 mouse strains.

To test this hypothesis, we generated vectors containing both subunits of IL-12 and found that supernatants of cells containing constructs expressing the SJL/J-derived variant of IL-12p40 synthesized significantly greater amounts of IL-12p70 than did the constructs expressing the C57BL/6-derived variant of IL-12p40. These differences are most likely attributable to posttranslational events, because the transcriptional and translational regimens applying to the two constructs are identical. In addition, the differences cannot be ascribed to preferential changes in secretion, as suggested by studies showing the mutations in the IL-12p40 chain can influence secretion rates of this protein (24), because we determined that cells expressing both constructs exhibited excellent correlation of intra- and extracellular IL-12p70/p40 ratios. Finally, we also determined that the IL-12p70 expressed by cells bearing both transgenes produced IL-12 that had equivalent capacity to induce synthesis of IFN-γ, indicating that although cells exhibited quantitative differences in IL-12p70 production, they did not exhibit qualitative differences in IL-12 function.

Carra et al. (25) found that interference with N-glycosylation but not O-glycosylation results in alterations in binding of individual chains of heterodimers. Indeed, in studies involving specific inhibition of these forms of glycosylation, we confirmed this differential effect with respect to the IL-12p40/IL-12p35 heterodimer formation. However, there are two caveats to this conclusion. First, because the Met-Thr variation would predict an extra O-glycosylation site in the SJL/J strain IL-12p40, one cannot completely rule out the presence of an altered O-glycosylation site in this p40, even if this site does not influence heterodimer formation. Second, we noted that incubation of cells expressing the SJL/J-derived construct with kifunensin, an inhibitor of N-glycosylation, did not completely resolve the increased heterodimer formation seen with this construct. This implies that the inhibition was not complete or that other mechanisms are at play in SJL/J-type IL-12 p40/p35 (or IL-12p40/p19) heterodimer formation (e.g., amino acid-induced alterations in electrostatic interactions between the subunits).

The higher molecular mass of the SJL/J-derived IL-12p40 seen on Western blot may be the consequence of an altered structure in the SJL/J-derived chain that makes the N-glycosylated residues more accessible to secondary glycosylation in the Golgi apparatus. This mechanism applies to the formation of complex-type N-glycans, as opposed to the high-mannose-type N-glycans, because the sugar residues are inaccessible to secondary glycosylation as the result of protein folding (reviewed in Ref. 26). This structural difference is likely to be a consequence of the amino acid differences in the SJL/J strain, which are also responsible for the enhanced heterodimer formation involving SJL/J IL-12p40 and p35 (and possibly p19).

Final proof that the increased levels of IL-12p70 synthesis by SJL/J mice, compared with that of C57BL/6 mice, is due to differences in amino acid composition of the respective IL-12p40 chains came from studies of C57BL/6 transgenic mice bearing transgenes expressing the p40 chains from the two mouse strains. In particular, the mice bearing the transgene expressing the SJL/J variant of IL-12p40 synthesized significantly more IL-12p70 than did the mice bearing a transgene expressing the C57BL/6 variant of IL-12p40. Because the two types of transgenic mice were completely similar at the transcriptional and translational levels, as evidenced, for example, by the identical amount of free IL-12p40 synthesized by both transgenic mice after LPS challenge with the only difference being the amino acid composition, it follows that this difference in amino acid composition is the cause of the differences in the amounts of secreted IL-12p70. It is unlikely that the differences in IL-12p70 secretion observed in the IL-12 transgenic mice were attributable to different copy number insertion of the transgene, because the amount of p40 secreted and the level of mRNA were similar between the transgenic lines. In addition, we generated transgenic lines from different founders with potential different copy number inserts, and the results were similar in these lines (data not shown). Although not directly studied, a similar mechanism may be responsible for the fact that we observed a higher synthesis rate of IL-23 in LPS-stimulated SJL/J mice compared with C57BL/6 mice.

Mucosal homeostasis is a dynamic process that depends on the balance between pro- and anti-inflammatory signals, most of which are under the control of genetic factors. Thus, in an individual or strain bearing a genetic trait resulting in altered regulation of the immunologic response, one can expect to see a change in susceptibility to inflammation. In the current study, we formally verified this principle by showing that a genetic trait controlled by a locus on chromosome 11 and leading to an increased capacity of IL-12p40 to form heterodimers with IL-12p35 or IL-23p19 to form IL-12 or IL-23, respectively, is associated with a greater susceptibility to the induction of TNBS colitis. Thus, in the study relevant to this point, we showed that C57BL/6 mice, normally highly resistant to TNBS colitis but in this case bearing a transgene expressing SJL/J IL-12p40, developed histological signs of colon inflammation upon administration of TNBS, whereas C57BL/6 mice bearing a transgene expressing the C57BL/6 IL-12p40 developed little or no colitis.

It should be kept in mind that C57BL/6 mice bearing the SJL/J transgene IL-12p40 developed colitis that was less severe than that observed in the same study of SJL/J mice. It is reasonable to explain this apparent discrepancy by the fact that the genetic-susceptibility locus in the transgenic mice was only one of at least two susceptibility loci previously identified as important in the induction of TNBS colitis. The other identified locus was on chromosome 9, and it was subsequently shown to affect the expression of the gene encoding claudin-18, a protein involved in epithelial barrier integrity (27). Thus, it seems likely that a full degree of susceptibility to TNBS colitis requires at least two factors: decreased epithelial barrier function and increased proinflammatory effector cytokine synthesis. Ongoing studies with congenic C57BL/6 transgenic mice harboring the chromosome 9 region derived from the SJL/J strain, as well as the IL-12p40 chain, will be necessary to provide a definitive verification of this hypothesis.

There has been ongoing interest in the relationship between polymorphisms in cytokine genes and the expression of these genes to a large number of immune-mediated diseases. For example, this is seen in the case of IL-12, where various polymorphisms have been found that correlate with variations in IL-12 levels (28–30), and a substantial number of human diseases have now been genetically linked to the IL-12 gene, including various infectious diseases and autoimmune diseases, such as psoriasis (7, 28, 29, 31–33). However, most, if not all, studies of polymorphisms relating to cytokine genes relate to polymorphisms in regulatory sequences (i.e., the promoter region or the 3′ untranslated region) (34). Thus, to our knowledge, the current study of IL-12 is the first one to demonstrate that a polymorphism in the coding region of a cytokine gene directly dictates the biological availability of that cytokine and that this, in turn, leads to changes in disease susceptibility. Thus, although earlier, more limited studies did not find an association between IBD and IL12B, recent GWAS found an association between human IBD and IL12B (35). Whether the mechanism identified in this study underlies this association, and, thus, influences human dis-
ease, requires further investigation and should be taken into consideration when interpreting findings from GWAS.

In conclusion, we found ample evidence that polymorphisms in the SLJ/J3-derived IL-12p40 chain lead to an IL-12p40 subunit of IL-12 and IL-23 with a higher affinity for its p35 or p19 subunit counterpart, respectively. This results in an inherently greater synthesis rate of the mature cytokine, which contributes to the greater susceptibility for TNBS colitis found in the SLJ/J strain.

Disclosures
The authors have no financial conflicts of interest.

References


II12B POLYMORPHISMS AND IL-12 FORMATION

The authors have no financial conflicts of interest.