

# Non-Canonical Interleukin 23 Receptor Complex Assembly

## P40 PROTEIN RECRUITS INTERLEUKIN 12 RECEPTOR $\beta$ 1 VIA SITE II AND INDUCES P19/INTERLEUKIN 23 RECEPTOR INTERACTION VIA SITE III\*

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**Background:** The heterodimeric cytokine IL-23 binds to a receptor complex consisting of IL-23R and IL-12R $\beta$ 1.

**Results:** Binding of IL-23 to IL-12R $\beta$ 1 is mediated by domains 1 and 2 of p40.

**Conclusion:** The IL-23·IL-23R·IL-12R $\beta$ 1 complex formation does not follow the classical “site I-II-III” architectural paradigm.

**Significance:** The p40 subunit is shared by IL-23 and IL-12 and interacts directly with IL-12R $\beta$ 1.

IL-23, composed of the cytokine subunit p19 and the soluble  $\alpha$  receptor subunit p40, binds to a receptor complex consisting of the IL-23 receptor (IL-23R) and the IL-12 receptor  $\beta$ 1 (IL-12R $\beta$ 1). Complex formation was hypothesized to follow the “site I-II-III” architectural paradigm, with site I of p19 being required for binding to p40, whereas sites II and III of p19 mediate binding to IL-12R $\beta$ 1 and IL-23R, respectively. Here we show that the binding mode of p19 to p40 and of p19 to IL-23R follow the canonical site I and III paradigm but that interaction of IL-23 to IL-12R $\beta$ 1 is independent of site II in p19. Instead, binding of IL-23 to the cytokine binding module of IL-12R $\beta$ 1 is mediated by domains 1 and 2 of p40 via corresponding site II amino acids of IL-12R $\beta$ 1. Moreover, domains 2 and 3 of p40 were sufficient for complex formation with p19 and to induce binding of p19 to IL-23R. The Fc-tagged fusion protein of p40<sub>D2D3</sub>/p19 did, however, not act as a competitive IL-23 antagonist but, at higher concentrations, induced proliferation via IL-23R but independent of IL-12R $\beta$ 1. On the basis of our experimental validation, we propose a non-canonical topology of the IL-23·IL-23R·IL-12R $\beta$ 1 complex. Furthermore, our data help to explain why p40 is an antagonist of IL-23 and IL-12 signaling and show that site II of p19 is dispensable for IL-23 signaling.

IL-23 is a member of the IL-12 family of proinflammatory cytokines, which includes IL-12, IL-23, IL-27, and IL-35 (1). Structural homology and shared cytokine and receptor subunits also link the IL-12 to the IL-6 cytokine family (2).

IL-23 regulates the development of Th17 cells, which produce a spectrum of cytokines, including IL-17A and IL-17F

(3–5). Th17 cells are involved in the pathogenesis of autoimmune diseases and chronic inflammatory disorders (6), and targeting of IL-23 has become a promising strategy in these disorders (7).

IL-23 is a disulfide-linked composite cytokine comprising p19 and p40 (8). p19 belongs to the gp130 class of long-chain cytokines, and the p40 subunit is similar in the domain structure of typical class I cytokine receptors, such as the non-signaling IL-6 receptor  $\alpha$  (IL-6R) (9). IL-23-induced JAK/STAT signaling is mediated by the  $\beta$  receptor chains IL-12 receptor  $\beta$ 1 and the IL-23 receptor (IL-12R $\beta$ 1/IL-23R) (10). The IL-23R is composed of an N-terminal Ig-like domain (domain 1 (D1)),<sup>3</sup> a cytokine binding module (CBM) consisting of two fibronectin type III domains (domains 2 (D2) and 3 (D3)), and a long stalk region, followed by the transmembrane region and the cytoplasmic region (11). The IL-12R $\beta$ 1 lacks an N-terminal Ig-like domain and starts with the CBM followed by three fibronectin type III-like domains, the transmembrane, and the cytoplasmic region (12). The assembly of the IL-12 and IL-23 receptor signaling complexes has been predicted to be mediated by the “site I-II-III” paradigm found in all other members of this receptor family, such as IL-6/IL-6R/gp130, CNTF·CNTFR·gp130·LIFR and LIF·gp130·LIFR (13). IL-6 alone has no affinity to gp130, but complex formation of IL-6 with IL-6R $\alpha$  via site I induces a conformational shift and results in binding site activation of IL-6 toward gp130 via site II of IL-6 to the CBM (D2 and D3) of the first gp130 receptor and via site III of IL-6 to the D1 Ig-like domain of the second gp130 receptor (14). In analogy, in the IL-23 cytokine-receptor complex, p19 should interact with p40, IL-12R $\beta$ 1, and IL-23R via binding sites I, II, and III. Because the IL-12R $\beta$ 1 lacks an Ig-like domain, site II of p19 should interact with the CBM of IL-12R $\beta$ 1 and site III with the Ig-like domain of IL-23R (1, 13, 15).

Interestingly, p40 interacts directly with IL-12R $\beta$ 1 and acts mainly as an antagonist of IL-12 and IL-23 signaling (16–21). The direct interaction of p40 with IL-12R $\beta$ 1 is unique among the  $\alpha$  receptors of the IL-6/IL-12 family (2), and we suggest that

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<sup>3</sup> The abbreviations used are: D1, domain 1; CBM, cytokine binding module; IP, immunoprecipitation; CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; LIFR, leukemia inhibitory factor receptor; LIF, leukemia inhibitory factor.

## IL-23R Complex Assembly

the IL-23 receptor complex is not assembled via the classical “site I-II-III” paradigm. Unfortunately, only the structure of IL-23, highlighting site I of p19 (13) but not of the IL-23·IL-23R·IL-12R $\beta$ 1 complex is available. To test our hypothesis, we characterized the binding sites of p19 to p40 and of IL-23 to IL-12R $\beta$ 1 and IL-23R using molecular modeling-based site-directed mutagenesis and deletion variants. We identified the “hot spot” amino acid residues of p19<sub>site I</sub> and p19<sub>site III</sub> and showed that p40 has adopted site II from p19 and interacts directly with the CBM of IL-12R $\beta$ 1.

### EXPERIMENTAL PROCEDURES

**Cells and Reagents**—COS-7 (ACC-60) and CHO-K1 (ACC-110) cells were from the Leibniz Institute German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Murine Ba/F3 cell lines have been described previously (22). FLAG polyclonal antibody was from Sigma-Aldrich (St. Louis, MO). Streptavidin-HRP and p40 polyclonal antibody were from R&D Systems (Minneapolis, MN). c-myc monoclonal antibody (9E10) was produced and purified as described in Ref. 22. Peroxidase-conjugated secondary antibodies and human Fc antibody were from Pierce.

**Cloning**—cDNA for murine IL-12p40 (gene ID 16160) was fused to murine IL-23p19 (gene ID 83430) by a linker peptide, resulting in p40-p19-FP (also named Hyper-IL-23, HIL-23) and cloned into the pcDNA3.1 expression vector (Invitrogen) containing an N-terminal FLAG tag and a C-terminal His<sub>6</sub> tag. Standard cloning procedures were used for the generation of pcDNA3.1-FLAG-p40-His and respective deletion variants, pcDNA3.1-FLAG-p19-His, and cDNAs without an N-terminal tag. Mutations within p40, p19, or IL-12R $\beta$ 1 were generated by PCR followed by DpnI digestion of methylated template DNA. cDNAs coding for murine IL-23R (gene ID 209590) and IL-12R $\beta$ 1 (gene ID 16161) have been described previously (22). The extracellular domains of IL-23R (amino acids 1–315) comprising D1, D2, and D3 and IL-12R $\beta$ 1 (amino acids 1–566) containing D1–D5 were C-terminally fused with human Fc tags (soluble (s)IL-23R and sIL-12R $\beta$ 1). Deletion variants of sIL-12R $\beta$ 1 were generated by PCR and splicing by overlapping extension PCR. The resulting cDNAs were subcloned into the eukaryotic expression vector p409 (23). cDNA of the Fc-tagged fusion protein p40<sub>D2D3</sub>-p19Fc was generated by PCR. The sequences of the oligonucleotides used for cloning of expression vectors will be provided upon request. All expression constructs were verified by sequencing and test expression in COS-7 cells (data not shown). Amino acids are numbered according to the database entry starting with the signal peptide.

**Modeling of the IL-23R Complex**—A fold recognition algorithm (ProHit package, ProCeryon Biosciences GmbH, Salzburg, Austria) was used to generate the alignments used in the following model studies. The template with the highest score of the pair potential was used to generate the corresponding alignments. According to these alignments, amino acid residues were exchanged in the template. Insertions and deletions in CYP17A1 were modeled using a database search approach included in the software package WHATIF (24). The complex of human p19 and human p40 (IL-23) was solved by x-ray crystallography (13) (PDB code 3DUH). These coordinates were

used as a template to generate a model of the murine p19 and murine p40 complex. This complex was superimposed onto the IL-6·IL-6R complex within IL-6·IL-6R·gp130<sub>2</sub> (25) (PDB code 1P9M). The coordinates of the Ig-like domain of gp130 were used as a template to build a model of the Ig-like domain of IL-23R and the cytokine-binding region of gp130 (26) (PDB code 1BQU) to build the model of the cytokine-binding region of IL-12R $\beta$ 1. The model of the Ig-like domain of IL-23R and the model of the cytokine-binding region of IL-12R $\beta$ 1 were superimposed on the corresponding domains of IL-6·IL-6R·gp130<sub>2</sub>, thereby generating the full murine p19·p40·IL-12R $\beta$ 1·IL-23R complex.

**Transfection**—Transfections of COS-7 ( $1 \times 10^6$ ) or CHO-K1 ( $2.5 \times 10^5$ ) cells with the indicated plasmids were performed using TurboFect™ transfection reagent (Fisher Scientific GmbH, Schwerte, Germany) according to the instructions of the manufacturer. Transfected CHO-K1 cells were selected in 1.1 mg/ml G-418 sulfate (Genaxxon Biosciences, Ulm, Germany). The cell clone with the highest concentration of p40<sub>D2D3</sub>-p19Fc (p40 ELISA) in the supernatant was selected.

**ELISA**—The concentrations of p40, p40-p19-Fp, and p40<sub>D2D3</sub>-p19Fc in cell culture supernatants were determined by ELISA DY499 (R&D Systems) following the protocol of the manufacturer.

**Cell Viability Assay**—Viability of Ba/F3 cell lines was determined as described in Ref. 22. Cells were cultured for 3 days in a final volume of 100  $\mu$ l in the presence of 10% cell culture supernatant of transiently transfected COS-7 cells secreting the indicated cytokines, the purified p40<sub>D2D3</sub>-p19Fc, or defined concentrations of p40-p19-Fp, or cells were left unstimulated. All variants were measured in triplicates per experiment, and mean  $\pm$  S.D. is indicated. The *n*-fold proliferation was calculated by setting fluorescence values of cells growing without cytokine to a value of 1. All experiments were performed at least three times, and one representative experiment was selected.

**Coimmunoprecipitation (Co-IP)**—For co-IP, transiently transfected COS-7 cells were lysed in IP buffer (20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, and 1 complete protease inhibitor mixture tablet/50 ml buffer (Roche Diagnostics) supplemented with 1% Triton X-100 for 1.5 h at 4 °C. For co-IP, cytokine-containing lysates were mixed with those containing the soluble Fc-tagged receptors. As a negative control, the cytokine variant was incubated without receptors. For IP of C-terminal c-myc-tagged p19 or p40 with the indicated cytokine variants, cell lysates of cotransfected COS-7 cells were used and incubated with 5  $\mu$ g of the c-myc antibody 9E10. As a negative control, the lysate was incubated without antibody. Co-IP of purified p40<sub>D2D3</sub>-p19Fc was performed using 5  $\mu$ g of protein and cell lysates containing the C-terminal c-myc-tagged IL-23R. As a negative control, the receptor was incubated without purified protein. All samples were incubated overnight at 4 °C under gentle agitation. 50  $\mu$ l of protein A-agarose (Roche Diagnostics) was added and incubated at 4 °C for 4 h under gentle agitation. The samples were washed five times with IP buffer, and proteins were eluted by adding 50  $\mu$ l of 5 $\times$  Laemmli buffer, followed by incubation for

10 min at 95 °C. The resulting supernatants were subjected to Western blot analysis.

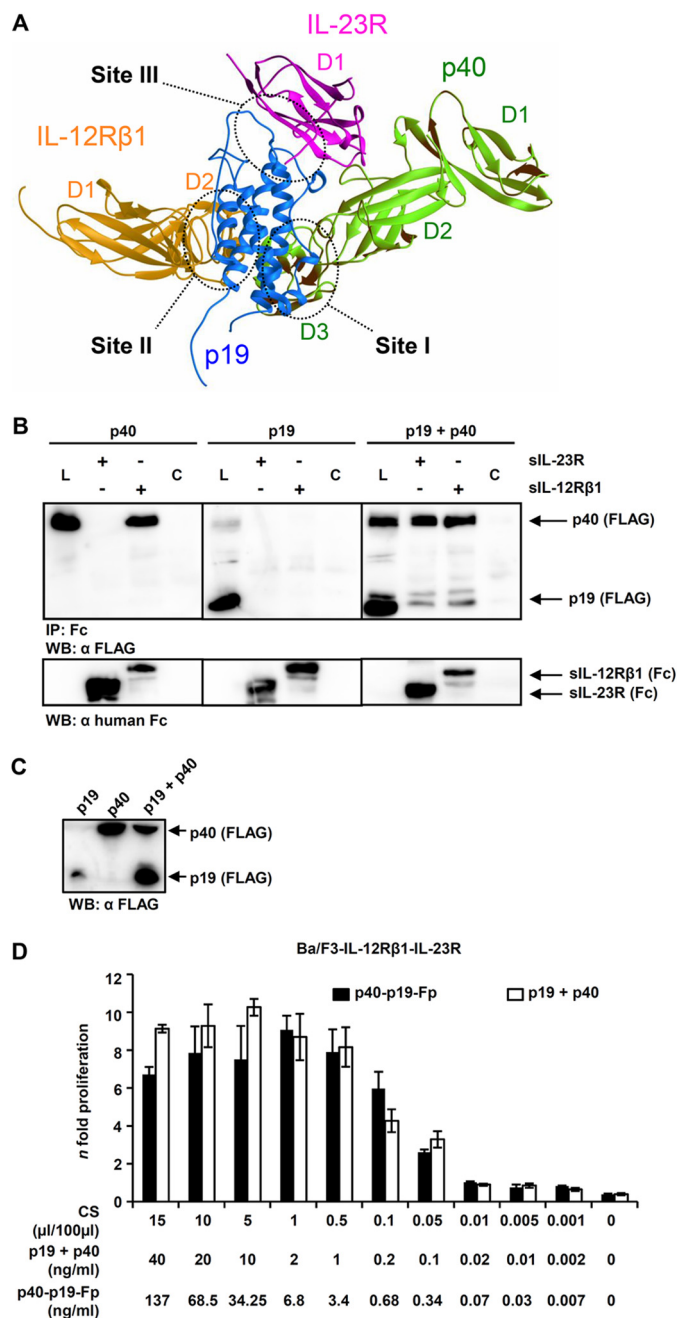
**Western Blotting**—Equal amounts of proteins (cell culture supernatants, 50  $\mu$ l/lane; cell lysates, 15  $\mu$ l/lane; coimmunoprecipitates, 35  $\mu$ l/lane) were loaded and separated by SDS-PAGE, and Western blotting was performed as described previously (22).

**Purification of Recombinant p40<sub>D2D3</sub>-p19Fc**—CHO-K1 cell culture supernatant containing p40<sub>D2D3</sub>-p19Fc was subjected to affinity chromatography (protein A-agarose (Roche Applied Science) and eluted by 5 column volumes of 50 mM citric acid (pH 4). p40<sub>D2D3</sub>-p19Fc was concentrated, and buffer was exchanged for 50 mM Tris, 150 mM NaCl (pH 8.0) using Amicon® Ultra-15 centrifugal filters (Merck Millipore). p40<sub>D2D3</sub>-p19Fc concentration was determined by absorption at 280 nm.

## RESULTS

**p19 Fails to Interact with IL-12R $\beta$ 1 and IL-23 in the Absence of p40**—The structure of IL-12R $\beta$ 1 and IL-23R allow the formation of a quaternary signaling complex composed of p19, p40, IL-23R and IL-12R $\beta$ 1 in a 1:1:1:1 stoichiometry. Interaction of p19 with p40 is supposed to be mediated by site I, of p19 with CBM of IL-12R $\beta$ 1 by site II, and of p19 with the Ig-like domain of IL-23R by site III (13) (Fig. 1A). In detail, if p19<sub>site II</sub> is located in an orientation similar as the IL-6-CBM-gp130 complex, then binding of the A and B helices of p19 (site II) interact with IL-12R $\beta$ 1 (13). The loop connecting the C-D helices of p19 represents the site III interface with IL-23R because it contains the characteristic hot spot site III tryptophan 157 (human, Trp-156; murine, Trp-157) conserved among all gp130 cytokines, including Trp-157 in human IL-6 or Trp-144 in viral IL-6 (27).

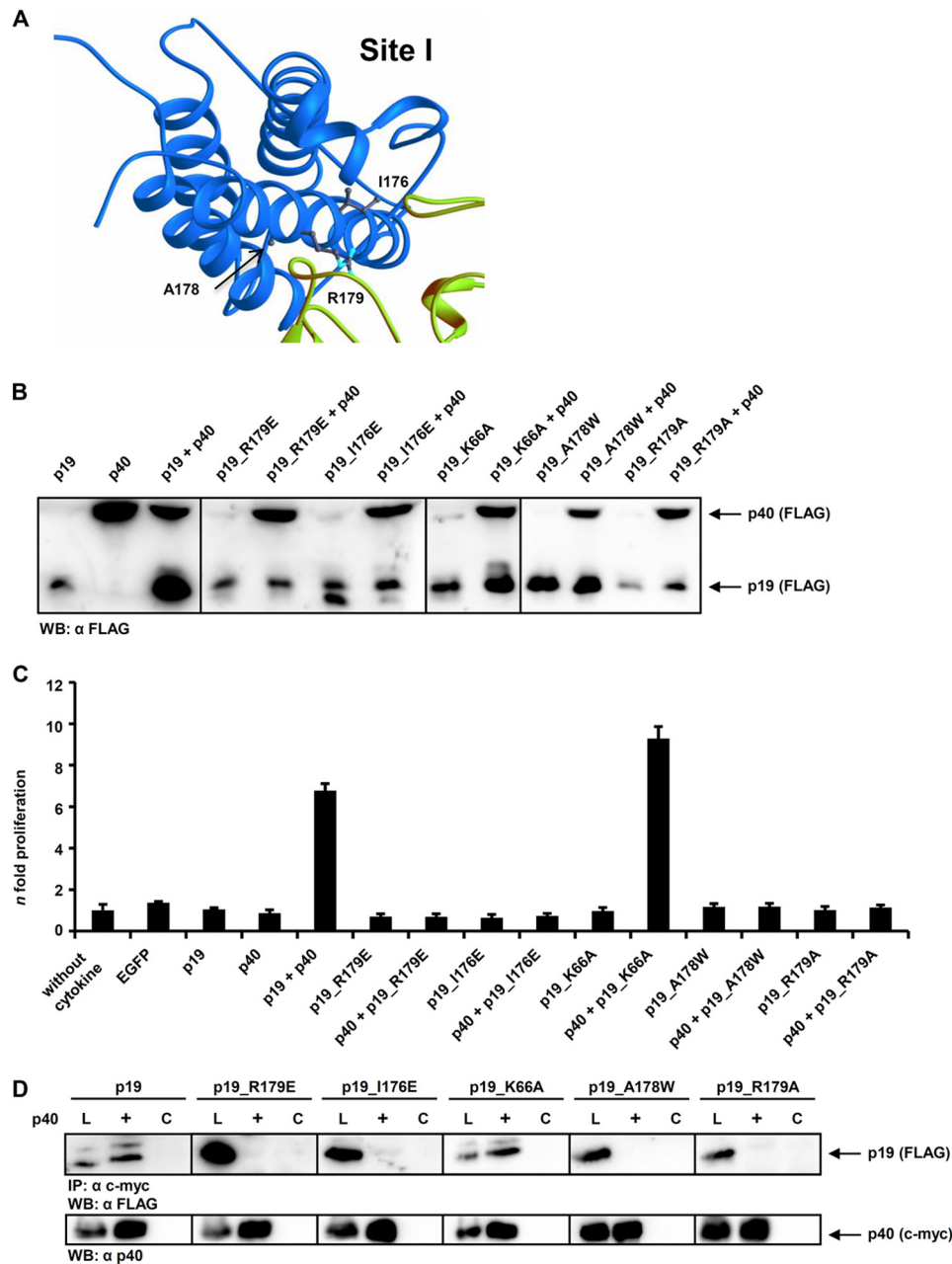
Initially, we verified that p40 binds to IL-12R $\beta$ 1 but not to IL-23R and showed that binding of p19 to IL-12R $\beta$ 1 or IL-23R is dependent on p40 (Fig. 1B). Fc-tagged soluble IL-12R $\beta$ 1 or IL-23R were precipitated with protein A-agarose, and coprecipitated FLAG-tagged p40 and p19 were detected by Western blotting using FLAG mAbs. As a control, precipitated soluble IL-12R $\beta$ 1 or IL-23R was detected using Fc-Abs. p19 was detected as a major lower and a minor higher protein, which likely reflects different glycosylation forms. However, degradation/processing cannot be excluded. To the best of our knowledge, it is not known whether IL-12p35 needs p40 for interaction with IL-12R $\beta$ 1 and IL-12R $\beta$ 2, but binding of the other cytokine members of the IL-6/IL-12 type cytokine family to their  $\beta$  receptors depends on the preceding complex formation with the  $\alpha$  receptor subunit (28–30). Although, a recent report showed that bacterially expressed and refolded p19 is able to bind to IL-23R in the absence of p40 (31), this study lacks the general demonstration of the biological activity of bacterially produced p19 in complex with p40 and IL-23R/IL-12R $\beta$ 1. As observed previously by Oppmann *et al.* (8), secretion of p19 was promoted by p40 coexpression (Fig. 1C). Moreover, p40 enhanced the stability, intracellular trafficking, and export of p35 (32). Here the biological activity of secreted IL-23 (p19/p40) from COS-7 cells was verified using Ba/F3 cells stably



**FIGURE 1. Binding of p19 to IL-12R $\beta$ 1 and IL-23R is dependent on p40.** A, IL-23<sub>p40</sub> (green, domains D1–D3) and IL-23<sub>p19</sub> (blue) bind to IL-12R $\beta$ 1 (orange, domains D1 and D2) and IL-23R (pink, domain D1). B, coimmunoprecipitation of FLAG-tagged p40 or p19 and Fc-tagged sIL-12R $\beta$ 1 or sIL-23R using protein A-agarose. One of three independent experiments is shown. L, lysates; +, coimmunoprecipitates; C, controls (without sIL-12R $\beta$ 1 or sIL-23R); WB, Western blot. C, Western blotting of secreted p40 and/or p19 from transfected COS-7 cells. D, cell viability assay of Ba/F3-IL-12R $\beta$ 1-IL-23R cells stimulated with increasing amounts of the indicated cytokine-conditioned supernatants (CS). Data are mean  $\pm$  S.D. The concentration of p19/p40 and p40-p19-Fp was 200 ng/ml and 685 ng/ml, respectively, as determined by p40-ELISA.

transduced with murine IL-12R $\beta$ 1 and murine IL-23R (Fig. 1D). Proliferation of parental Ba/F3 cells depends on IL-3 and activation of STAT5. After stable transduction with cDNAs coding for murine IL-12R $\beta$ 1 and murine IL-23R, Ba/F3-IL-12R $\beta$ 1-IL-23R cells proliferated in the presence of p19/p40 and p40-p19-Fp, which is a fusion protein of p40 and p19 (Fig. 1D)

## IL-23R Complex Assembly

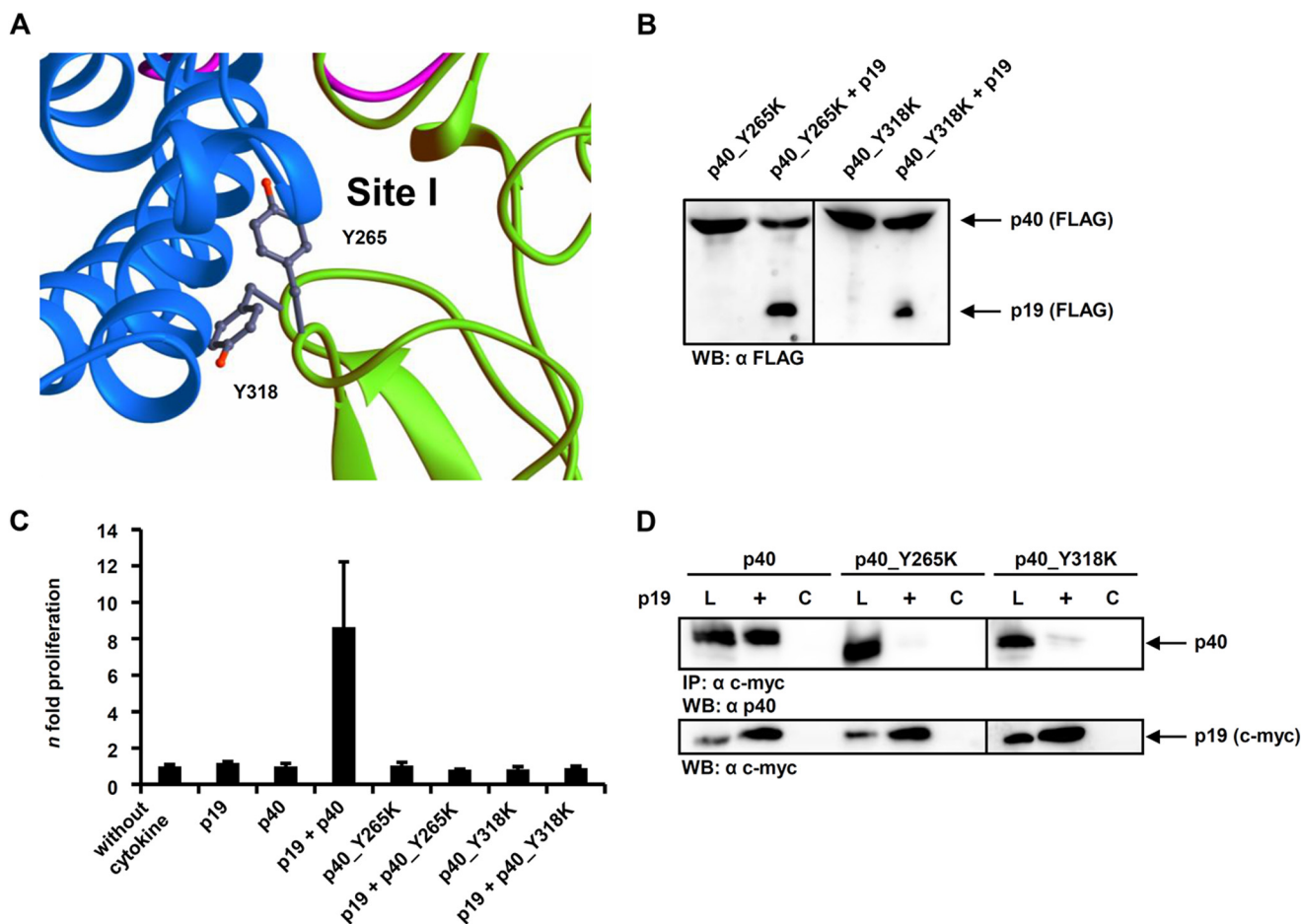


**FIGURE 2. Characterization of site I in p19.** *A*, model of site I of p19 (blue) toward p40 (green). *B*, Western blotting (WB) of secreted p40 and/or p19\_R179E, K66A, I176E, A178W, and R179A from transfected COS-7 cells. *C*, cell viability assay of Ba/F3-IL-12R $\beta$ 1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean  $\pm$  S.D. *D*, co-IP of c-myc-tagged p40 and FLAG-tagged p19 or p19\_R179E, K66A, I176E, A178W, and R179A. L, lysates; +, coimmunoprecipitates; C, controls (without mAbs).

(22). Maximal cell growth was achieved with 0.5  $\mu$ l of p19/p40 and p40-p19-Fp-conditioned supernatants, which corresponds to 1 and 3.4 ng of cytokine/ml, respectively, as determined by ELISA (Fig. 1D). All further biological activity assays of p19/p40, p40-p19-Fp, and variants thereof were performed with a 20-fold excess (10  $\mu$ l of conditioned supernatant, corresponding to 20–68 ng/ml) over the minimal dose required to ensure maximal proliferation of Ba/F3-IL-12R $\beta$ 1-IL-23R cells.

**Targeted Site I Mutations in p19 or p40 Prevent the Formation of IL-23**—The crystal structure of IL-23 highlighted the binding interface of p19 with p40 (13). Arg-178 in human p19 and Arg-179 in murine p19 have been suggested to be critical hot spot amino acid residues in site I of p19 (Fig. 2A). Moreover,

a graphical inspection of the complex suggested additional hot spot mutations: K66A, I176E, and A178W. COS-7 cells were cotransfected with p40 and p19\_R179E and R179A, p19\_K66A, and I176E and A178W. With the exception of p19\_K66A, secretion of p19\_R179E/A and p19\_I176E and A178W was not promoted by p40 coexpression (Fig. 2B), and the remaining secreted p19\_R179E/A, p19\_I176E and A178W plus p40, failed to induce IL-23-dependent proliferation of Ba/F3 cells stably transduced with murine IL-12R $\beta$ 1 and murine IL-23R (Fig. 2C). Co-IP of wild-type p19 and p19\_K66A, but not of the p19\_R179E/A, p19\_I176E, and A178W mutants with p40, suggested that the formation of biological active IL-23 was abolished by these site I mutations (Fig. 2D). Our data indicated that Arg-



**FIGURE 3. Targeted site I mutations in p40 prevent the formation of IL-23.** *A*, model of p40 (green) toward site I of p19 (blue). *B*, Western blotting (WB) of secreted p19, p40\_Y265K, and p40\_Y318K. One of three independent experiments is shown. *C*, cell viability assay of Ba/F3-IL-12R $\beta$ 1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean  $\pm$  S.D. *D*, co-IP of c-myc-tagged p19 and FLAG-tagged p40 or the p40 mutants Y265K and Y318K. L, lysates; +, coimmunoprecipitates; C, controls (without mAbs). One of three independent experiments is shown.

179, Ile-176, and Ala-178 are the critical hot spot amino acid residues of site I in p19.

Next, we introduced the mutations Y265K and Y318K into domain D3 of p40 to interrupt binding to p19 (Fig. 3A). Both p40 mutants were secreted from transiently transfected COS-7 cells irrespective of p19 coexpression (Fig. 3B). In the presence of p19, p40\_Y265K and p40\_Y318K failed to induce proliferation of IL-23-responsive Ba/F3 cells, suggesting that the formation of biologically active IL-23 was abrogated (Fig. 3C). Moreover, p40\_Y265K and p40\_Y318K were not precipitated with p19 (Fig. 3D). These data demonstrated that the residues Tyr-265 and Tyr-318 in p40 are essential for p19-p40 complex formation.

For human IL-6R and EBI3, only two domains are sufficient for binding to IL-6 or p28 and to induce biological activity of IL-6 and IL-27\_p28. Importantly, the responsible domains 2 and 3 of the IL-6R and domains 1 and 2 of EBI3 are structurally homologous to D2 and D3 of p40 (33–35). We expected that domains 2 and 3 of p40 might be sufficient for binding of p19 and biological activity of IL-23. Therefore, we generated cDNAs coding for p40 deletion variants consisting of D1, D2, D3, D1D2, or D2D3. All p40 deletion variants were secreted into the supernatant of transiently transfected COS-7 cells (Fig.

4A). When compared with cosecretion of p19 with full-length p40, cosecretion of p19 with the p40 deletion variants was reduced but clearly detectable (Fig. 4A). Importantly, none of the p19/p40 deletion variant combinations facilitated IL-23-induced proliferation of Ba/F3-IL-12R $\beta$ 1-IL-23R cells, suggesting that all domains of p40 are needed for secretion and biological activity of p19 (Fig. 4B). Co-IPs revealed that p40\_D2, p40\_D1D2, and p40\_D2D3, but not p40\_D1 or p40\_D3 alone, interact with p19, demonstrating that D2 of p40 was sufficient for interaction with p19 (Fig. 4C). This is an apparent paradoxical situation because p40\_Y265K and p40\_Y318K also did not heterodimerize with p19, showing that these hot spot amino acid residues within domain 3 also critically contribute to the interaction of p19 and p40 (Fig. 3D). We assume that the exchange of the hydrophilic but uncharged tyrosine residues into positively charged lysine residues in p40\_Y265K and p40\_Y318K resulted in repulsion of p19 and p40, thereby preventing heterodimerization of p19 and full-length p40. Yoon *et al.* (9) described that binding of p40 and p35 leads to rotation of the p40D3 domain with respect to D2. A rotation of p40D3 might also occur upon binding of p19 and, therefore, enable the interaction of Tyr-265 or Tyr-318 with p19.

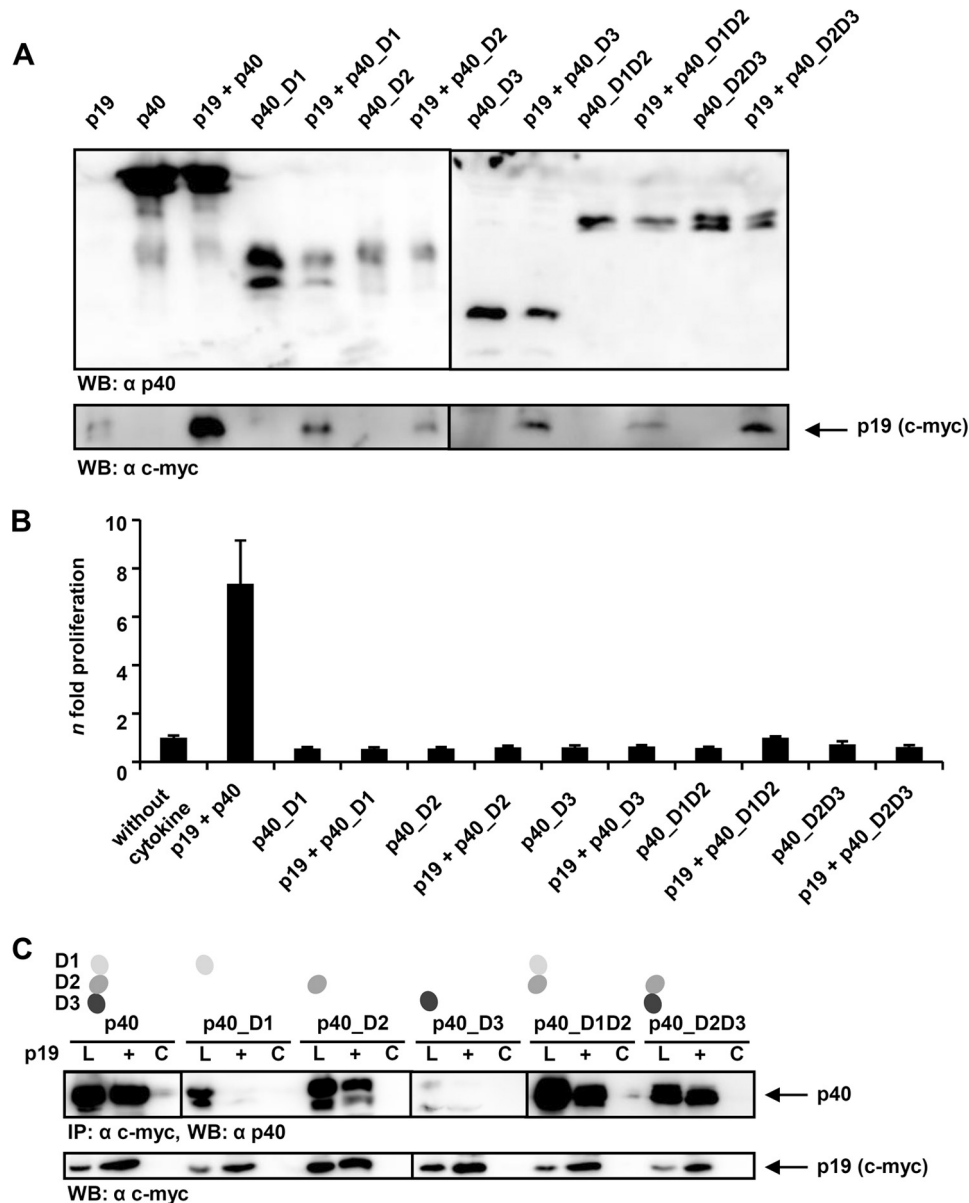


FIGURE 4. **Binding of deletion variants of p40 to p19.** *A*, Western blotting (WB) of secreted p19 and p40, p40\_D1, p40\_D2, p40\_D3, p40\_D1D2, and p40\_D2D3. One of three independent experiments is shown. *B*, cell viability assay of Ba/F3-IL-12R $\beta$ 1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean  $\pm$  S.D. *C*, co-IP of FLAG-tagged p40 variants and c-myc-tagged p19. *L*, lysates; +, coimmunoprecipitates; C, controls (without mAbs). One of three independent experiments is shown.

The p19<sub>site III</sub> W157A Hot Spot Mutation Disrupted the Interaction of IL-23 with IL-23R—Human p19 contains a conserved hot spot site III tryptophan at position 156 (13) (murine p19, position 157). Accordingly, we introduced a single point mutation into p19, resulting in the amino acid exchange Trp-157 to Ala-157 (Fig. 5A). p19 and p19\_W157A were efficiently secreted after coexpression with p40, which was also true for the fusion proteins p40-p19-Fp, in which p40 is linked to p19 by a short peptide chain (8), and p40-p19\_W157A-Fp (Fig. 5B). However, only wild-type p19/p40 and p19-p40-Fp, but not p19\_W157A/p40, induced proliferation of Ba/F3-IL-12R $\beta$ 1-IL-23R cells (Fig. 5C). Co-IPs showed that the heterodimeric p19\_W157A/p40 and the p40-p19\_W157A-Fp interact with IL-12R $\beta$ 1 but not with IL-23R, demonstrating that the interaction of IL-23 and IL-23R is mediated via a classical site III (Fig.

5, D and E). As a control, the interaction of the p19-p40-Fp and the single components p19 and p40 with IL-12R $\beta$ 1 and IL-23R was shown by co-IP (Fig. 5, D and E).

Amino Acid Exchanges in the Predicted Site II of p19 Did Not Affect Binding of IL-23 to IL-12R $\beta$ 1—Using molecular modeling and homology-based approaches, we deduced Leu-36, Arg-38, and Leu-135 as hot spot amino acid residues within the putative site II of p19 (see “Experimental Procedures”). We expected that mutation of these amino acid residues into negatively charged glutamic acid abrogate the interaction of p19 with IL-12R $\beta$ 1 (Fig. 6A). COS-7 cells were cotransfected with cDNAs coding for p40 and p19, p19\_L36E, p19\_R38E, or p19\_L135E. Western blotting revealed that all p19 mutants were secreted into the cell culture supernatant (Fig. 6B). Although production of p19\_L36E was reduced markedly com-

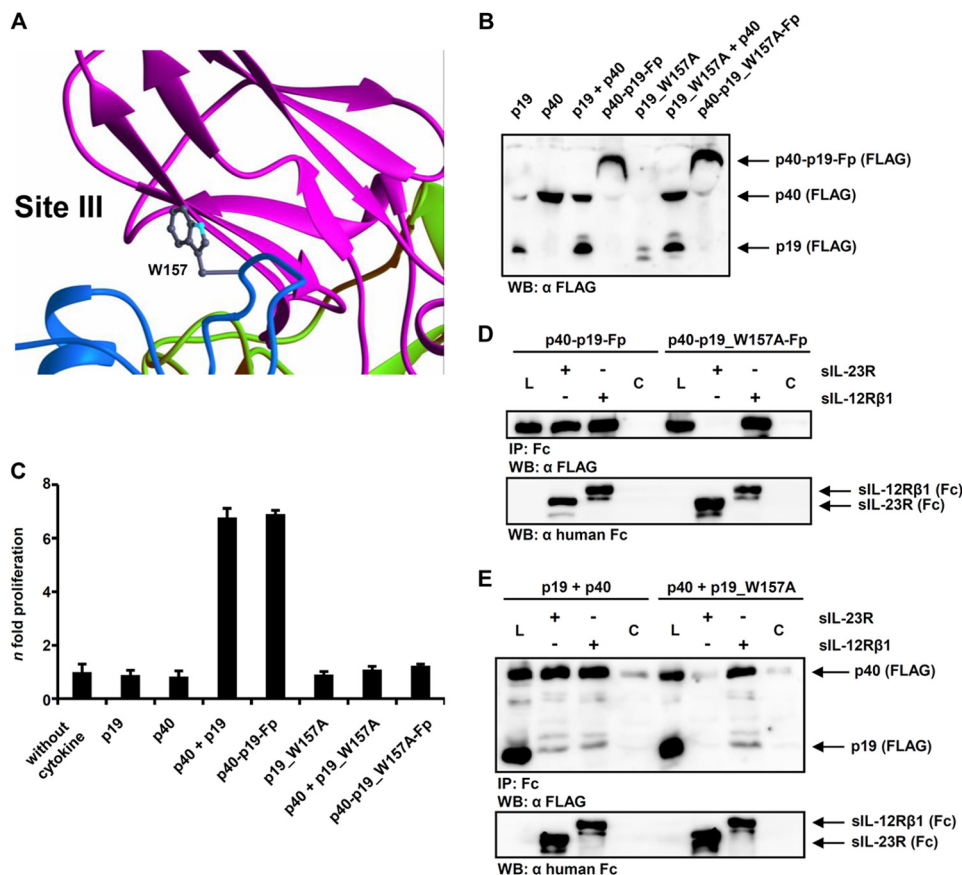


FIGURE 5. **Characterization of p19 site III.** *A*, model of the predicted site III of p19 (blue) toward IL-23R (pink). *B*, Western blotting (WB) of secreted p40 and p19 or p19\_W157A, p40, p40-p19-Fp, or p40-p19\_W157A-Fp. One of three independent experiments is shown. *C*, cell viability assay of Ba/F3-IL-12Rβ1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean  $\pm$  S.D. *D*, co-IP of FLAG-tagged p40 and p19 or p19\_W157A and Fc-tagged sIL-12Rβ1 or sIL-23R. One of three independent experiments is shown. *E*, co-IP of FLAG-tagged p40-p19\_wt and \_W157A fusion proteins and Fc-tagged sIL-12Rβ1 or sIL-23R. L, lysates; +, coimmunoprecipitates; C, controls (without sIL-12Rβ1-Fc or sIL-23R-Fc). One of three independent experiments is shown.

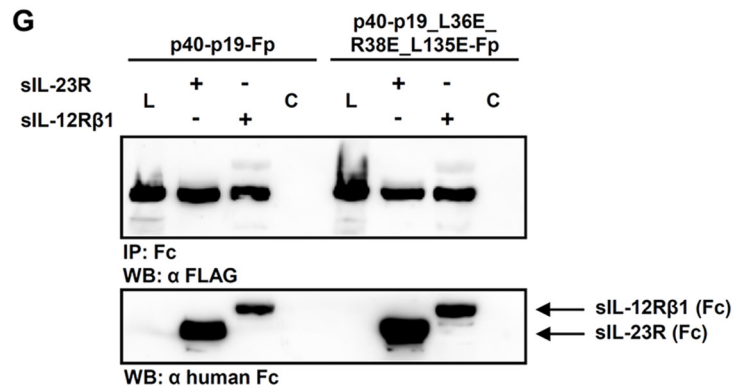
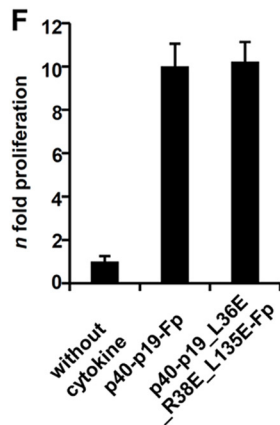
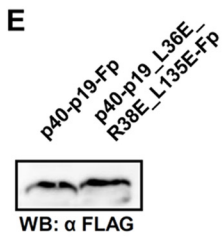
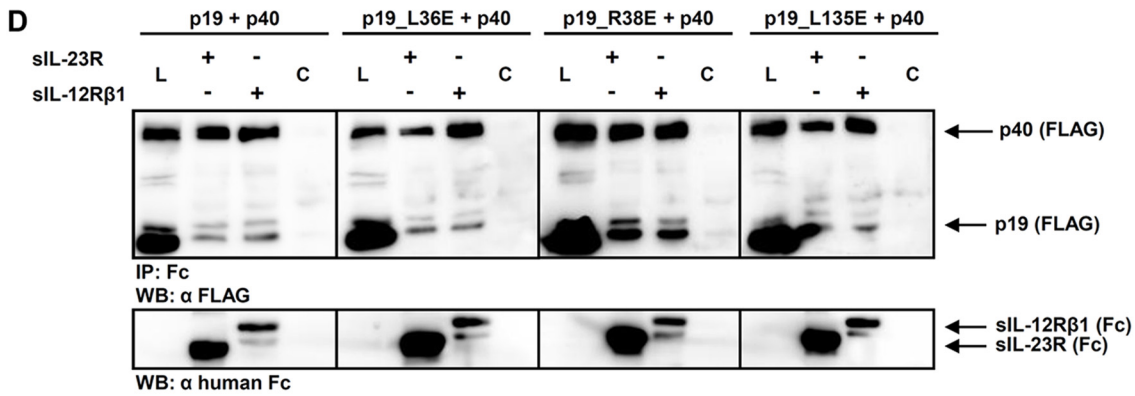
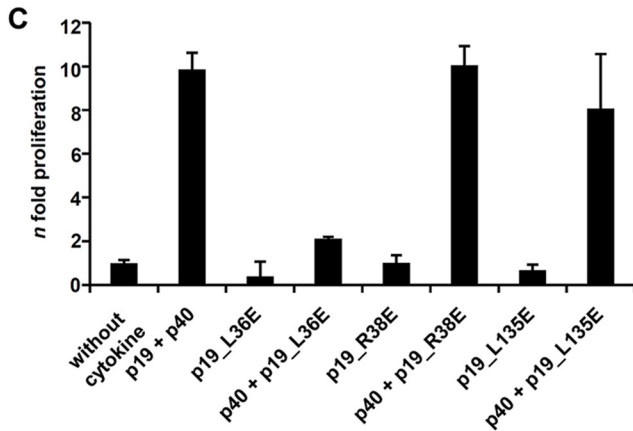
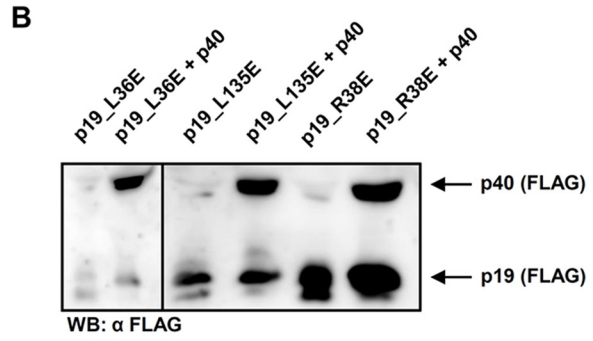
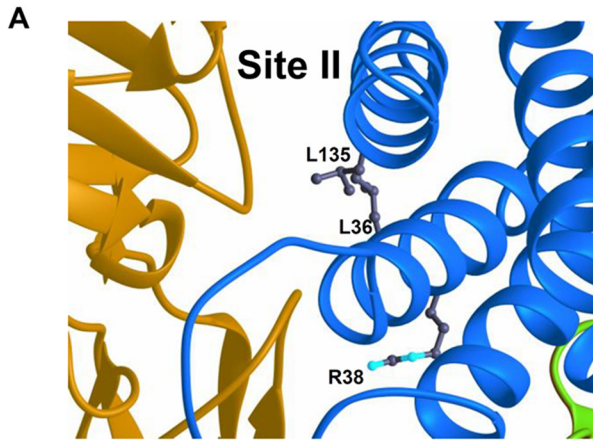
pared with p19\_L135E and p19\_R38E, conditioned supernatants containing the p19 mutants and p40 induced proliferation of Ba/F3-IL-12Rβ1-IL-23R cells (Fig. 6C), suggesting that formation of the biological active IL-23 receptor complex was not affected by these putative site II mutations, which was confirmed by co-IP of the predicted p19\_site II mutants and p40 with IL-12Rβ1 and IL-23R (Fig. 6D). Next, we combined the three single mutants in a p19\_L36E\_R38E\_L135E triple mutant fused to p40 (p40-p19\_L36E\_R38E\_L135E-Fp), which was secreted from transiently transfected COS-7 cells (Fig. 6E). As observed for p40-p19-Fp, p40-p19\_L36E\_R38E\_L135E-Fp also induced proliferation of Ba/F3-IL-12Rβ1-IL-23R cells (Fig. 6F) and was precipitated by soluble IL-12Rβ1 and IL-23R (Fig. 6G). These data demonstrated that mutations within the predicted site II did not abolish the binding of p19/p40 to IL-12Rβ1, and, therefore, we questioned the existence of a classical site II in p19.

**Interaction of p40 with IL-12Rβ1 Is Mandatory for IL-23 Receptor Complex Formation**—The antagonistic properties of p40 rely on the direct interaction with IL-12Rβ1 (18, 21). As described previously (21) and as depicted in Fig. 1B, p40 binds to IL-12Rβ1, but not to IL-23R, in the absence of p19. Because we were not able to identify a canonical site II in p19, we hypothesized that the interaction of IL-23 with IL-12Rβ1 mainly depends on p40 rather than on p19.

The aforementioned p40 deletion variants were used to analyze the binding of p40 to IL-12Rβ1. Only the p40 variant D1D2 was able to bind to IL-12Rβ1, although to a much lesser extent compared with wild-type p40, whereas all other deletion variants lost the ability to interact with IL-12Rβ1 (Fig. 7A). These results demonstrate that p40\_D1D2 is sufficient for the interaction with IL-12Rβ1.

Next, we analyzed which domain of IL-12Rβ1 is needed for the interaction with p40. As depicted in Fig. 7B, IL-12Rβ1\_D1D2 (CBM) but not D1 or D2 alone or D3-D5, was able to precipitate p40, suggesting that the functional arrangement of both domains of the CBM of IL-12Rβ1 is critical for p40 binding. According to our model of the p40-IL-12Rβ1 complex, the amino acid residues Val-116, Leu-117, and Ser-118 (D1) and Tyr-143 (D2) of IL-12Rβ1 should be in close contact with p19 and, therefore, were chosen as candidates for site-directed mutagenesis (Fig. 7C). However, our data indicate that p19 did not interact with IL-12Rβ1 via site II, and, therefore, we tested whether these predicted binding interface amino acids in IL-12Rβ1 might be responsible for site II interaction with p40. We introduced point mutations in IL-12Rβ1, resulting in the amino acid exchanges Y143R and V116E/L117E/S118E and in the combination Y143R/V116E/L117E/S118E. The IL-12Rβ1 V116E, L117E, and S118E triple mutant and the IL-12Rβ1 V116E, L117E, S118E, Y143E quadruple mutant completely

# IL-23R Complex Assembly





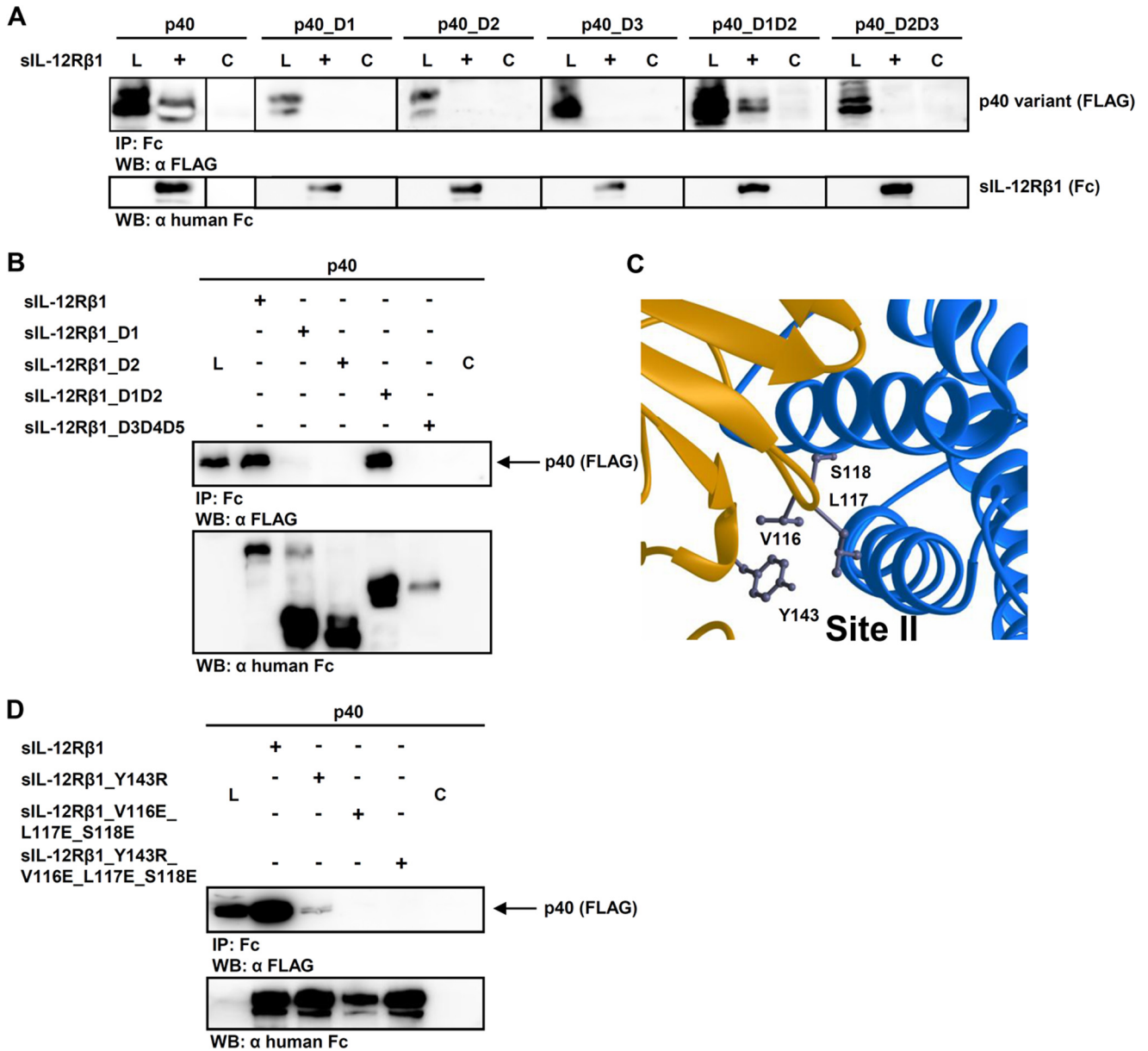


FIGURE 7. **p40 substitutes for the site II interaction of p19 toward IL-12Rβ1.** *A*, co-IP of FLAG-tagged p40 variants and Fc-tagged sIL-12Rβ1. One of three independent experiments is shown. *WB*, Western blot. *B*, co-IP of FLAG-tagged p40 and Fc-tagged sIL-12Rβ1 deletion variants. One of three independent experiments is shown. *C*, model of the predicted site II of p19 (blue) toward IL-12Rβ1 (orange). *D*, co-IP of FLAG-tagged p40 and Fc-tagged sIL-12Rβ1 point mutation variants. One of three independent experiments is shown. *L*, p40 lysate; +, coimmunoprecipitates; *C*, control (without sIL-12Rβ1 variant).

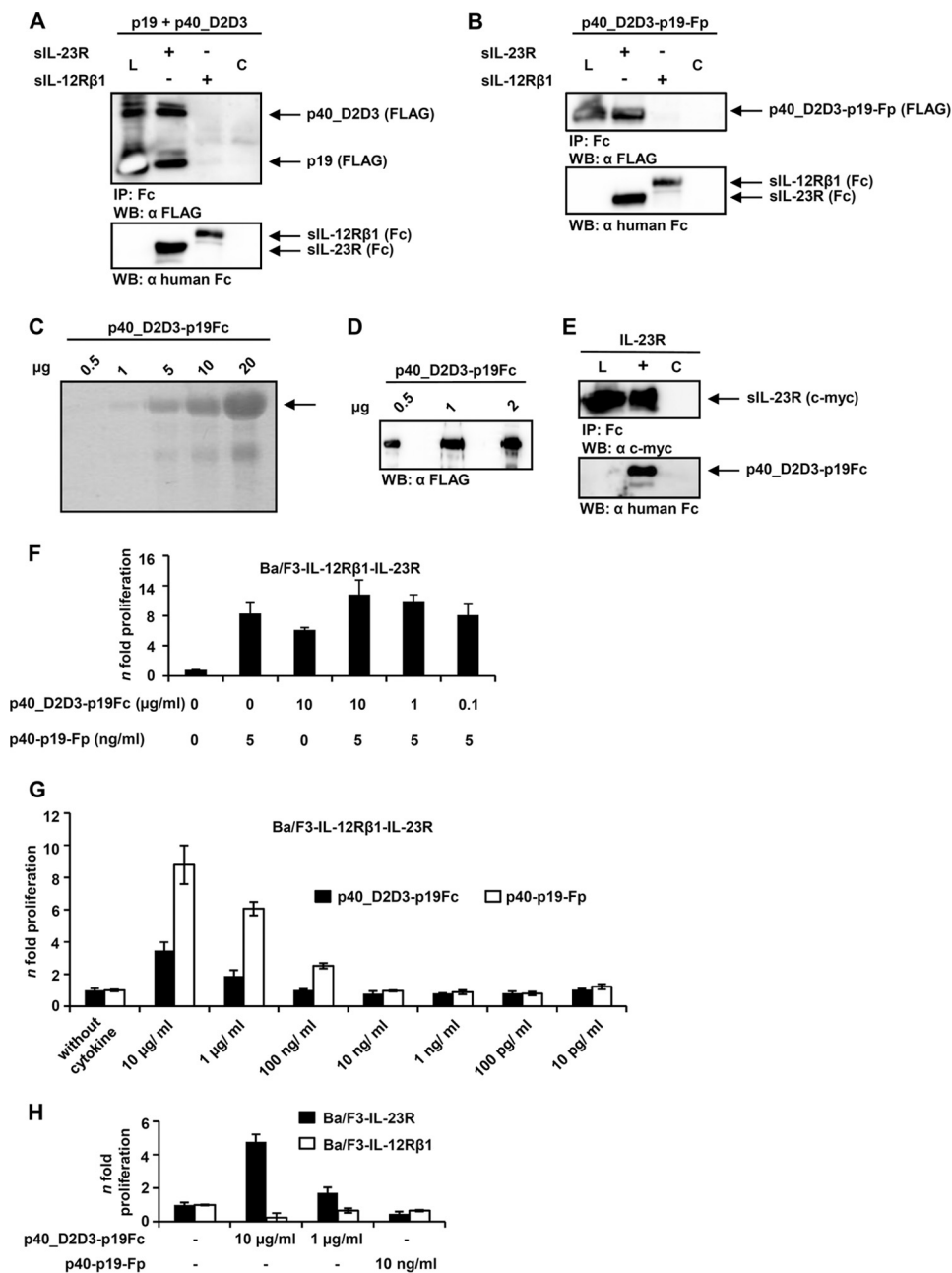
failed to interact with p40 in coimmunoprecipitation experiments. However, the IL-12Rβ1 Y143E single mutant largely, but not completely, lost its binding capacity to p40 (Fig. 7D). Taken together, these data show that p40 has overtaken the functional site II interaction capacity from p19. In all other IL-6 type cytokines, interaction of site II of the cytokine is mediated

with this functional arrangement of both domains of the CBM but not by isolated domains of the CBM (36).

*p40\_D2D3-p19 Is Not a Competitive Antagonist of IL-23 Signaling*—Because p40 was mandatory to induce p19 binding of IL-23R, we wondered whether p19/p40\_D2D3, which should not bind to IL-12Rβ1, was still able to interact with IL-23R.

FIGURE 6. **Absence of a classical site II in p19.** *A*, model of the predicted site II of p19 (blue) toward IL-12Rβ1 (orange). *B*, Western blotting (WB) of secreted p40 and p19\_L36E, p19\_L135E, or p19\_R38E. One of three independent experiments is shown. *C*, cell viability assay of Ba/F3-IL-12Rβ1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean ± S.D. *D*, co-IP of FLAG-tagged p40 and p19, p19\_L36E, p19\_L135E, or p19\_R38E with Fc-tagged sIL-12Rβ1 or sIL-23R. *L*, lysates; +, coimmunoprecipitates; *C*, controls (without sIL-12Rβ1-Fc or sIL-23R-Fc). One of three independent experiments is shown. *E*, Western blotting of secreted p40-p19-Fp and p40-p19\_L36E\_R38E\_L135E-Fp. *F*, cell viability assay of Ba/F3-IL-12Rβ1-IL-23R cells stimulated with the indicated cytokines (10% conditioned cell culture supernatant). One of three independent experiments is shown. Data are mean ± S.D. *G*, co-IP of FLAG-tagged p40-p19-Fp or p40-p19\_L36E\_L135E\_R38E-Fp with Fc-tagged sIL-12Rβ1 or sIL-23R. One of three independent experiments is shown.

## IL-23R Complex Assembly



**FIGURE 8. p40\_D2D3-p19 specifically interacts with IL-23R but not with IL-12R $\beta$ 1 and acts as an agonist independently of IL-12R $\beta$ 1.** *A*, co-IP of FLAG-tagged p40\_D2D3 and p19 and Fc-tagged sIL-12R $\beta$ 1 or sIL-23R. *L*, lysates; +, coimmunoprecipitates; *C*, controls (without sIL-12R $\beta$ 1-Fc or sIL-23R-Fc); *WB*, Western blot. One of three independent experiments is shown. *B*, co-IP of the fusion protein p40\_D2D3-p19-Fp with Fc-tagged sIL-12R $\beta$ 1 or sIL-23R. One of three independent experiments is shown. *C* and *D*, Coomassie staining (*C*) and Western blotting (*D*) of purified p40\_D2D3-p19Fc. *E*, co-IP of myc-tagged sIL-23R and recombinant p40\_D2D3-p19Fc. *L*, lysates; +, coimmunoprecipitates; *C*, controls (without sIL-23R). One of three independent experiments is shown. *F–H*, cell viability assay of Ba/F3-IL-12R $\beta$ 1-IL-23R cells (*F* and *G*), Ba/F3-IL-12R $\beta$ 1 cells (*H*), and Ba/F3-IL-23R cells (*H*) stimulated with the indicated recombinant cytokines. One of three independent experiments is shown. Data are mean  $\pm$  S.D.

Co-IP experiments revealed that p19 plus p40\_D2D3 and the fusion protein p40\_D2D3-p19-Fp still bind to IL-23R but not to IL-12R $\beta$ 1 (Fig. 8, *A* and *B*).

Therefore, we hypothesized that p40\_D2D3-p19-Fp might serve as a competitive antagonist of IL-23 by binding to IL-23R but not to IL-12R $\beta$ 1. Accordingly, CHO-K1 cells stably expressing an Fc-tagged p40\_D2D3-p19-fusion protein (p40\_D2D3-p19Fc) were generated. Recombinant p40\_D2D3-p19Fc was purified by affinity chromatography. The purity and identity of p40\_D2D3-p19Fc was verified by SDS-PAGE (Fig. 8*C*)

and Western blotting (Fig. 8*D*). IL-23R was coprecipitated by purified p40\_D2D3-p19Fc (Fig. 8*E*). Surprisingly, p40\_D2D3-p19Fc was not able to inhibit IL-23-induced proliferation of Ba/F3-IL-12R $\beta$ 1-IL-23R cells, suggesting that p40\_D2D3-p19Fc has no antagonistic properties (Fig. 8*F*). As a positive control, cellular proliferation was stimulated by p40-p19-Fp. Concentrations above 1  $\mu$ g/ml p40\_D2D3-p19Fc, however, led to cellular proliferation of Ba/F3-IL-12R $\beta$ 1-IL-23R cells (Fig. 8*G*). Next, we analyzed whether p40\_D2D3-p19Fc-induced cellular proliferation was dependent on IL-12R $\beta$ 1 and/or IL-23R.

Therefore, Ba/F3-IL-12R $\beta$ 1 and Ba/F3-IL-23R cells were stimulated with 1 and 10  $\mu$ g/ml p40\_D2D3-p19Fc. As depicted in Fig. 8H, Ba/F3-IL-23R cells, but not Ba/F3-IL-12R $\beta$ 1 cells, proliferated in the presence of 1 and 10  $\mu$ g/ml p40\_D2D3-p19Fc, suggesting the formation of biological active IL-23R homodimers.

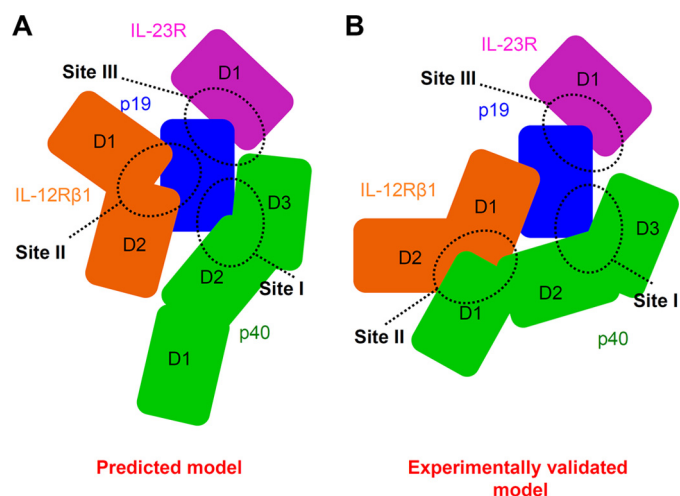
## DISCUSSION

In this study we defined critical hot spot amino acid residues for the formation of the IL-23 receptor complex in sites I and III of p19 and in p40. We showed that the putative binding site II of p19 did not contribute to IL-12R $\beta$ 1 binding, which was adopted by interaction of p40\_D1D2 with IL-12R $\beta$ 1 for receptor complex formation.

Our results confirmed that the cytokine p19 interacts via the classical site I with its  $\alpha$  receptor p40. Among the IL-6/IL-12 cytokine family, cytokines with a corresponding  $\alpha$  receptor did not bind to their  $\beta$  receptor in the absence of the  $\alpha$  receptor. The interaction of the cytokine with its  $\alpha$  receptor induces a conformational shift that activates binding sites II and III toward the  $\beta$  receptors (14). Although one recent report has suggested that p40 was not needed for the binding of p19 to IL-23R (31), our data clearly showed that only p19 in complex with p40 was able to bind to IL-23R. A conserved hot spot tryptophan among IL-12/IL-6 type cytokines is a critical binding determinant of site III (37). Here the amino acid exchange p19\_W157A, which was predicted as hot spot amino acid in p19 (13), completely abolished IL-23/IL-23R interaction and biological activity of IL-23, suggesting that IL-23 also has a typical site III.

Surprisingly, p19 variants with the single and combined amino acid exchanges L36E, R38E, and L135E within the postulated site II were biologically active. Because p40 binds to IL-12R $\beta$ 1 in the absence of p19, we hypothesized that binding of IL-23 to IL-12R $\beta$ 1 is simply mediated by p40 and not by p19. Interestingly, binding of IL-12R $\beta$ 1 to p40 was mediated by the CBM (D1D2) of IL-12R $\beta$ 1 but not by D1 or D2 alone or the fibronectin-type III domains D3–D5. Moreover, typical corresponding site II amino acids in IL-12R $\beta$ 1 were mandatory for interaction of IL-12R $\beta$ 1 and p40. Our data show that p40 has adopted the functional interaction site with IL-12R $\beta$ 1 from p19 because, in IL-6 type cytokines, site II is located on the cytokine and not on the  $\alpha$ -receptor subunit (36). Therefore, functionally, there is no need and no place for an additional site II interaction site of p19 toward IL-12R $\beta$ 1. Furthermore, the binding of p40 to the CBM of IL-12R $\beta$ 1 explains why p40 is an antagonist of IL-23 and IL-12 signaling.

Finally, we generated the fusion protein p40\_D2D3-p19-Fp, which specifically interacted with IL-23R but not with IL-12R $\beta$ 1. Because p40\_D2D3-p19-Fp should compete with IL-23 for binding to IL-23R, p40\_D2D3-p19-Fp was expected to have antagonistic properties. Fusion to the Fc domain of a human antibody is a common approach to improve the *in vivo* circulation half-life of protein therapeutics and increase proteolytic stability (38). Surprisingly, p40\_D2D3-p19Fc did not act as a competitive antagonist of IL-23 signaling. At high concentrations, the fusion protein p40\_D2D3-p19Fc induced signaling, most likely via an IL-23R homodimer, because p40\_D2D3-



**FIGURE 9. Predicted and experimentally validated model of the IL-23 receptor complex.** A, predicted model with canonical sites I, II, and III of p19. B, experimentally validated model with sites I and III in p19 and major interaction of p40 with IL-12R $\beta$ 1. The proposed binding site II of p19 did not contribute to IL-12R $\beta$ 1 binding. Instead, the interaction of p40\_D1D2 with IL-12R $\beta$ 1 was sufficient for receptor complex formation. Shown are p40 (green, D1–D3), p19 (blue, site I), IL-12R $\beta$ 1 (orange, domains D1 and D2), and IL-23R (pink, D1).

p19Fc induced cellular proliferation of Ba/F3-IL-23R cells. Because these cells lack IL-12R $\beta$ 1, our results indicate that signal transduction of p40\_D2D3-p19Fc was independent of IL-12R $\beta$ 1. In line with these findings, our unpublished data showed that ligand-independent forced dimerization of IL-23R leads to IL-12R $\beta$ 1-independent receptor activation.<sup>4</sup>

On the basis of our results, we propose an alternative model of the IL-23 cytokine/cytokine receptor complex topology on the basis of canonical site I and site III but suggested a non-canonical binding site II that is mainly localized in D1/D2 of p40 toward the CBM of IL-12R $\beta$ 1 (Fig. 9, A and B).

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<sup>4</sup> D. M. Floss and J. Scheller, unpublished results.

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