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Cutting Edge: IL-23 Cross-Regulates IL-12 Production in T Cell-Dependent Experimental Colitis

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Although IL-12 and IL-23 share the common p40 subunit, IL-23, rather than IL-12, seems to drive the pathogenesis of experimental autoimmune encephalomyelitis and arthritis, because IL-23/p19 knockout mice are protected from disease. In contrast, we describe in this study that newly created LacZ knockin mice deficient for IL-23 p19 were highly susceptible for the development of experimental T cell-mediated TNBS colitis and showed even more severe colitis than wild-type mice by endoscopic and histologic criteria. Subsequent studies revealed that dendritic cells from p19-deficient mice produce elevated levels of IL-12, and that IL-23 down-regulates IL-12 expression upon TLR ligation. Finally, in vivo blockade of IL-12 p40 in IL-23-deficient mice rescued mice from lethal colitis. Taken together, our data identify cross-regulation of IL-12 expression by IL-23 as novel key regulatory pathway during initiation of T cell dependent colitis. The Journal of Immunology, 2006, 177: 2760–2764.

I L-12 is a key regulatory cytokine in cell-mediated immunity and induces the differentiation of naïve T cells toward a TH1 phenotype (1). The pathogenic activity of IL-12 has been demonstrated in various TH1-mediated experimental models of autoimmune diseases, including inflammatory bowel disease (2–5). Moreover, a recent clinical trial showed that targeting of IL-12 p40 by neutralizing Abs suppresses gut inflammation in patients with Crohn’s disease (6). However, two additional cytokines closely related to IL-12, denoted IL-23 and IL-27, were recently discovered (7). Because IL-23 shares the p40 subunit with IL-12, the role of IL-12 may have been overestimated in studies using neutralizing p40 Abs. The discovery that IL-23, but not IL-12, is essential in some models of chronic inflammation and autoimmunity led to a model in which IL-12 is required to induce IFN-γ-producing TH1 cells, whereas IL-23 mediates TH1 effector functions (8, 9).

Materials and Methods

Generation of IL-23 p19-deficient LacZ knockin mice

Clones were derived from a mouse bacterial artificial chromosome library as described previously (10). IL-23 p19-deficient C57BL6 × 129 F1, embryonic stem cell clones were then injected into C57BL6/J blastocysts to generate chimeric mice. Male chimeras were then mated with female C57BL6/J mice to generate F1, breeders. Mice were back-crossed to C57BL6 and genotyped by PCR. The strain has been formally denoted B6;129-IL23tm1Gdy.

Induction of colitis and mouse endoscopy

Inducible mouse models for colitis were used as described previously (3, 11). In brief, colitis was induced by either giving mice 2.5% dextran sodium sulfate (DSS)3 in drinking water for 1 wk or by intrarectal administration of 0.5% TNBS. For continuous monitoring of colitis, the Coloview high-resolution mouse video endoscopic system (Karl Storz) was used. Colitis in Rag1 knockout mice was induced as described previously (12).

Isolation and culture of primary cells

Mouse bone marrow-derived dendritic cells (BMDCs) were generated as described previously (13). In brief, BM cells were isolated from the femurs of 6–to 10-wk-old mice and cultured in serum-free X-Vivo-15 medium (Cambrex) supplemented with 10 ng/ml murine GM-CSF (PeproTech). DCs were then purified using immunomagnetic beads specific for CD11c (Miltenyi Biotec). Intestinal lamina propria mononuclear cells (LPMCs) were isolated as described previously (3). For activation of cells the following reagents were used: 1 μg/ml bacterial LPS (Sigma-Aldrich), 1 μM CpG (MWG-Biotech), lipoteichoic acid (Sigma-Aldrich), double-stranded RNA (MWG-Biotech), and 5 ng/ml mouse IL-23 (R&D Systems).

Isolation of mRNA and RT-PCR

Total RNA was isolated using the RNA micro kit (Qiagen). Generation of cDNA was performed with Stratascript II reverse transcriptase (Invitrogen Life Technologies). Real-time quantitative PCR was performed on the iQ iCycler (Bio-Rad). For detection of IL-23 p19, IL-12 p40, IL-12 p35, and β-actin, mRNA-specific cDNA primers and fluorescent-labeled probes (Quantitect; Qiagen) were used. PCR were performed with the qRT-PCR Mix (Abgene).

ELISA

To measure IL-12 and IL-6 protein production, 106 BMDCs per well were seeded out in 1 ml of culture medium. After 48 h, cell-free culture supernatants

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3 Abbreviations used in this paper: DSS, dextran sodium sulfate; BMDC, bone marrow dendritic cell; LPMC, lamina propria mononuclear cell; gal, galactosidase; MEICS, murine endoscopic index of colitis severity.

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were removed and assayed for IL-12 p70 and IL-6 concentration by specific ELISA (14).

**Immunohistochemistry and β-galactosidase (β-gal) assay**

Immunofluorescence of cryosections was performed using the TSA Cy3 system (PerkinElmer) and a fluorescence microscope (Olympus) as described (14). In brief, cryosections were fixed in paraformaldehyde and incubated overnight with primary Abs specific for either CD11c (Santa Cruz Biotechnology) or IL-23 p19 (polyclonal rabbit anti-mouse peptide Ab; Eurogentec). Subsequently, the slides were incubated with biotinylated secondary Abs (Dianova), streptavidine-HRP, and finally, with tyramide (Cy3 or FITC) according to the manufacturer’s instructions (PerkinElmer). Nuclei were counterstained with Hoechst 3342 (Molecular Probes).

**Statistical analysis**

Endoscopic data were analyzed by Student’s t test using the program Excel (Microsoft).

**Results and Discussion**

**Mice lacking the p19 subunit of IL-23 are highly susceptible to colitis**

Proinflammatory cytokines produced by APCs play a major role in chronic intestinal inflammation (15, 16), although the precise role of IL-23 in this context remains to be determined. However, constitutive expression of IL-23 and p40 in DCs of the terminal ileum was recently observed in healthy mice (14). Such expression of p40 was stimulated by bacterial uptake in DCs with subsequent induction of p40 via NF-kB. To investigate whether bacteria-induced IL-23 p19 is expressed in the colonic lamina propria of mice suffering from experimental Th1-mediated colitis, we performed immunohistochemistry using Abs against p19. Colitis was induced by the adoptive transfer of CD4+CD62L+ T cells into immunocompromised Rag1 knockout mice. Although no p19 was detectable in the colons of healthy unreconstituted animals, a specific cytoplasmic staining was seen in lamina propria cells of mice with experimental colitis (data not shown). These data suggested a role of IL-23 in the pathogenesis of colitis, and we further addressed this point using p19-deficient mice.

Previous studies have shown that mice deficient for IL-23 were protected from a variety of experimental animal models known to be driven by pathogenic T cell populations (8, 9). To investigate whether IL-23 plays an active role in the pathogenesis of colitis, we next created mice with a targeted deletion of the endogenous IL-23 p19 gene. Hereby, the endogenous gene was replaced by an in-frame insertion of the reporter gene LacZ with nucleotide precision (Fig. 1A), thereby allowing analysis of the activity of the endogenous IL-23 p19 promoter in the resulting knockin mice. IL-23 p19-deficient mice did not produce any detectable p19 mRNA in the gut (Fig. 1B) and elsewhere in the body (data not shown). However, β-gal was induced by BMDCs upon stimulation with TLR ligands, implicating that lacZ reporter gene activity was properly regulated by the endogenous IL-23 p19 promoter (data not shown). To investigate whether IL-23 p19 develop colitis, we next performed experiments with p19-deficient mice and wild-type littermates in the DSS model of experimental colitis. Accordingly, p19-deficient, heterozygous, and wild-type (littermates) mice were given a low dose of DSS in drinking water. Although wild-type mice lost little weight during low-dose DSS treatment, knockout mice showed a constant decline of their body weight starting at day 3 after DSS treatment (Fig. 1C). Furthermore, knockout mice did not recover even after withdrawal of DSS, indicating a chronic state of colitis in these mice. Heterozygous

**FIGURE 1.** Colitis in wild-type and newly generated IL-23 p19-deficient mice. A, IL-23 p19-deficient knockin mice were created using the Velocigene approach in which the endogenous IL-23 p19 gene was replaced by an in-frame insertion of the reporter gene LacZ (left panel), thereby allowing the analysis of the activity of the endogenous IL-23 p19 promoter in the resulting mice. The strain received the formal notation B6;129-IL23tm1Gdy. Mice were genotyped using a PCR-based approach with a common primer located upstream of exon 1 and a genotype-specific primer within the coding region of p19 (wild-type allele) or within the LacZ region (targeted allele), respectively (right panel). B, Real-time quantitative PCR indicating loss of p19 expression in the gut of IL-23 targeted mice. Gut samples of wild-type and knockout animals were homogenized and isolated RNA was used to generate cDNA. Data were normalized to expression of HPRT. C, Fifteen mice were treated with DSS for 1 wk as indicated in Materials and Methods to induce colitis. Weight loss in wild-type, heterozygous, and IL-23 p19-deficient mice was monitored by daily measurements. Weight is indicated as percentage of initial weight and is shown as percentage of the initial weight ± SEM.
mice also lost body weight, but to a lesser extent, compared with knockout animals. To investigate whether the IL-23 p19 promoter is induced in mice suffering from colitis, we analyzed β-gal activity in colon lysates of heterozygous mice. Colitis-induced weight loss correlated well with the level of reporter gene activity detected in these mice (data not shown), implicating that IL-23 is induced during the course of colitis.

To investigate the role of IL-23 in T cell-dependent colitis, we next analyzed colitis induced by low dosages of TNBS. Wild-type mice developed a mild colitis, as indicated by a moderate weight loss throughout the course of the experiment (Fig. 2A). In contrast, mice deficient for IL-23 p19 showed a much stronger wasting syndrome with severe diarrhea after rectal administration of TNBS. This difference was even more pronounced when TNBS treatment was repeated on day 7 of the experiment.

To confirm that the wasting disease was indeed due to TNBS colitis, we performed mini endoscopy. Whereas wild-type mice showed only mild alterations of the mucosal surface with a moderate murine endoscopic index of colitis severity (MEICS), knockout mice displayed signs of severe TNBS colitis with significantly (p < 0.05) higher MEICS score (Fig. 2B). Accordingly, the endoscopy of knockout mice showed a thickening of the bowel wall, alterations of the vascular pattern, and a more granular mucosa with high amounts of fibrin in the lumen. The endoscopic findings were confirmed by histopathological analysis of colon samples. Wild-type mice treated with TNBS showed only low histologic scores (Fig. 2C). In contrast, knockout mice demonstrated scores indicative of severe colitis, as shown by epithelial erosions and massive infiltrations into the mucosa.

In summary, we demonstrate that in contrast to experimental models of arthritis and multiple sclerosis, IL-23 is not essential for the development of DSS colitis and TH1-mediated TNBS colitis. In fact, we found that IL-23 p19 knockout mice are highly susceptible for development of TH1-mediated colitis upon TNBS administration and had even more severe colitis than wild-type mice. Thus, our data implicate important differences between certain TH1-mediated mucosal immune response in the gut and TH1-mediated diseases in other organs.

**IL-23 down-regulates IL-12 by inhibition of IL-12 p35 and p40 expression**

To investigate the cause of aggravated colitis in mice deficient for IL-23, we next investigated cytokine production by BMDCs isolated from wild-type and p19-deficient mice. Because cytokines such as IL-6, TNF-α, and IL-12 play a major role in the pathogenesis of colitis, we measured expression of these cytokines in BMDC supernatants by ELISA (Fig. 3A). IL-6 and TNF-α were strongly induced by stimulation in both wild-type and IL-23 p19-deficient DCs, and no significant differences could be detected. In contrast, IL-12 heterodimer was detectable in the supernatant of LPS-stimulated p19-deficient, but not wild-type, DCs. In contrast, CpG stimulation led to IL-12 release in both wild-type and knockout DCs, although the level of IL-12 released by wild-type cells was lower, compared with p19-deficient cells. Thus, our data demonstrate an overexpression of IL-12 by DCs in the absence of IL-23. To confirm overexpression of IL-12 in the gut immune system, we found that for IL-23 p19, we next isolated LPMCs from wild-type and knockout animals and stimulated them with LPS. LPMCs lacking p19 produced IL-12 upon stimulation with LPS, whereas IL-12 levels in wild-type LPMCs were below the detection limit (Fig. 3B). Thus, LPMCs from IL-23 p19 knockout mice produced enhanced levels of IL-12.

To confirm cross-regulation of IL-12 related cytokines by IL-23, we next investigated the expression of IL-12 and IL-23 subunits by quantitative PCR. Accordingly, BMDCs from wild-type and IL-23 p19-deficient mice were stimulated with LPS.
Weight loss was monitored by daily measurements.

Inhibition of IL-12 p40 rescues IL-23 p19-deficient mice from lethal colitis

To investigate whether overproduction of IL-12 could be responsible for the aggravated colitis seen in IL-23 p19-deficient mice, we neutralized IL-12 by using an IL-12 p40-neutralizing Ab. Accordingly, IL-23 p19 knockout mice were treated with TNBS and then separated into two groups (Fig. 4A). One group received 1 mg of neutralizing anti-IL-12 p40 Ab in PBS, whereas the second group was treated with PBS alone. The development of wasting disease was monitored throughout the course of the experiment. Mice treated with PBS alone developed wasting disease leading to the death of all animals by day 22 of the experiment (Fig. 4B). In contrast, IL-23 p19-deficient mice treated with two doses of anti-IL-12 p40 Abs (day 0 and day 7) were all rescued from lethal colitis. Accordingly, Ab-treated animals showed only a mild weight loss not leading to an irreversible and fatal wasting syndrome. In agreement with the weight analysis, endoscopy of mice treated with PBS showed signs of strong colitis characterized by a thickened mucosa, loss of the normal vessel pattern, and bleedings (Fig. 4C). In contrast, mice treated with IL-12 p40 Ab showed only very mild endoscopic signs of colitis. Thus, IL-12 emerges as a key player in the severe TNBS colitis seen in the absence of IL-23 p19.

Cross-regulation of IL-12 levels by IL-23

IL-12 has been proposed as a key cytokine in the pathogenesis of TH1-mediated intestinal inflammation, because neutralizing IL-12 p40 Abs caused suppression of established experimental colitis in various animal models (2–4). Furthermore, transgenic mice for the IL-12 inducible transcription factor STAT-4 were highly susceptible to TH1-driven colitis (17, 18). These studies suggested the potential use of IL-12 inhibitors for therapy of Crohn’s disease, because this disease is characterized by T-bet-expressing TH1 T lymphocytes in the gut. In fact, a recent phase 2 study reported significantly higher remission rates in anti-IL-12 Ab-treated patients, compared with controls (6). Thus, IL-12 may be a promising target for future therapeutic intervention in patients with Crohn’s disease. However, all of the above investigations were performed using Abs against the p40 subunit of IL-12 that neutralizes IL-12 and IL-23 simultaneously, and experimental colitis in IL-10-deficient mice is associated with increased IL-23 levels (19). Therefore, studies on the role of IL-23 deficiency in intestinal inflammation are required. In this study, we provide evidence that suppression of both cytokines is required for prevention of TH1-mediated TNBS colitis in the gut. Our data are consistent with a model in which overproduction of IL-12 p35/p40 in p19-deficient mice is the cause of aggravated TNBS colitis in these mice, compared with wild-type mice. Thus, although p35 and p40 subunits are not generally affected by the absence of IL-23 in all experimental systems (20), these findings demonstrate the existence of cross-regulation of IL-12 levels by IL-23 in the gut and demonstrate the in vivo relevance of such cross-regulation for TH1-mediated TNBS colitis. Our data thus support a model in which the production of IL-12 is regulated by other IL-12 family members, such as IL-23, during experimental colitis.

Disclosures

The authors have no financial conflict of interest.

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