Interleukin-35 Mediates Mucosal Immune Responses That Protect Against T-Cell–Dependent Colitis

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BACKGROUND & AIMS: The soluble hematopoietin receptor Epstein–Barr virus–induced protein (EBI)-3 is an immune regulator that has been associated with the pathogenesis of inflammatory bowel disease. However, the concept that EBI3 is part of an interleukin (IL)-27 heterodimer that mediates chronic inflammatory and autoimmune diseases has been challenged by the description of IL-35, a bioactive cytokine comprising EBI3 and IL-12 p35. We investigated the roles of IL-27 and IL-35 in chronic inflammation of the intestine. **METHODS:** We analyzed EBI3-deficient mice and IL-27p28–deficient mice with spontaneous or T-cell transfer-induced colitis and compared outcomes with wild-type mice (controls). We constructed vectors that express EBI3 covalently linked to the IL-12p35 chain (recombinant [r]IL-35). **RESULTS:** Intestines of EBI3-deficient mice had increased pathologic features of colitis, compared with IL-27p28–deficient or control mice; they also had shorter survival times, indicating that IL-35, rather than IL-27, protects the intestine from immune responses in mice. The mucosa of EBI3-deficient mice accumulated subsets of activated CD4+ T cells that produced T-helper (Th)1 and Th17 cytokines. Adoptive transfer of these T cells induced colitis in RAG-deficient mice. The rIL-35 significantly reduced the development of several forms of experimental colitis and reduced levels of markers of Th1 and Th17 cells. **CONCLUSIONS: IL-35 controls the development of T-cell–dependent colitis in mice. It might be developed as a therapeutic target for patients with chronic intestinal inflammation.**

Keywords: IBD; Mouse Model; Immune Regulation; Suppression.

The etiology of inflammatory bowel disease (IBD) still remains incompletely understood, but it is generally agreed that a complex interplay between genetic, environmental, and immunologic factors contributes to disease initiation and progression.[1] Numerous studies in animal models clearly identified members of the family of interleukin (IL)-12–related cytokines as central mediators of mucosal inflammation.[2] In both animal models and human Crohn's disease there is evidence for dominance of a polarized T-helper 1 (T_H1) pathway that depends on increased mucosal expression of IL-12 (p40:p35), which is the key T-cell differentiation factor driving cellular immune responses characterized by the production of proinflammatory cytokines interferon (IFN)-γ and tumor necrosis factor-α.[3] Consistently, targeting IL-12 may be effective in treating CD and such strategies currently are evaluated.[4] However, previous concepts on the role of T-helper cells in inflammatory and autoimmune diseases have been challenged by the recent description of the highly proinflammatory T_H17 subset characterized by production of IL-17 and IL-22.[5] IL-23 (p40:p19), a cytokine sharing the p40 subunit with IL-12, is essential for maintenance of the T_H17 phenotype and is associated with disease in colitis models.[6] Taken together with the discovery of the protective Arg381Gln polymorphism in the IL-23R gene in Crohn's disease, these findings strongly suggest a major role of this cytokine in IBD.

More recently, computational studies identified IL-27, a heterodimer composed of Epstein–Barr virus–induced protein (EBI)-3, and an IL-27p28 subunit that binds to a receptor formed by the WSX-1 and gp130 chains.[7] Initially, IL-27 was described as a T_H1-promoting factor synergizing with IL-12 to drive proliferation and IFN-γ production of naive but not memory $CD4^+$ T cells. However, subsequent studies elucidated that the biological functions of IL-27 are more complex, and a role for IL-27 as a negative regulator of T_H2 and particularly T_H17 responses emerged.[8] IL-27 exerts these functions by inducing IL-10 production in T cells and directly blocking ROR-γT production in a signal transducer and activator of transcription (STAT)1-dependent manner.[9] In addition, we and others have identified EBI3 as a regulator of innate immune cells such as granulocytes and macrophages.[10,11] Such immunoregulatory potential of EBI3 is implicated further by the recent discovery that EBI3 is expressed in $Foxp3^+CD25^+$ T_{reg} and constitutes with the IL-12p35 chain the novel cytokine IL-35.[12–14] IL-35 is induced in $CD4^+CD25^+$ T_{reg} on contact with $CD4^+CD25^-$ effector cells and contributes to their suppressive activity in vitro and in vivo.[15,16] Furthermore, rIL-35 suppressed the proliferation of $CD4^+$ effector cells and inhibited T_H17 polarization. In vivo, recombinant (r)IL-35 was shown to

Abbreviations used in this paper: DSS, dextrane sodium sulfate; EBI, Epstein–Barr virus–induced protein; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; LPMC, lamina propria mononuclear cell; MLN, mesenteric lymph nodes; PCR, polymerase chain reaction; rIL, recombinant interleukin.

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dramatically inhibit the onset of collagen-induced arthritis and, in contrast to rIL-27, even was able to modulate already established disease, suggesting that IL-35 administration could be a potential therapeutic strategy in inflammation and autoimmunity.[17] However, the lack of knowledge about the IL-35 receptor, current limitations in its detection, and the fact that EBI3$^{-/-}$ mice lack with IL-27 and IL-35, 2 cytokines with largely redundant biological functions, is currently limiting the understanding of its precise biological function. This is reflected by the data on the role of IL-27 and IL-35 in the context of IBD, in which studies exist showing that IL-27R deficiency protects from colitis in IL-10$^{-/-}$ mice,[18] whereas the same strain was shown to be affected in dextrane sodium sulfate (DSS) colitis.[11,19] On the other hand, EBI3 deficiency does not lead to changes in intestinal pathology in trinitrobenzene sulfonic acid colitis, but is protective in oxazolone-induced colitis.[20]

Here, we analyzed the differential role of IL-27 and IL-35 for the development of intestinal pathology by direct comparison of EBI3 and IL-27p28–deficient mice in models of T-cell–dependent colitis. Although EBI3-deficient mice developed early severe intestinal disease, IL-27p28$^{-/-}$ mice were phenotypically similar to wild-type mice, suggesting that IL-35 rather than IL-27 is a critical factor limiting intestinal inflammation in these models. Furthermore, administration of a single-chain IL-35 fusion-protein led to suppression of colitis activity, indicating that IL-35 is able to suppress pathologic intestinal immune responses in vivo.

Material and Methods

Mice

EBI3-deficient mice were described previously.[20] UbC-luc mice were obtained from Caliper Lifesciences (Mainz, Germany). For generation of IL-27p28–deficient mice clones containing the IL-27 genomic locus were derived from a mouse BAC library as described previously.[21] IL-27p28–deficient C57BL/6x129 F1 ES-cell clones were injected into C57BL6/J blastocysts to generate chimeras. Male chimeras were mated with female C57BL6/J mice to generate F1 breeders. Both strains were backcrossed to the C57BL/6 background for at least 10 times. Some mice were back-crossed to a RAG1-knockout background. To generate IL-27/IL-35–deficient mice with conditional ablation of STAT3 in myeloid cells, EBI3$^{-/-}$ and p28$^{-/-}$ mice were mated with LysmCre mice obtained from Jackson Laboratories (C56BL/6-background). Littermate Cre-negative wild-type or STAT3 heterozygous mutants were used as controls. Induction of DSS and trinitrobenzene sulfonic acid colitis was performed as previously described.[22] All mice used in experiments were bred and maintained in microisolator cages and all mouse procedures were performed using committee-approved protocols.

Primary Cells

Murine lamina propria mononuclear cells (LPMCs) were isolated as previously described.[22] Briefly, colons were mechanically dissected into small pieces and intestinal epithelial cells were removed by incubation in 5 mmol/L ethylenediaminetetraacetic acid. Remaining tissue was digested using collagenase D, DNaseI, and DispaseII (all Roche Diagnostics, Mannheim, Germany). Digested tissue was passed through a 40-μm cell strainer, and the remaining cellular content was separated from debris using a 40%/80% Percoll gradient. In some experiments, CD4$^+$ T cells from mesenteric lymph nodes (MLN) were isolated by MACS separation (Miltenyi-Biotec, Bergisch-Gladbach, Germany) to a purity degree of more than 95%, as evaluated by FACS.

Histologic Scoring of Inflammation

Inflammation was graded semiquantitatively on a scale from 0 to 6 in a blinded fashion. Two subscores grading the degree of inflammatory cell infiltrations (0–3) and tissue damage (0–3) were determined, resulting in a combined score ranging from 0 (no changes) to 6 (widespread cellular infiltrations and extensive tissue damage). For grading infiltration of inflammatory cells, an infrequent presence of inflammatory cells in the lamina propria was classified as 0; increased numbers of inflammatory cells, including neutrophils, as 1; submucosal presence of inflammatory cell clusters as 2; and a score of 3 was applied for transmural cell infiltrations. For grading of epithelial damage, a normal mucosal structure was classified as 0, isolated focal epithelial damage was counted as 1, the presence of mucosal erosions/ulcerations was counted as 2, and a score of 3 was given if extensive mucosal damage and extension through deeper structures of the bowel wall was present.

Mouse Endoscopic Procedures

Colitis activity was monitored with a high-resolution video endoscopic system (Karl Storz, Tuttlingen, Germany) at indicated time points in anesthetized mice. Endoscopic scoring of colitis activity was based on the evaluation of mucosal translucency, vascularity, granularity, fibrin deposition, and stool consistency as previously described.[22]

Measurement of Cytokines

For measurement of cytokines in supernatants from cell preparations or full-thickness organ cultures we used the FlowCytomix (Ebioscience, Frankfurt, Germany) according to the manufacturer's instructions using a FACSCantoII (Becton Dickinson, Heidelberg, Germany). IL-27 levels were determined by enzyme-linked immunosorbent assay (Ebioscience).

Analysis of Gene Expression

Total RNA was extracted with RNeasy columns (Qiagen, Hilden, Germany) including DNaseI digestion. RNA was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) using random hexamers. Quantitative polymerase chain reaction (PCR) analysis was performed using Quantitect Primer assays from Qiagen in a CFX96 system (Biorad). Relative differences between samples were calculated with the Pfaffl-model-based Rest2009 software using HPRT as the reference gene.[23]

IL-35 Expression Vector

For construction of IL-35, complementary DNAs encoding for EBI3 and p35 were cloned from lipopolysaccharide-stimulated splenocytes by reverse-transcription PCR. For cloning of single-chain IL-35, fragments encoding EBI3, followed by a $(GlyGlyGlySer)_4$ linker and the mature coding sequence of p35 were generated by PCR and cloned in a expression plasmid containing regulatory regions from the ubiquitous EF1α promoter and HTLV enhancer. To ensure efficient secretion, the EBI3 leader sequence was replaced by the signal peptide of IgGκ.