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Oral *Escherichia coli* Colonization Factor Antigen I Fimbriae Ameliorate Arthritis via IL-35, Not IL-27

Irina Kochetkova,* Theresa Thornburg,* Gayle Callis,* Kathryn Holderness,* Massimo Maddaloni, and David W. Pascual†

A *Salmonella* therapeutic expressing enterotoxigenic *Escherichia coli* colonization factor Ag I (CFA/I) fimbriae protects against collagen-induced arthritis (CIA) by eliciting two regulatory T cell (Treg) subsets: TGF-β–producing Foxp3+CD39+CD4+ T cells and IL-10–producing Foxp3+CD39+CD4+ T cells. However, it is unclear whether CFA/I fimbriae alone are protective and whether other regulatory cytokines are involved, especially in the context for the EBV3-sharing cytokines, Treg-derived IL-35 and APC-derived IL-27, both capable of suppressing Th17 cells and regulating autoimmune diseases. Subsequent evaluation revealed that a single oral dose of purified, soluble CFA/I fimbriae protected against CIA as effectively as did *Salmonella*-CFA/I and found that Foxp3+CD39+CD4+ T cells were the source of secreted IL-35, whereas IL-27 production by CD11c+ cells was inhibited. Inquiring into their relevance, CFA/I fimbriae–treated IL-27R–deficient (WSX-1−/−) mice were equally protected against CIA as were wild-type mice, suggesting a limited role for IL-27. In contrast, CFA/I fimbriae–mediated protection was abated in EB13−/− mice, accompanied by the loss of TGF-β and IL-10–producing Tregs. Adoptive transfer of C57BL/6 CD39+CD4+ T cells to EB13−/− mice with concurrent CFA/I plus IL-35 treatment effectively stimulated Tregs suppressing proinflammatory collagen II–specific Th cells. In contrast, recipients cotransferred with C57BL/6 and EB13−/− CD39+CD4+ T cells and treated with CFA/I plus IL-35 were not protected, implicating the importance of endogenous IL-35 for conferring CFA/I-mediated protection. Thus, CFA/I fimbriae stimulate IL-35 required for the coinduction of TGF-β and IL-10.

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Rheumatoid arthritis is an incurable, systemic inflammatory disease of the joints, leading to cartilage loss, bone erosion, ankylosis, and consequent disability. A chronic inflammation involves a complex network of pathogenic cells from the innate and adaptive immune system, as well as a number of soluble factors that contribute to joint destruction (1, 2). TNF-α is a major facilitator of this disease progression (3, 4), and TNF-α antagonists can effectively diminish inflammation and attenuate destruction of cartilage and bone (5–8). However, prolonged treatment with TNF-α antagonists can have deleterious side effects, making patients more susceptible to opportunistic infections (9, 10).

In this same vein, efforts also have focused on adapting regulatory T cells (Tregs) and their cytokines, particularly IL-10 and TGF-β, to treat arthritis (11–14) and other autoimmune diseases (15–17). Recently, the spectrum of cytokines with regulatory properties has expanded to include the newly discovered CD4+ T cell cytokine, IL-35 (18), and it was found that the APC-derived cytokine, IL-27, can also be anti-inflammatory (19). IL-35 is formed by the EBV-induced gene 3 (EBI3) subunit dimerizing with the IL-12p35 subunit (20). It is produced by Tregs and potentially suppresses inflammatory diseases (18, 21). IL-35 binds to its receptor, the heterodimer IL-12RB2 and gp130, signaling through transcription factors STAT1 and STAT4 (22). In addition to stimulating Foxp3+CD25+CD4+ T cells, we showed (23) that IL-35 stimulated alternative Treg subsets encompassed within CD39+CD4+ T cells and was capable of conferring protection against collagen-induced arthritis (CIA). It was suggested that IL-35 can act independently of IL-10 or TGF-β (24), but our studies (23) showed that, in the absence of IL-10, IL-35 was ineffective.

In contrast, dimerizing with p28, the EB13 subunit forms IL-27, which is produced primarily by APCs. IL-27 binds to its receptor, WSX-1, and can promote Th1 cell polarization as a consequence of T-bet and STAT1 activation (25, 26). Yet, WSX-1−/− mice showed impaired resistance to *Trypanosoma cruzi*, leading to a prolonged parasitemia and higher mortality (27), supported by greater TNF-α, IL-6, and Th2-type cytokines during established *T. cruzi* infection. Given these findings, IL-27 signaling also appeared important for regulation of antiparasitic immune responses in WSX-1−/− mice (27). Initially found to support Th1 cell development, IL-27p28 neutralization diminished IFN-γ production, resulting in reduced disease severity in adjuvant-induced arthritis in rats (28), as well as in experimental autoimmune encephalomyelitis (EAE) in mice (29). IL-27R−/− mice were also resistant to proteoglycan-induced arthritis and showed reduced IFN-γ production (30). In contrast, IL-27 is studied mostly for its described immunoregulatory properties (19, 31–34). Activating CD4+ and CD8+ effector T cells, IL-27 suppresses Th17 cell–transferred EAE, implicating the importance of stimulating IL-10–producing T cells (31).

Colonization factor Ag I (CFA/I) is a virulence factor for enterotoxigenic *Escherichia coli* to enable intestinal colonization of humans (35). In an effort to generate a vaccine, this fimbria was...
expressed by an attenuated Salmonella vaccine vector and showed protection in animals, stimulating elevated mucosal IgA and serum IgG Abs following oral vaccination (36, 37). Interestingly, this vaccine was found to inhibit proinflammatory cytokine production, presenting the possibility of serving as an anti-inflammatory vaccine (38). It was subsequently found to ameliorate such inflammatory diseases as EAE (15, 39) and CIA (40, 41). With regard to CIA, two functionally distinct, but complementary, subsets of Tregs are induced with Salmonella-CFA/I: TGF-β–producing Foxp3+ CD39+ and IL-10–producing Foxp3+CD39+ CD4+ T cells (41). Together, these subsets suppress type II collagen (CII)-specific Th1 and Th17 cells (41). Stimulation of TGF-β by CFA/I fimbriae is also important for upregulation of CD39 apyrase in a CREB-dependent fashion by CD4+ T cells (41).

In this study, we demonstrate that purified soluble CFA/I fimbriae, when given orally, can recapitate our findings using Salmonella-CFA/I by the stimulation of TGF-β/Foxp3+ CD39+CD4+ and IL-10/Foxp3+CD39+CD4+ T cells to suppress CIA. In addition to these relevant findings is the observation that soluble CFA/I fimbriae induce IL-35–producing Foxp3+ Tregs in mice protected from CIA, as well as diminish IL-27 production by CD11c+ cells. Supporting the importance of endogenous IL-35 for protection against CIA, EBI3+/− mice are unresponsive to oral CFA/I fimbriae treatment. In contrast, WSX-1+/− mice, although showing less severe disease, were protected from arthritis upon oral CFA/I fimbriae treatment, as reflected by the enhanced production of TGF-β and IL-10 and increased numbers of CD39+ Tregs. Furthermore, these data show that endogenous IL-35 is essential for resolving arthritis by CFA/I fimbriae, because CIA EBI3+/− recipients were responsive to adoptive transfer with wild-type (WT) CD39+CD4+ T cells and subsequent cotreatment with CFA/I fimbriae and rIL-35. Exogenous IL-35 was only effective in EBI3+/− mice while treatments continued; upon ceasing its administration, arthritis returned. Donor cells diluted with EBI3+/−CD39+CD4+ T cells failed to confer protection. Thus, this study shows that CFA/I fimbriae can stimulate IL-35, not IL-27, for protection against CIA, and an endogenous source of IL-35 is required for CFA/I fimbriae to abate disease.

Materials and Methods

Mice

Male C57BL/6 (B6) mice (Charles River Laboratories) and breeding colonies of EBI3+/− and WSX-1+/− mice (The Jackson Laboratory) were maintained at Montana State University Animal Resources Center. All mice were kept in ventilated cages with a high-efficiency particulate abatement filter. Sterile food and water were supplied ad libitum. All experimental protocols were concordant with institutional policies for animal health and well-being.

Purification of CFA/I fimbriae

The CFA/I fimbriae producing E. coli strain H695 were grown on Minca agar in pans at 37°C for 48–60 h. Cells were harvested from the agar surface and smeared for 15 min on ice. Cell debris was removed by centrifugation at 10,000 rpm for 20 min. The fimbriae were precipitated overnight in a final concentration of 20% 4.1 M ammonium sulfate and 20 mM Tris-HCl, resuspended in 5 ml deionized/distilled water, and dialyzed overnight into deionized/distilled water to remove residual salts. The next day, insoluble proteins were separated via ultracentrifugation at 18,000 rpm (40,000 x g) for 1 h. CFA/I fimbriae were again precipitated from supernatant overnight using 20% final concentration of 4.1 M ammonium sulfate and resuspended in PBS, and the quality was evaluated by SDS-PAGE and Western blot analyses. Western blots were probed with rabbit anti-CFA/I fimbriae Ab (developed in-house). Endotoxin was removed via anion exchange chromatography using an Uno Q column (Bio-Rad, Hercules, CA). Endotoxin levels were below biologically relevant levels, as assessed by the Limulus Amebocyte Lysate assay (Associates of Cape Cod, East Falmouth, MA).

CIA

CIA was induced in C57BL/6, EBI3+/−, or WSX-1+/− mice with 100 µg chicken CII emulsified in CFA (both from Chondrex, Redmond WA) (23, 41), and 100 µl emulsion was given s.c. ~0.5 cm from the base of tail. Mice were observed daily beginning on day 21 post-CII challenge at the onset of disease. Each limb was evaluated using a scale of 0–3, as previously described (23): 0, no clinical signs; 1, mild redness of a paw or swelling of single digits; 2, significant swelling of ankle or wrist with erythema; and 3, severe swelling and erythema of multiple joints. The maximum score per mouse is 12.

CFA/I fimbriae and IL-35 treatment

Mice were orally gavaged with 200 µl sterile 50% saturated sodium bicarbonate solution, followed by a single dose of 80 µg purified soluble CFA/I fimbriae or sterile PBS on day 14 post-CII challenge. Recombinant mouse IL-35 was expressed and purified as previously described (23). IL-35 treatments of EBI3+/− mice consisted of 1-µg doses of recombinant mouse IL-35 given i.p. from days 21 through 25 post-CII challenge.

Histopathology

Paws from front and hind limbs and knees were fixed in 10% neutral-buffered formalin, decalcified in 5% formic acid, processed, embedded in paraffin, and cut into 5-µm sections. Adjacent sections were stained with H&E to evaluate inflammation and bone destruction or with toluidine blue to evaluate cartilage matrix and chondrocyte loss. H&E-stained sections were scored: 0, no changes; 1, synovial hyperplasia and moderate inflammatory infiltration; 2, pannus formation with cartilage degeneration; and 3, severe inflammatory infiltration, cartilage and bone degeneration. Toluidine blue–stained sections were scored: 0, no cartilage loss; 1, superficial zone proteoglycan and chondrocyte loss; 2, proteoglycan and chondrocyte loss into the middle zone of the layer above the tidemark; and 3, severe chondrocyte and proteoglycan matrix loss through tidemark (23). Paw and knee joint sections were evaluated, with a total maximum score of 18 possible per mouse.

Ab and cytokine ELISAs

To evaluate CII-specific or CFA/I fimbriae–specific serum IgG titers, flat-bottom, 96-well plates (MaxiSorb; Nunc) were coated with 2 µg/ml ELISA Grade CII (Chondrex) or 2 µg/ml purified CFA/I fimbriae overnight at 4°C. Sera samples collected from individual mice on day 35 post-CII challenge were serially diluted and added to coated wells, as previously described (40). Ag-specific reactivity was detected with goat anti-mouse HRP-labeled IgG1, IgG2a, and IgG2b Abs (Southern Biotechnology Associates, Birmingham, AL) and then developed with ABTS (Moss, Pasadena, CA). End point titers represent the reciprocal dilutions of the last dilution yielding an absorbance at OD415 ≥ 0.100 U above negative controls.

To evaluate CII-specific T cell responses, on day 40 or 42 post-CII challenge, CD4+ T cells were sorted by negative selection on magnetic beads (Invitrogen) from popliteal, axillary, inguinal, and iliac lymph nodes. CD4+ T cells was restimulated with 50 µg/ml T Cell Proliferation CII (Chondrex) in the presence of syngeneic irradiated (3000 rad) APCs for 4 d at 37°C and 5% CO2. Supernatants were subjected to cytokine-specific ELISAs, as previously described (15, 23, 41). Briefly, diluted standards and CD4+ T cell supernatants were added to wells precoated with cytokine-specific capture mAb (FN-γ, IL-6, IL-10, IL-17 [BD Pharmingen, San Diego, CA]; TGF-β [R&D Systems, Minneapolis, MN]). Reactions were detected with biotinylated cytokine-specific Abs and, after incubation with anti–biotin–HRP (Vector Laboratories), were developed with ABTS. To measure IL-35 production, wells were coated with anti-human/mouse p35 mAb (clone 27537, R&D Systems). Rabbit IgG anti–IL-35 Ab (developed in-house) was used as secondary Ab in a capture ELISA. Reaction was detected with HRP-conjugated donkey anti-rabbit IgG (adsorbed against mouse, rat, hamster, human, bovine serum proteins; Jackson ImmunoResearch Laboratories, West Grove, PA). To measure IL-27 secretion, whole LN lymphocytes from PBS- or CFA/I fimbriae–treated mice were restimulated with 50 µg/ml T Cell Proliferation Grade CII (Chondrex) for 3 d, and supernatants were subjected to capture IL-27 ELISA. An anti-mouse IL-27 EBI3 capture (R&D Systems) and biotinylated anti-mouse IL-27 p28 (R&D Systems) detection mAb pair was used in this ELISA.

Flow cytometry

Upon termination of the disease course, spleen, LN, and mesenteric LN (MLN) lymphocytes were stained with fluorochrome-labeled anti-CD39 and anti-CD4 mAbs (eBioscience, San Diego, CA). For analysis of surface
expressed TGF-β, biotin-labeled anti-TGF-β Ab (R&D Systems), paired with fluorochrome-conjugated streptavidin (BD Pharmingen), was used. For analysis of Foxp3 intracellular expression, after staining of surface markers, cells were fixed in 2% paraformaldehyde, permeabilized with ice-cold methanol, and stained with fluorochrome-labeled anti-Foxp3 mAb (eBioscience). For detection of intracellular IL-35 and IL-27, cells were incubated with 25 ng/ml PMA, 1 µg/ml ionomycin, and 10 µg/ml brefeldin A for 6 h. Following surface immunofluorescent staining, cells were fixed in 2% paraformaldehyde for 10 min, permeabilized with BD Perm/Wash buffer, and stained directly with anti-mouse EBI3 mAb (R&D Systems or LifeSpan Biosciences) labeled with Alexa Fluor 488 using an Alexa Fluor 488 Microscale Protein Labeling Kit (Molecular Probes, Invitrogen) or its isotype control labeled with Alexa Fluor 488 (R&D Systems), and with anti-mouse p35–Alexa Fluor 647 mAb (eBioscience), anti–IL-27p28-PE mAb (BioLegend, San Diego, CA), or with their isotype controls labeled with Alexa Fluor 647 or PE (eBioscience).

Adoptive transfer of CD39+CD4+ T cells
CIA was induced in EBI3–/– mice on day 0. Naïve WT and EBI3–/– spleen and combined peripheral LN CD4+ T cells were enriched by negative selection using Dynabeads (Invitrogen). After staining with anti–CD4–FITC (eBioscience) and anti–CD39–PE (BioLegend), CD39+CD4+ T cells were further sorted using a BD FACSArIA II (>95% purity). On day 14, 2 × 105 WT or a mixture of WT and EBI3–/– (10^5:10^5) CD39+CD4+ T cells were adoptively transferred to CII-challenged EBI3–/– recipients, followed by oral CFA/I fimbriate treatment. On days 21–25, recombinant mouse IL-35 (1.0 µg/dose) (23) was given i.p. daily.

Statistics
The Mann–Whitney U test was applied to analyze clinical scores, histology scores, and level of cartilage loss. Differences in arthritis incidence between experimental groups was checked with the Fisher exact probability test. One-way ANOVA was performed to analyze ELISA and flow cytometry results. Data were considered statistically significant at *p < 0.05.

Results
Soluble CFA/I fimbriae can ameliorate CIA
It was shown that E. coli CFA/I fimbriae, when expressed by an attenuated Salmonella, resulted in suppression of CIA (41). To ascertain whether purified, soluble CFA/I fimbriae are effective against CIA, C57BL/6 mice were challenged with CII on day 0 and were treated with a single 80-µg oral dose of purified endotoxin-free CFA/I protein on day 14. Control mice were given sterile PBS. By day 31, all PBS-treated mice showed clinical symptoms of arthritis. In the CFA/I fimbriae–treated group, 70% of mice remained protected over the course of the experiment (40 d) (Fig. 1A). The average clinical score for the CFA/I fimbriae–treated group was significantly less than for PBS-treated mice (Fig. 1A). Histopathological analysis of H&E-stained joint sections revealed significantly less inflammation and joint destruction, confirming the clinical findings. CFA/I fimbriae–treated mice also had less chondrocyte and proteoglycan matrix loss, as evident from the toluidine blue–stained sections (Fig. 1B, 1C).

Upon conclusion of the study, CII-specific CD4+ T cell cytokine responses were measured using collected supernatants from Ag-restimulated popliteal, axillary, inguinal, and iliac LN CD4+ T cells. Soluble CFA/I fimbriae treatment markedly inhibited Th1 and Th17 cell responses, as well as stimulated IL-10– and TGF-β–producing CD4+ T cells (Fig. 1D). Cytokine responses by CD4+ T cells were also evaluated 1 wk after CFA/I fimbriae treatment, and similar cytokine profiles were observed (data not shown). The results of these studies show that orally administered CFA/I fimbriae alone can mitigate CIA by suppressing Th1 and Th17 cell responses.

Soluble CFA/I fimbriae stimulate CD39+CD4+ T cells with regulatory phenotypes
Protection against CIA by Salmonella-CFA/I was associated with induced CD39+CD4+ T cells (41). The frequency of CD39+ Tregs in spleens, MLNs, and LNs from CFA/I fimbriae–treated CIA mice was evaluated. By flow cytometry, all three tissues showed significantly elevated percentages of CD39+CD4+ T cells (Fig. 2A). The MLN and LN CD39+CD4+ T cells from CFA/I fimbriae–treated mice also showed a significantly greater frequency of Foxp3-expressing CD39+CD4+ T cells (Fig. 2B). Concordant with our previous findings for Salmonella-CFA/I (41), TGF-β associated primarily with Foxp3+ CD39+CD4+ T cells, and IL-10 associated with Foxp3+CD39+CD4+ T cells (Fig. 2C). The percentage of LN TGF-β–Foxp3+CD39+CD4+ T cells in CFA/I fimbriae–treated mice was enhanced nearly 2-fold compared with PBS-treated mice (Fig. 2C). The frequency of IL-10–expressing Foxp3+CD39+CD4+ T cells was also significantly increased after treatment with soluble fimbriae (Fig. 2C).

Soluble CFA/I fimbriae stimulate IL-35 production by Foxp3+CD39+CD4+ T cells and suppress IL-27 production by CD11c+ cells
Exogenously administered IL-35 was shown to mitigate CIA by stimulating protective CD39+CD4+ T cells (23). Thus, we queried whether CFA/I fimbriae can stimulate endogenous IL-35 by these induced Tregs. At the conclusion of the study, draining LN lymphocytes were isolated and gated on CD39+CD4+ T cells to measure the extent of EBI3 and p35 expression by flow cytometry. In protected CFA/I fimbriae–treated mice, the percentage of CD39+CD4+ T cells simultaneously expressing EBI3 and p35 subunits was 4-fold greater than in PBS-treated mice and nearly 15-fold greater than in naïve mice (Fig. 3A, 3C). Moreover, the EBI3- and p35-specific mean fluorescence intensities (MFIs) were correspondingly increased in CFA/I fimbriae–treated mice (Fig. 3B).

Soluble IL-35 production by CD4+ T cells correlated with the flow cytometry analysis for IL-35 (Fig. 3A, 3D). CD4+ T cells from CFA/I fimbriae–treated mice showed a 4-fold increase in IL-35 production following CII restimulation. Subsequently, gating on Foxp3+ and Foxp3+CD39+CD4+ T cells, IL-35 predominantly associated with the Foxp3+CD39+CD4+ T cells (Fig. 3E). These results show that CFA/I fimbriae stimulate endogenous IL-35 in mice protected from CIA.

Because IL-27 and IL-35 share the EBI3 subunit, we wondered whether soluble CFA/I fimbriae can also stimulate IL-27. IL-27 is produced by dendritic cells (DCs) and was shown to have immunoregulatory properties with regard to adjuvant-induced arthritis (27) and EAE (28, 30). To assess its expression, flow cytometry was used to analyze splenic and draining LN cells from PBS- and CFA/I fimbriae–treated mice at the termination of the study (day 40). Relatively very few CD4+ T cells expressed EBI3 and IL-27p28 subunits simultaneously (Fig. 4A, 4C). Gated LN CD11c+ cells from CFA/I fimbriae–protected mice showed significantly less EBI3 and p28 coexpression relative to those cells from PBS control mice (Fig. 4A, 4C). Notably, the MFI for p28 was suppressed by CFA/I fimbriae (Fig. 4B). In contrast, the MFI for EBI3 was slightly enhanced (Fig. 4B). To confirm these results, an IL-27 ELISA was performed for CII-restimulated LN cells, and collected supernatants were analyzed, revealing nearly 3-fold less IL-27 secretion by lymphocytes from CFA/I fimbriae–protected mice than from PBS control mice (Fig. 4D). Thus, these data show that IL-27 is not being augmented by CFA/I fimbriae, rather IL-27 is being suppressed.

Protection elicited by soluble CFA/I fimbriae is IL-35 dependent, not IL-27 dependent
Because TGF-β and IL-10 are important for the protection conferred by CFA/I fimbriae, we examined the role of endogenous IL-35 following fimbriae treatment. Mice deficient in IL-35 (EBI3–/–)
were induced with CIA and treated with CFA/I fimbriae on day 14. Interestingly, the disease severity and incidence in EBI3−/− mice were not significantly different from B6 mice; however, protection by CFA/I fimbriae was completely abated (Fig. 5A). To ascertain the contribution of endogenous IL-27 to protection against CIA, subsequent studies tested the therapeutic effect of CFA/I fimbriae in WSX-1−/− mice. As with EBI3−/− mice, WSX-1−/− mice were induced with CIA and treated on day 14 with CFA/I fimbriae. Although PBS-treated mice developed arthritis with a slightly delayed onset and essentially lower average clinical score, CFA/I fimbriae treatment was as effective as in B6 mice in ameliorating CIA (Fig. 5A), suggesting that endogenously induced IL-27 is not important for protection conferred by CFA/I fimbriae. These data implicate the relevance of endogenously induced IL-35 for protection mediated by CFA/I fimbriae, because EBI3−/− mice were unable to resolve CIA. Although EBI3−/− mice are deficient in both IL-27 and IL-35, the failure of IL-27 signaling in WSX-1−/− mice suggests that IL-27 has a lesser or no role in CFA/I fimbriae–conferred protection.

To determine whether the immunodeficient mice could recognize CFA/I fimbriae, end point serum IgG anti-CFA/I fimbriae Ab titers were measured. Both B6 and EBI3−/− mice showed very similar IgG Ab titers, unlike WSX-1−/− mice, which exhibited significantly reduced CFA/I fimbriae–specific IgG1, IgG2a, and IgG2b serum titers (Fig. 5B). The absence of IL-27 signaling correlated with less recognition of the fimbriae, whereas Ab production by EBI3−/− mice was not significantly different from B6 mice.

CII-specific IgG Ab responses were also tested among the three mouse strains on day 35 post-CII challenge. Correlating with clinical and histological findings, significantly less IgG Ab titers were measured in B6 mice treated with CFA/I fimbriae compared with PBS-treated mice (Fig. 5C). In WSX-1−/− mice, only the IgG2a titer was significantly lower than in PBS-treated controls (Fig. 5C). In EBI3−/− mice, CFA/I fimbriae treatment failed to reduce CII-specific IgG subclass Ab titers. Relative to B6 or WSX-1−/− mice, CFA/I fimbriae–treated EBI3−/− mice had elevated levels of CII-specific IgG1, IgG2a,
and IgG2b Abs (Fig. 5C). Thus, these data show that the strength of the CIA-specific IgG Ab responses, as well as average arthritis scores, were muted upon CFA/I fimbriae treatment in mice deficient in IL-27 signaling.

**CFA/I fimbriae suppress Th1 and Th17 cell responses in B6 and WSX-1<sup>−/−</sup> mice while sustaining Treg responses**

To assess the impact of CFA/I fimbriae on Th cell responses as a consequence of the loss of IL-27 and IL-35 function, Ag-specific LN CD4<sup>+</sup> T cells from B6, EBI3<sup>−/−</sup>, and WSX-1<sup>−/−</sup> mice were isolated and restimulated with CII in vitro at day 40 post-CII challenge. Supernatants were subsequently evaluated for the production of proinflammatory and regulatory cytokines. CFA/I fimbriae–treated B6 mice showed a 3–4-fold reduction in Th1 and Th17 cell responses relative to PBS-treated mice (Fig. 6A). In contrast, CFA/I fimbriae–treated EBI3<sup>−/−</sup> mice did not exhibit a reduction in IFN-γ and IL-17 production relative to PBS-treated mice, although IL-6 was partially diminished (by 1.6-fold) (Fig. 6A). CFA/I fimbriae–treated WSX-1<sup>−/−</sup> mice also showed significant reductions in IFN-γ and IL-6, but not IL-17, relative to PBS-treated WSX-1<sup>−/−</sup> mice (Fig. 6A). Thus, in the absence of EBI3, proinflammatory cytokine responses go unabated upon CFA/I fimbriae treatment, and, in the absence of IL-27 signaling, IFN-γ and IL-6 are still repressed by CFA/I fimbriae.

IL-10 and TGF-β were measured after CFA/I fimbriae treatment in B6, EBI3<sup>−/−</sup>, and WSX-1<sup>−/−</sup> mice. IL-10 levels were significantly enhanced, by 12.3- and 5.2-fold in B6 and WSX-1<sup>−/−</sup> mice, respectively, relative to their respective PBS control group, whereas EBI3<sup>−/−</sup> mice showed no significant change upon treatment (Fig. 6A). TGF-β levels exhibited modest 4.7- and 2.1-fold increases in B6 and EBI3<sup>−/−</sup> mice, respectively, upon CFA/I fimbriae treatment (Fig. 6A). Notably, WSX-1<sup>−/−</sup> mice exhibited a large increase (12.7-fold) in TGF-β production (Fig. 6A). Thus, as with B6 mice, WSX-1<sup>−/−</sup> mice are responsive to CFA/I fimbriae treatment, as reflected by the enhanced IL-10 and TGF-β stimulation and concomitant reductions in IFN-γ and IL-6 that correlate with the reduced disease severity in these mice.

It was demonstrated that TGF-β contributes to the upregulation of CD39 because neutralization of TGF-β ultimately dampens the phosphorylation of CREB, decreasing CD39 apyrase expression by CD4<sup>+</sup> T cells (41). To assess the impact of soluble CFA/I fimbriae on CD39 expression, CD39<sup>CD4+</sup> T cell frequencies were measured in PBS- and CFA/I fimbriae–treated B6, EBI3<sup>−/−</sup>, and WSX-1<sup>−/−</sup> mice. The elevated TGF-β in B6 and WSX-1<sup>−/−</sup> mice treated with CFA/I fimbriae correlated with a significant increase in the percentages of CD39<sup>CD4+</sup> T cells present in draining LNs (Fig. 6B). In contrast, CFA/I fimbriae–treated EBI3<sup>−/−</sup> mice failed to show modulation of CD39 expression by their CD4<sup>+</sup> T cells (Fig. 6B). Interestingly, LN CD4<sup>+</sup> T cells showed enhanced expression of CD39 in PBS-treated WSX-1<sup>−/−</sup> mice compared with B6 mice (Fig. 6B), suggesting that WSX-1<sup>−/−</sup> mice may be more resistant to CIA. PBS-treated WSX-1<sup>−/−</sup> mice exhibited reduced disease severity, and this correlated with the enhanced frequency of Foxp3<sup>+</sup> CD39<sup>CD4+</sup> T cells in draining LNs relative to similarly treated B6 mice (Fig. 6C). CFA/I fimbriae treatment enhanced Foxp3 expression in B6 mice but not in WSX-1<sup>−/−</sup> mice because they already had enhanced Foxp3 expression (Fig. 6C). In contrast, the percentage of Foxp3<sup>+</sup> CD39<sup>CD4+</sup> T cells in EBI3<sup>−/−</sup> mice remained unchanged compared with PBS-treated controls, and it was reduced by 30% relative to B6 and WSX-1<sup>−/−</sup> mice (Fig. 6C). Thus, these data show that the protection conferred by CFA/I fimbriae against CIA is not IL-27 dependent, because IL-10 and TGF-β remained to be stimulated; increases in CD39 expression were evident in WSX-1<sup>−/−</sup> mice and, in fact, were augmented by their CD4<sup>+</sup> T cells. In EBI3<sup>−/−</sup> mice, the loss of protection against CIA by CFA/I fimbriae...
correlated with the abolished induction of regulatory cytokines and of CD39+CD4+ T cells.

Endogenous IL-35 is required for protection against CIA because EBI3−/− mice are unable to sustain protection without exogenous IL-35 intervention

Because IL-35 cannot be produced by EBI3−/− mice, we queried whether exogenously administered IL-35 could restore protection. CIA was induced in EBI3−/− mice, and IL-35 treatments were initiated at disease onset (day 21) for 5 consecutive days, similar to those described for WT mice (23). Although IL-35 treatments reduced disease severity and incidence of arthritis (Fig. 7A), the disease course resumed shortly after cessation of treatment, suggesting that an endogenous source of IL-35 is required for continued protection against CIA. Thus, to assess the capacity of Treg-derived IL-35 to suppress arthritis, adoptive-transfer studies were performed in EBI3−/− recipients. Groups of EBI3−/− mice were induced with CIA, as described above, on day 0, followed by adoptive transfer with naive WT or a 1:1 mix of WT and EBI3−/− CD39+CD4+ T cells on day 14 to demonstrate the significance of
this subset’s ability to generate IL-35 following in vivo CFA/I fimbrial stimulation. On the same day following adoptive transfer, recipients were given a single, oral dose of CFA/I fimbriae. One week later, at the time of clinical onset, five consecutive daily doses (1 μg) of rIL-35 were give, similar to those described previously (23) (Fig. 7B). Clinical improvement was evident in EBI3−/− recipients treated with IL-35 or CFA/I fimbriae plus IL-35 over the course of the disease observation (maximum average
clinical score, 2.8 and 3.2, respectively, versus 8 for PBS-treated group, Fig. 7C). Interestingly, over the course of IL-35 treatment and through day 29, 60–80% of the B6 CD39+CD4+ T cell recipients (not treated with CFA/I fimbriae) did not exhibit clinical symptoms of arthritis, although disease incidence reached 80% after day 35 (Fig. 7C). Over the same time frame, 60% of CFA/I fimbriae plus IL-35–treated recipients showed protection early, which decreased to 40% over the course of the disease (Fig. 7C). Notably, in one of three experiments is depicted. *p < 0.05, versus PBS-treated mice. (B) CFA/I fimbriae–specific IgG production is reduced in the absence of IL-27 signaling but is not affected in EBI3-deficient mice. Sera samples were analyzed by ELISA after the clinical study. *p < 0.001, versus EBI3+/− mice, † p < 0.005, versus WT C57BL/6 mice. (C) CIA-specific IgG responses correlate with clinical responses in WT, EBI3−/−, and WSX-1−/− mice. *p < 0.001, **p < 0.005, † p < 0.05, versus corresponding PBS control mice.

Discussion

The pathogenesis of autoimmune arthritis involves autoreactive Th1 and Th17 cells mediating inflammation of the joints, resulting in cartilage and bone destruction. In addition to autoreactive T cells, autoreactive B cells and the recruitment of innate cells into the joints further perpetuate tissue destruction and contribute soluble mediators, including TNF-α, IL-1β, and IL-6 (42, 43). Current methods rely primarily on treating the symptoms and not the cause of the disease, and, arthritis, in general, is plagued by the lack of efforts focusing on the development of Ag-specific interventions (44). Thus, drug-driven induction of Tregs and/or DCs may offer a viable alternative for treating arthritis. In furthering such efforts, CIA is used to test novel therapeutics because it

FIGURE 5. IL-27 signaling is not essential for protection against autoimmune arthritis by CFA/I fimbriae, whereas the lack of the shared EBI3 subunit results in loss of protection. (A) On day 14 after CII challenge, EBI3−/− or WSX-1−/− mice (5/group) were treated with CFA/I fimbriae, and clinical symptoms were monitored. One of three experiments is depicted. *p < 0.05, versus PBS-treated mice. (B) CFA/I fimbriae–specific IgG production is reduced in the absence of IL-27 signaling but is not affected in EBI3-deficient mice. Sera samples were analyzed by ELISA after the clinical study. *p < 0.001, versus EBI3+/− mice, † p < 0.005, versus WT C57BL/6 mice. (C) CIA-specific IgG responses correlate with clinical responses in WT, EBI3−/−, and WSX-1−/− mice. *p < 0.001, **p < 0.005, † p < 0.05, versus corresponding PBS control mice.

recipients given the WT plus EBI3−/− mixture showed no change in IFN-γ or IL-17 production relative to untreated mice (Fig. 7D). Improvement of arthritis was associated with a significant increase in IL-10 in WT Treg recipients treated with either IL-35 or CFA/I fimbriae plus IL-35, whereas TGF-β was greatly augmented only in WT Treg recipients treated with CFA/I fimbriae plus IL-35 (Fig. 7D). In support of this cytokine-secretion analysis, the number of TGF-β+ Tregs, as analyzed by flow cytometry, was significantly elevated for all EBI3−/− recipients given the WT Tregs and treated with CFA/I fimbriae plus IL-35 (Fig. 7E). Likewise, all intervention groups showed an increase in Foxp3+ Tregs (Fig. 7E). Thus, these data show that CFA/I fimbriae–induced IL-35 is required for protection against CIA and that an endogenous source of IL-35 is required to sustain this protection; cessation of IL-35 treatment or dilution of CD39+CD4+ Tregs resulted in continued susceptibility to CIA.

Subsequent analyses aimed to evaluate the cytokine profiles following the intervention in these EBI3−/− mice. Exogenous IL-35 was responsible for significantly suppressing IFN-γ and IL-17 production by CII-restimulated LN CD4+ T cells (Fig. 7D), and this was significantly augmented upon in vivo cotreatment with CFA/I fimbriae, resulting in an even greater reduction in IFN-γ production; the groups treated with IL-35 or CFA/I fimbriae plus IL-35 showed a similar reduction in IL-17 (Fig. 7D). The EBI3−/− mice
shares features with human rheumatoid arthritis with regard to joint pathology and the T cell aspects of this disease (45, 46). Exploiting this model, we previously showed the potency of CFA/I fimbriae, when delivered by an attenuated Salmonella vector, to stimulate increased immune responses. When applied to mice with CIA, the fimbriae also stimulated T-cell-specific Tregs suppressing Th1 and Th17 cells (40, 41). Interestingly, the induced Tregs showed a CD39+CD4+ phenotype. Further analysis revealed that these Tregs could be divided into two subsets based on whether they expressed Foxp3: TGF-β–producing Foxp3+CD39+CD4+ T cells and IL-10–producing Foxp3+CD39+CD4+ T cells (41). Although each subset could confer protection upon adoptive transfer, each provided suboptimal protection compared with CFA/I fimbriae–treated WT mice.**

**Figure 6.** A CII–specific CD4+ T cell cytokine responses in C57BL/6, EBI3−/−, and WSX-1−/− mice upon termination of clinical studies. Bars indicate differences within species tested. *p < 0.001, **p < 0.005, versus similarly treated WT and WSX-1−/− mice, ***p ≤ 0.01, versus similarly treated WT and EBI3−/− mice, †p < 0.05, versus WT mice, ‡p = 0.005, versus CFA/I fimbriae–treated WT mice. (B) LN CD39+CD4+ T cells are induced by CFA/I fimbriae in WT and WSX-1−/− mice. Animals were treated with CFA/I fimbriae on day 14 post-CII challenge, as described above. The frequencies of CD39+CD4+ T cells from individual mice were analyzed by flow cytometry on day 40; bars indicate differences within species tested. *p < 0.001, versus CFA/I fimbriae–treated EBI3−/− and WT mice, **p < 0.05, versus PBS-treated EBI3−/− and WT mice, †p < 0.005, versus CFA/I fimbriae–treated EBI3−/− mice. (C) Foxp3–expressing CD39+CD4+ T cells are induced by CFA/I fimbriae in LNs of WT and WSX-1−/− mice; bar indicates differences within WT mice. *p < 0.001, versus CFA/I fimbriae–treated WT and WSX-1−/− mice, ***p < 0.005, versus WT and EBI3−/− PBS-treated mice.

Given these previous findings, the objectives for the current study were to determine whether CFA/I fimbriae could be administered independently of Salmonella and to further elucidate CFA/I fimbriae’s mechanisms of protection. As shown, a single oral dose was sufficient to confer protection against CIA. Consistent with the Salmonella studies (41), soluble CFA/I fimbriae protected against CIA when given on day 14 post-CII challenge, the time when immune activation occurs in the joints. CFA/I fimbriae effectively offset the development of inflammation in the joints of 70% of the treated mice by suppressing Th1 and Th17 cells, with the concomitant stimulation of IL-10– and TGF-β–producing CD4+ T cells. The latter is important because we showed previously that TGF-β is responsible for promoting CD39 expression (41). Moreover, soluble CFA/I fimbriae stimulated Foxp3 expression by a portion of these CD39+CD4+ T cells. Although Salmonella-CFA/I was shown to activate Foxp3+CD25+ CD4+ T cells during EAE, our results with CIA consistently showed the induction of CD39+CD4 Tregs instead. This is believed to be a consequence of the disease, perhaps due to the increased apoptosis associated with CIA releasing ATP (47), which was shown to activate CD39 cells (48, 49). Expression of CD39 (ectonucleoside triphosphate diphosphohydrolase-1) on CD4+ T cells plays an important role in the immune suppression by induced Tregs (48, 49) and Langerhans cells (50) by hydrolyzing ATP into AMP. In turn, AMP is rendered into adenosine by CD73 (49). CD39 is transcriptionally regulated through cAMP/CREB (51). CFA/I fimbriae–stimulated TGF-β was shown to be involved in the activation of CREB upon its S133 phosphorylation and, thus, promoting CD39 expression (41). Along these lines, when rIL-35 is exogenously administered to mice with CIA, protection correlates with the CD39+CD4+ T cell subset as well; this property is lost in IL-10−/− mice, further implicating the importance of IL-10 in the IL-35 pathway against CIA (23). Because of this similarity in stimulating CD39+CD4+ T cells during CIA, we wondered whether CFA/I fimbriae can also stimulate endogenous IL-35, because previous studies (18, 21) showed that IL-35 is produced by Tregs. Upon examination of CD39+CD4+ T cells from CIA mice treated with soluble CFA/I fimbriae, flow cytometry analysis revealed the simultaneous expression of the EBI3 and p35 subunits. Consistent with what other investigators (21) found, IL-35 was predominantly derived from Foxp3+CD4+.
FIGURE 7. Endogenous IL-35 from CD39+CD4+ T cells is required for protection against arthritis in CFA/I fimbriae–treated mice. (A) Severity and incidence of CIA in EBI3−/− mice treated with five doses of rIL-35 from days 21–25 postchallenge (arrowheads). (B) Scheme of adoptive transfer of donor Tregs into EBI3−/− recipients. CIA was induced in EBI3−/− mice on day 0. On day 14, challenged EBI3−/− mice received naive WT or an equal mix of WT and EBI3−/− CD39+CD4+ T cells. On the same day, EBI3−/− recipients were orally treated with CFA/I fimbriae. Treatment with rIL-35 was performed upon disease onset, as described in (A). (C) Severity and incidence of CIA in recipient treatment groups. *p < 0.005, **p < 0.05, versus PBS control group. (D) Inflammatory and regulatory cytokine production by CII-specific LN CD4+ T cells on day 40 post-CII challenge. *p < 0.001, versus each experimental group, **p < 0.005, #p < 0.001, versus treated EBI3−/− mice. (E) The frequencies of surface TGF-β+ and intracellular Foxp3+ CD39+CD4+ T cells in treatment groups. *p < 0.005, versus treated EBI3−/− recipients given only donor WT CD39+CD4+ T cells, **p < 0.005, **p < 0.001, versus PBS-treated EBI3−/− mice.
T cells, but differed in being CD39+. With regard to the two Treg subsets induced by CFA/I fimbriae, IL-35 associated with the Foxp3+CD39+CD4+ T cells, again suggesting that IL-35 may be linked with the IL-10–producing Tregs and not a separate entity, as suggested by other investigators (52).

IL-35 shares the EB13 subunit with IL-27, which can also have immunoregulatory properties (19, 30–33); thus, it is important to assess the role of IL-27 in these studies. What aims in differentiating their role is that IL-27 and IL-35 are produced by different cells: IL-27 is primarily secreted by APCs (reviewed in Ref. 53), and IL-35 is produced primarily by Tregs (18, 21). Flow cytometry analysis revealed that IL-27 was derived from CD11c+ cells from diseased mice and not from Tregs. In contrast, IL-27 levels were reduced in CFA/I fimbriae–treated CIA mice, as measured by flow cytometry and in Ag-restimulation assays. Finally, CFA/I fimbriae conferred protection in mice with compromised IL-27 signaling, further implicating that the observed protection in B6 mice is independent of IL-27 but dependent on IL-35. In this respect, the CFA/I–driven increase in IL-35 was associated with B6 Foxp3+CD39+CD4+ Tregs and was not derived from CD11c+ cells, although CD11c+ cells from protected mice expressed significantly more EB13, but not simultaneously with p35 (data not shown). Additional FACS analyses failed to reveal B cells expressing IL-35 (data not shown), unlike that shown with EBV–transformed human cells (20), but our analyses do not exclude a potential nonlymphoid source of IL-35 (20). Nonetheless, the IL-35 secretion analysis performed with Ag-restimulated, purified CD4+ T cells obtained from CFA/I fimbriae–treated CIA mice (Fig. 3D) confirmed production from CD4+ T cells. In the absence of IL-35 (EB13+ mice), mice lost the capacity to induce elevated quantifies of IL-10 and TGF-β following CFA/I fimbriae treatment. In fact, the disease progressed unabated, with enhanced IFN-γ and IL-17 production.

IL-27’s mode of action is believed to be attributed to its ability to inhibit RORγt (31) and control Th17 cell development at the priming stage by inducing the expression of PD-L1 on CD4+ T cells (19). IL-27 has potent effects in controlling EAE (19, 30, 31), and this protective response is lost in EB13+ mice (32). In fact, EAE was exacerbated in WSX-1+ mice (33). Similar to EAE, exogenously administered IL-27 was found to be therapeutic for CIA (53, 54); however, few studies have examined the role of endogenous IL-27 (55, 56) or IL-35 (18) in arthritis. Of particular interest in the present studies is the observation that CFA/I fimbriae induced IL-35 production by CD39+CD4+ T cells while comitantly suppressing IL-27 production by CD11c+ cells. CFA/I fimbriae lost their protective capacity in EB13+ mice, but not in WSX-1+ mice, implicating the importance of endogenous IL-35 in mediating CFA/I fimbriae’s protection. Interestingly, the EB13 deficiency did not affect disease severity or incidence of CIA, and disease onset and clinical scores were similar in B6 and EB13+ mice, supporting the notion that unprotected diseased mice produce minimal to no IL-35. This was further substantiated by the analysis of CD39+CD4+ T cells, showing minimal IL-35 production in untreated CIA mice. In contrast, WSX-1+ mice exhibited less severe disease compared with B6 mice, perhaps implicating the importance of IL-27 for effector T cell survival, as recently suggested by the absence of colitis in Rag2−/− mice adoptively transferred with WSX-1−/−CD45RB+CD4+ T cells (57). As in WSX-1+ mice, in vivo neutralization of IL-27p28 in B6 mice had no impact upon CFA/I fimbriae’s capacity to protect against CIA (data not shown). Thus, these collective findings further substantiate the importance of IL-35, rather than IL-27, in CFA/I–mediated protection. Although it remains unclear how CFA/I fimbriae ultimately affect the stimulation of CD39+CD4+ T cells, past studies (38) with macrophages showed that CFA/I fimbriae dampen proinflammatory cytokines (IL-1, IL-6, and TNF-α), suggesting that fimbriae interact with APCs, and these, in turn, stimulate IL-35 production by CD39+CD4+ T cells. Such a possibility is currently being explored.

For the most part, the observed Th1 and Th17 cell responses corresponded to the clinical findings in B6, EB13+ mice, and WSX-1−/− mice. Unprotected B6 and EB13+ mice showed increased production of IFN-γ, IL-6, and IL-17, as well as reduced regulatory cytokines, IL-10 and TGF-β. Following CFA/I fimbriae treatment, B6 and WSX-1−/− mice, but not EB13+ mice, showed corresponding reductions in IFN-γ, IL-6, and IL-17. In contrast, WSX-1−/− mice showed marked increases in TGF-β compared with similarly treated B6 mice. This resistance to CIA may be attributed, in part, to the increases in CD39+ Tregs, because they were increased in both PBS- and CFA/I fimbriae–treated WSX-1−/− mice. TGF-β was shown to augment CD39 expression (41), and the increased presence of Tregs and/or decreased activation of effector T cells (56) could account for these findings. In contrast, CFA/I fimbriae–treated EB13+ mice showed elevated IFN-γ and IL-17 relative to PBS-treated CIA mice, and IL-10 and TGF-β remained unchanged despite treatment.

The importance of endogenous IL-35 in CFA/I fimbriae–mediated protection against arthritis became evident when EB13+ mice showed no response to CFA/I fimbriae treatment. Such a finding compelled testing whether exogenous IL-35 could substitute for CFA/I fimbriae in the absence of endogenous IL-35; the data showed that such treatment subduced disease as long as IL-35 treatments were maintained, and once interventions ceased so did protection. These results evoked whether WT Tregs responding to CFA/I fimbriae treatment could restore protection in EB13+ mice. It was shown that intervention with rIL-35 at the onset of CIA strongly protected B6 mice during the entire disease course (23). In the current study, EB13+ mice required continuous administration of IL-35, because disease quickly developed upon cessation of treatment. Likewise, soluble CFA/I fimbriae protected immunocompetent mice against CIA, or as long mice had an endogenous source of IL-35. To restore protection against CIA in EB13+ mice, WT CD39+CD4+ T cells were adoptively transferred as a source of endogenous IL-35 when induced by CFA/I fimbriae. Because naive Tregs were used in the adoptive transfer, exogenous IL-35 was also administrated and compared with EB13+ recipients treated only with IL-35. EB13+ recipients treated with CFA/I fimbriae plus IL-35 or IL-35 alone showed similar clinical disease, but mice treated with IL-35 alone showed greater disease incidence. These data suggest that EB13+ recipients treated with CFA/I fimbriae alone would be insufficient for sustaining IL-35 signaling to thwart further disease, and collectively suggest that another cell source of IL-35 is required. Such a possibility is being investigated. Collectively, these data further implicate the importance of endogenous IL-35, whether derived solely from Tregs and/or other unknown sources. This was more prominent in EB13−/− recipients given equal amounts of WT and EB13−/− CD39+CD4+ T cells, diluting the WT Treg effect, and for which no protection was observed even upon optimal intervention by CFA/I fimbriae plus IL-35. Minimal, these findings point to the fact that a sufficient source of endogenous IL-35 must be present to attain a qualitative therapeutic effect. This loss of protection correlated with enhanced IFN-γ and IL-17 production, whereas protected EB13−/− recipients treated with CFA/I fimbriae plus IL-35 exhibited marked reductions in proinflammatory cytokines and increased TGF-β and IL-10 production, with concomitant increases in Tregs. These findings are consistent
with those reported previously for CFA/I fimbriae stimulating TGF-β and IL-10 production by CD39+ Tregs for protection against CIA (23, 41). Based on the results of this study, coupled now is the additional requirement for IL-35 to guard against arthritis, and the sequence of each cytokine’s release during protection remains to be determined. Clearly, in the absence of IL-35, TGF-β and IL-10 are markedly reduced; conversely, in the absence of IL-10, IL-35 cannot render protection against CIA (23). It remains to be determined how these three cytokines interact, as well as whether IL-35 serves as an anti-inflammatory amplifier (adjuvant) to augment, and possibly sustain, TGF-β and IL-10 production.

In conclusion, these studies demonstrate that soluble CFA/I fimbriae have potent immunomodulatory activity, eliminating the requirement for a live vector. Importantly, single, low oral dose of CFA/I fimbriae conferred protection against CIA, and prior stimulation with CFA/I fimbriae protected against arthritic progression. The requirement for IL-35 to guard against arthritis is evident in EBI3−/− mice, which do not produce IL-35. Furthermore, studies have shown that IL-35 production depends on the cytokines that are produced by the CFA/I fimbriae stimulation. IL-35 overexpression has been shown to confer protection against CIA in EBI3−/− mice, and the lack of IL-35 production is associated with increased IL-17 and TNF-α production.

Disclosures
The authors have no financial conflicts of interest.

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