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*J Immunol* 2010; 184:7144-7153; Prepublished online 10 May 2010;
doi: 10.4049/jimmunol.0902739
http://www.jimmunol.org/content/184/12/7144

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IL-35 is produced by regulatory T cells, and this novel cytokine can downregulate Th17 cell development and inhibit autoimmune inflammation. In this work, an rIL-35, as a single-chain fusion between murine IL-12p35 and EBV-induced gene 3, was expressed in yeast. This rIL-35 inhibited OVA-specific cellular and Ab responses in OVA-challenged recipients of DO11.10 CD4+ T cells. Likewise, IL-35 inhibited clinical manifestation of collagen-induced arthritis or could cease further disease exacerbation upon initiation of IL-35 treatment. Exogenous IL-35 treatments suppressed Th1 and Th17 cells and promoted CD39 expression by CD4+ T cells. Sorted CD25+CD39+CD4+ T cells from IL-35-treated mice produced IL-10 and, upon adoptive transfer, were sufficiently potent to inhibit subsequent development of inflammation in mice with collagen-induced arthritis, whereas sorted CD25+CD39− CD4+ T cells showed reduced potency. IL-35 treatments of IL-10−/− mice failed to induce protective CD39+CD4+ T cells, demonstrating the effector role of IL-10 by IL-35 immunosuppression.

E pstein-Barr virus-induced gene 3 (EBI3) encodes the 34-kDa protein that shows structural similarity with the class I cytokine receptor family and can heterodimerize with p28 to form IL-27 (1) or with IL-12p35 subunit to form IL-35 (2). IL-27, produced primarily by APCs, binds WSX-1 receptor and promotes Th1 cell development (1), as evident in a number of studies. Blocking IL-27p28 suppresses adjuvant-induced arthritis in rats (3) and experimental autoimmune encephalomyelitis (EAE) accompanied by diminished IFN-γ (4). EBI3−/− CD4+ T cells feature significantly higher IFN-γ but lower IL-4 production than EBI3+/+ CD4+ T cells (5). In contrast, mice deficient in the IL-27R subunit WSX-1 show enhanced EAE (6), perhaps suggesting a regulatory dependency by IL-27R. Such regulatory potential is further implicated by the recent discovery of EBI3 expression by regulatory CD4+ T cells and its association with IL-12p35, forming IL-35 (7, 8). Accordingly, IL-35 could inhibit experimental colitis (7) and dampen collagen-induced arthritis (CIA) in DBA/1 mice via suppression of Th17 cells (8).

Natural regulatory CD4+ T cells designated by coexpression of CD25 have been shown to be potent inhibitors of autoimmune inflammation in a contact-dependent fashion and express FoxP3 (9). Other regulatory CD4+ T cell subsets are induced upon immunization with Ag; one such subset similar to natural regulatory T cells (Treg) is capable of suppressing autoimmune responses to unrelated Ags (10, 11); another subset (Th3 cells) is characterized by production of TGF-β (12); and a third subset (Tr1 cells) suppresses via IL-10 and is phenotypically diverse among CD25+, CD25−, FoxP3+, and FoxP3− CD4+ T cells (13). Recently, a new Treg cell subset has been described based on its CD39 expression, an extracellular ectonucleoside triphosphate diphosphohydrolase-1 enzyme, capable of controlling activated lymphocytes through conversion of extracellular ATP (14). Enhanced CD39 expression has recently been found associated on both CD25− and CD25+ CD4+ T cells following therapeutic intervention with a Salmonella vaccine carrying enterotoxigenic Escherichia coli colonization factor Ag I in DBA/I mice with CIA (15). Unlike our previous observations in which the same vaccine stimulated Treg cells capable of suppressing EAE in an Ag-independent fashion (11), when a similar approach was adopted for CIA, only partial protection was conferred by Treg cells, but complete protection was conferred by total CD4+ T cells containing the CD39+ subset (15).

Inquiring into the potency of IL-35 as a therapeutic for arthritis, this current study examines the regulatory function of exogenously applied rIL-35 when it is expressed as a single polypeptide fusion between the murine IL-12p35 subunit with EBI3. This rIL-35 was tested in two different models: DO11.10 CD4+ T cells adoptively transferred with subsequent challenge with OVA and CIA in C57BL/6 mice. IL-35 inhibited cellular and anti-OVA Ab responses. IL-35 treatments of C57BL/6 mice inhibited CIA via IL-17 and IFN-γ suppression by CD39+ CD4+ T cells, producing IL-10. Thus, these studies identify a mode of in vivo action by IL-35.

Materials and Methods

Mice

BALB/c and C57BL/6 6- to 8-wk-old males (The Jackson Laboratory, Bar Harbor, ME, or Charles River Laboratories, Wilmington, MA), breeding colonies of DO11.10 TCR-transgenic mice, and IL-10−/− mice were maintained at Montana State University Animal Resources Center (Bozeman, MT) in individual ventilated cages. All procedures were compliant with institutional policies for animal health and well being.

Acknowledgments

rIL-35

IL-35 was produced as a single polypeptide fusion between mouse IL-12p35 subunit and mouse EBI3 via a bovine elastin linker (VPGVGVPGVG), as previously used for IL-12 (16). p35-eb3 from pGT146mIL-12 (Invivogen, San Diego, CA) was amplified with primers containing 5’ EcoRI and 3’ SalI restriction sites. EBI3 from pORF mIL-27 (Invivogen) was amplified with primers containing 5’ SalI and 3’ KpnI restriction sites. Fragments were cloned into Topo vectors (Invitrogen/Life Technologies, Carlsbad, CA), excised, and then a tripartite ligation was performed on p35-eb3 EcoRI/Sall, EBI3 SalI/KpnI, and the Pichia pastoris expression vector pPICZB EcoRI/KpnI. EcoRI primer bears an ATG initiation codon. Primers maintained an open reading frame between the two active components, as well as into the His-tag borne on the expression vector for protein purification. P. pastoris was transformed with a linearized construct via electroporation (Electro Cell Manipulator ECM630, BTX Harvard Apparatus, Holliston, MA). Yeast colonies were screened, and the final clone was grown following Easy Select Expression kit (Invitrogen).

For expressed protein purification, cells were disrupted by bead beating (BioSpec Products, Bartlesville, OK) and spun to remove debris, and filtered (0.45 μm) supernatant was applied to a Talon affinity resin column (BD Biosciences, San Jose, CA) per manufacturer’s instructions. The purified protein was probed by Western blot using anti-mouse EBI3 mAb (BD Biosciences, San Jose, CA) and was subsequently dialyzed against PBS and stored at −80°C.

OVA-specific delayed-type hypersensitivity and Ab responses

CD4+ T cells were purified using Dynal Mouse CD4 Negative Isolation kit (Invitrogen) from DO11.10 spleens and lymph nodes (LNs). Purified CD4+ T (3 × 10⁶) cells were adoptively transferred to sex- and age-matched BALB/c mice. One day later, recipients were challenged with 0.5 μg OVA, and mice were treated daily with 0.75 μg IL-35 or half with 200 μg PBS daily until day 5, when a delayed-type hypersensitivity (DHT) test was performed, as previously described (17). DHT response was the difference in ear swelling between OVA- and PBS-challenged pima.

On day 7, collected sera were evaluated by OVA-specific ELISA (17). Endpoint titers were expressed as the reciprocal logarithm of two for serum dilutions with an absorbance ≥0.1 above negative control.

OVA-specific T cell assays

DO11.10 CD4+ T cell recipients were challenged with OVA and treated with IL-35 or sterile PBS as described above. On day 7, mice were sacrificed, and splenic and LN CD4+ T cells were purified by negative isolation to >95% of purity. Purified CD4+ T cells were resuspended in complete medium, and 2.5 × 10⁶ cells/well in 200 μl were restimulated with 1 μg/ml OVA23–339 peptide with syngenic irradiated (3000 rad) APCs. After 48 h, cells were pulsed with [3H]thymidine (0.5 μCi/well) for an additional 18 h. Cultured cells were harvested, and incorporated radioactivity was measured by scintillation counting. Stimulation index (SI) was calculated as (CPMstim − CPMunstim)/CPMunstim.

For cytokine production, purified CD4+ T cell cultures were restimulated for 4 d; supernatants were collected and analyzed by cytokine-specific ELISA.

CIA induction and IL-35 treatments

To induce CIA in C57BL/6 mice, lyophilized chicken collagen II (CII) (Chondrex, Redmond, WA) was solubilized in 0.05 M acetic acid at 2 mg/ml at 4°C 24 h prior to use, and then 100 μg chicken CII emulsified in CFA containing 4 mg/ml Mycobacterium tuberculosis (Chondrex) were injected s.c. in the tail 1.5–2 cm from the mouse body (Chondrex protocol; 15, 18). Using this method, by day 35, nearly 100% of mice consistently showed clinical symptoms (15). Treatments with 0.75 μg IL-35 or sterile PBS were performed daily beginning day 21 post CIA challenge to the study termination. Mice were scored using a scale of 0–3 for each limb for a maximal total score of 15 (15): 0, no signs of inflammation; 1, mild redness or swelling of single digits; 2, significant swelling of ankle or wrist with erythema; and 3, severe swelling and erythema of multiple joints. Incidence of arthritis was the percent of animals with CIA in the group. Severity of the

FIGURE 1. rIL-35 suppresses OVA-specific immune responses. A. Schematic of murine IL-35 and Western blot of purified protein detected with anti-histag mAb, goat anti-IL-12 Ab, or anti-EBI3 mAb. B. OVA-specific DTH response. Sorted DO11.10 CD4+ T cells (>95% purity) were adoptively transferred to sex- and age-matched BALB/c mice. One day later, recipients were challenged with 100 μg OVA in IFA s.c.; half of the recipients was treated i.p. with 0.75 μg IL-35 and half with 200 μl sterile PBS. Mice were treated with IL-35 or PBS daily until day 5, when a delayed-type hypersensitivity (DHT)
disease was described by average clinical score. In addition, swelling in both knee joints and four paws was measured with an electronic digital caliper (World Precision Instruments, Sarasota, FL) in anesthetized mice on day 42 post CIA challenge, and cumulative swelling was calculated as a sum of all measurements per mouse.

**Histopathological analysis of CIA**

At study termination, joint sections were prepared, stained, and scored (15): 0, no changes; 1, synovial hyperplasia and mild inflammatory infiltration; 2, pannus formation with cartilage degeneration; and 3, heavy inflammatory infiltration, severe bone deformity and cartilage destruction. Cartilage degeneration was evaluated in toluidine blue-stained sections on a graded scale of 0–3: 0, no cartilage loss; 1, minimal chondrocytes and proteoglycan loss in superficial zone; 2, moderate chondrocytes and proteoglycan loss into middle zone above tidemark; and 3, severe cartilage degeneration through tidemark.

**CII-specific Ab responses**

Serum samples were collected from individual mice with CIA on days 21 and 35 (at peak of disease) postchallenge. Diluted sera were added to microtiter plates coated with 2 μg/ml ELISA-grade chicken CII (Chondrex) as previously described (15). Goat anti-mouse HRP-labeled IgG1, IgG2a, or IgG2b (Southern Biotechnology Associates, Birmingham, AL) were used as detecting Abs. Enzymatic reaction was developed with ABTS (Moss, Pasadena, MD). OD was read at 415 nm using ELx808 microplate reader (Bio-Tek Instruments, Winooski, VT). Endpoint titer represented a reciprocal logarithm of 2 for the serum dilution with an OD ≥0.1 above negative control.

**CII-specific T cell assays**

CD4+ T cells from axillary, popliteal, and inguinal LNs from IL-35– or PBS-treated C57BL/6 mice were purified (>95% purity) by magnetic bead separation using Dynal Mouse CD4 Negative Isolation kit (Invitrogen) at 42 d post CII challenge. The purified CD4+ T cells (10^6/ml) were restimulated with 50 μg/ml plate-bound anti-CD3 mAb (eBioscience, San Diego, CA) and 10 μg/ml soluble anti-CD28 mAb (eBioscience) for 4 d. Cytokine-specific capture ELISAs were performed on collected supernatants.

For CIA-specific T cell proliferation assay, CD39+ and CD39– CD4+ T cells from IL-35– or PBS-treated mice were isolated (>90% purity) as described above, and 10^3 cells/200 μl were restimulated with 50 μg/ml T-cell Proliferation Grade CII (Chondrex) in the presence of irradiated (3000 rad) syngenic APCs. During the last 18 h of culture, [3H]thymidine incorporation was measured, and SI was calculated as above.

**Flow cytometry**

Individual spleens and LNs from PBS- or IL-35–treated mice were analyzed on day 28 post CIA challenge. Lymphocytes were fluorescently labeled with fluorochrome-conjugated mAbs: CD25 (BD Pharmingen, San Diego, CA), CD4, CD39, and FoxP3 (eBioscience). Fluorescence was acquired on an LSR II flow cytometer (BD Biosciences) using BD FACSDiva software, and samples were analyzed using FlowJo (Tree Star, Ashland, OR) software.

**Adoptive transfer of CD39+ and CD39– CD4+ T cells**

CIA was induced in C57BL/6 mice on day 0. On day 7, daily 0.75 μg treatments with IL-35 were initiated in separate groups of IL-10+/+ and IL-10–/– mice for 1 wk. On day 15 post CIA challenge, 10^6 CD39+ CD4+ and CD39–CD4+ T cells, sorted as described above, from IL-35– or PBS-treated mice were adoptively transferred to mice with CIA.

**Adoptive transfer of CD25+ CD39+CD4+ and CD25–CD39–CD4+ T cells**

CIA was induced in C57BL/6 mice on day 0 as described. Treatments with rIL-35 were initiated on day 7 in a separate group of C57BL/6 mice and treated daily for 1 wk. Purified total CD4+ T cells from IL-35–treated mice were stained with FITC–anti-CD4, Alexa Fluor 647–anti-CD39
and then sorted to CD25−CD39+ and CD25+CD39+ T cell subsets by BD FACSAria flow cytometer (BD Biosciences). On day 15 post CII challenge, mice were given i.v. 2 × 10^7 IL-35–induced, FACS-sorted CD25−CD39+ CD4+ or CD25+CD39+ CD4+ T cells (>95% purity). Mice were subsequently monitored for CIA development.

Statistics

Mann-Whitney U test was used for statistical analysis of DTH response, clinical scores, joint cumulative swelling, histology scores, and cartilage destruction. Fisher’s exact probability test was applied for incidence of CIA. One-way ANOVA and Student t test were performed to analyze T cell proliferation, flow cytometry, OVA-, CII-, and cytokine-specific ELISA data. Results were considered statistically significant if the p value was <0.05.

**Results**

**Recombinant single-chain mouse IL-35**

Mouse IL-35 was expressed as a single polypeptide between murine IL-12p35 and EBI3 genetically fused via an elastin linker (Fig. 1A) for expression in P. pastoris. The IL-35 was purified via its histag and recognized by Western blot using mAbs specific for histag or mouse EBI3, as well as by a polyclonal goat anti-mouse IL-12 Ab (Fig. 1A).

rIL-35 suppresses OVA-specific CD4+ T cell and Ab responses

To test the functional activity of rIL-35, a model of OVA-specific immunity was used. DO11.10 CD4+ T cells were sorted and transferred to BALB/c mice, challenged with OVA, and subsequently treated daily i.p with IL-35 or sterile PBS. On day 5, a DTH test was performed and ear thickness measured, revealing a 3-fold suppression to OVA-induced ear swelling by the IL-35–treated mice relative to PBS-treated mice (Fig. 1B). OVA-specific serum IgG2a and IgG2b Abs were significantly lessened by the IL-35 treatments (Fig. 1C), demonstrating that in vivo IL-35 treatments inhibit OVA-specific responses.

To investigate the impact of IL-35 upon OVA-specific Th cell responses, splenic and LN CD4+ T cells were sorted and evaluated in a T cell proliferation assay. Greater than 3-fold and ∼2-fold reductions in T cell proliferation by OVA-restimulated splenic and LN CD4+ T cells from IL-35–treated mice, respectively, were observed (Fig. 1D). Cytokine analysis of OVA-restimulated CD4+ T cells revealed ∼2.5-, 4-, and 8-fold reductions in IFN-γ, IL-6, and IL-17 production, respectively, by IL-35–treated mice relative to PBS-treated mice (Fig. 1E). IL-35 treatment resulted in significant suppression of IL-4 and IL-13 and concomitant increases in IL-10 and IL-9, but no changes in TGF-β were observed (Fig. 1E). These data demonstrate the immunosuppressive properties of IL-35 in an OVA-specific fashion.

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**FIGURE 3.** IL-35 suppresses CII-specific Ab and CD4+ T cell responses. A, Serum CII-specific Ab titers from PBS- or IL-35–treated mice as described in Fig. 2. Individual serum samples were collected on day 21 (prior first treatment) and on day 35 (1 wk after last treatment). *p < 0.01; **p < 0.05 as compared with PBS group on day 35. B, Cytokine production by CII-specific LN CD4+ T cells. Purified CD4+ T cells (10^6 cell/ml, >95% purity) were restimulated with CII in presence of syngenic irradiated APCs for 4 d. Mean cytokine concentrations from triplicate cultures ± SEM are depicted. *p < 0.001; **p < 0.005. Data are representative of three experiments. ND, not detected.

**FIGURE 4.** IL-35 ceases further progression of established CIA. CIA was induced as described in Fig. 2, and mice were treated daily i.p. with sterile PBS or IL-35 (0.75 μg) beginning day 35 through day 38 post CII challenge. A, Average clinical score per group; arrows indicate treatment time points. *p < 0.05 between groups. B, ∆ Cumulative swelling calculated as a difference between cumulative swelling of individual mice in PBS- (n = 10 mice) or IL-35–treated (n = 11 mice) groups and average cumulative swelling in naïve mice. Measurements were performed in anesthetized mice on day 42 post CII challenge. *p < 0.05.
IL-35 inhibits development of CIA in C57BL/6 mice
IL-35 was tested for its ability to suppress CIA. Arthritis can be induced in H-2b mice upon immunization with heterologous CII emulsified in CFA (18, 19), showing similar time of onset, clinical disease, and histopathology as CIA in DBA/1 mice (15). Starting 21 d post CIA induction, treatments with PBS or IL-35 were initiated daily until day 28. Only 10% of IL-35–treated mice showed clinical symptoms of arthritis, unlike PBS-dosed mice, which developed CIA over the expected time course with 100% incidence and average clinical score of ~6.5 (Fig 2A). Upon study termination, joint sections were stained with H&E, and some synovial hyperplasia was observed in median joint section of IL-35–treated mice, unlike the severely damaged joints obtained in PBS-treated animals (Fig. 2B). Sections stained with toluidine blue revealed minimal chondrocytes and proteoglycan loss in cartilage of IL-35–treated mice, whereas in control mice, the cartilage layer was irreversibly destroyed (Fig. 2B). On average, significantly lessened histopathological scores and cartilage losses were found in IL-35–treated mice when compared with PBS-treated mice (Fig. 2C). Thus, these experiments demonstrate that IL-35 is capable of suppressing the development of CIA.

IL-35 suppresses CII-specific Ab responses
To investigate the effect of exogenous IL-35 on CII-specific B cell responses, serum IgG1, IgG2a, and IgG2b anti-CII Ab levels from IL-35– or PBS-treated mice were evaluated pre- and posttreatments.

**FIGURE 5.** IL-35 stimulates induction of regulatory CD39+CD4+ T cells. CIA was induced, and IL-35 or PBS treatments were conducted as described in Fig. 2. On day 28, flow cytometry analysis was performed on stained splenic and draining LN lymphocytes, assessing CD39, CD25, and Foxp3 expression. A, Enhanced CD39 expression by splenic CD4+ T cells following IL-35 treatments. B, Frequencies of splenic and LN CD39+CD4+ T cells (six individual mice per group), **p < 0.001; **p < 0.05. C, Foxp3 expression in LN CD39+CD4+ T cells. Shaded histogram is isotype control (rat IgG2a). Mean fluorescence intensity (MFI) is 1023 ± 79 and 1731 ± 52 for PBS- and IL-35–treated mice, respectively, **p = 0.002. D, Frequencies of Foxp3+CD39+CD4+ T cells in spleens and LNs of PBS- or IL-35–treated mice (six individual mice per group). **p < 0.001. E, CD39+CD4+ T cells include CD25+CD4+ and CD25+CD4+ T cell subsets in PBS- and IL-35–treated mice. Gated LN CD39+CD4+ T cells are depicted. F, Foxp3 expression by LN CD25+CD4+ and CD25+CD4+ T cells following treatment with PBS or IL-35. MFI for Foxp3+CD25+CD4+ T cells is 1127 ± 82 and 2108 ± 42 for PBS- and IL-35–treated mice, respectively, **p < 0.001 between treatment groups. MFI for Foxp3+CD25+CD4+ T cells is 494 ± 112 and 612 ± 105 for PBS- and IL-35–treated mice, respectively, **p < 0.001 between treatment groups. Shaded histogram is rat PE-IgG2a stained control. G, At 42 d post CIA challenge, a T cell proliferation assay using cell-sorted CD39+CD4+ and CD39+CD4+ T cells (>90% purity) from mice treated with IL-35 or PBS was performed. Mean SI ± SEM is depicted from triplicate cultures, **p < 0.001; ***p < 0.01.
treated mice did not worsen during and posttreatment until day 42, which showed an average clinical score of ∼4. In contrast, the clinical disease exhibited by the PBS-treated mice continued to progress to a maximum score of ∼7 by day 39 (Fig. 4A). Upon termination of the study, swelling of knee joints and paws in mice from both groups was measured and compared with age- and sex-matched naive C57BL/6 mice (Fig. 4B). The IL-35–treated mice showed lessened joint edema as compared with PBS-treated mice, further demonstrating that exogenous IL-35 is a potent inhibitor of autoimmune inflammation.

**IL-35 protects against CIA by the stimulation of regulatory CD39^+CD4^+ T cells**

Our previous studies suggest that increased CD39 expression by CD25^−CD4^+ T cells could be essential for conferring therapeutic intervention against CIA (15). To analyze the types of regulatory cells induced by IL-35 treatment, CD4^+ T cells from spleens and pooled draining LNs of individual CII-challenged, IL-35–treated mice were analyzed for CD39, CD25, and FoxP3 expression. IL-35 treatment significantly increased the frequency of CD39^+CD4^+ T cells in spleens and LNs (Fig. 5A, 5B) with enhanced FoxP3 expression (Fig. 5C, 5D). Average frequencies of CD39^+CD4^+ T cells in spleens of IL-35– and PBS-treated mice were 38.6 ± 1.05% and 23.9 ± 1.44% of total CD4^+ T cells, respectively (p < 0.001). Percent of FoxP3^+CD39^+CD4^+ T cells in spleens of IL-35–treated mice was 27.8 ± 0.80% and PBS-treated mice 14.1 ± 1.43% (p < 0.001). Frequencies of CD39^+CD4^+ T cells in draining LNs were 21.3 ± 1.1% and 16.7 ± 0.72% in IL-35– and PBS-treated mice, respectively (p < 0.05). Expression of FoxP3 in LN CD39^+CD4^+ T cells subset was notably enhanced: 55.3 ± 4.35% in IL-35–treated mice versus 19.6 ± 3.41% in PBS-treated mice (p < 0.001) (Fig. 5C, 5D). As a subset of CD39^+CD4^+ T cells, both PBS- and IL-35–treated groups displayed similar CD25^+CD4^+ T cell frequencies (Fig. 5E). Notably, both CD25^+CD4^+ and CD25^−CD4^+ T cells demonstrated higher FoxP3 expression after IL-35 treatment (Fig. 5F). CII-restimulated, cell-sorted total CD39^−CD4^+ and CD39^+CD4^+ T cells from PBS-treated and from protected mice revealed that IL-35 treatment supports proliferation of CD39^+CD4^+ T cells (Fig. 5G).

**In vivo IL-35 promotes IL-10 production**

Because FoxP3 associated with both Treg and CD39^+CD4^+ T cells and implicated the stimulation of a mixed regulatory T cell response, the cytokine profiles of sorted CD39^+CD4^+ and CD39^−CD4^+ T cells from C57BL/6 and IL-10^−/− mice treated with IL-35 or sterile PBS were investigated. Groups of mice were treated daily with IL-35 or PBS for 7 d and then sorted for their CD4^+ T cell subsets. CD39^−CD4^+ T cells from IL-35–treated C57BL/6 and IL-10^−/− mice produced significantly less IFN-γ in comparison with PBS-treated C57BL/6 and IL-10^−/− mice (Fig. 6A). IL-35 treatment did not alter IL-17 production by C57BL/6, but enhanced 5-fold IL-17 production by IL-10^−/− CD39−CD4+ T cells (Fig. 6A). IL-4 and IL-13 production by CD39−CD4+ T cells was suppressed in both PBS- and IL-35–treated IL-10^−/− mice and netted a 2-fold reduction in IL-4 by C57BL/6 mice, but IL-35 treatment augmented 4-fold IL-10 production by C57BL/6 CD39^+CD4^+ T cells (Fig. 6A).

**FIGURE 6.** Cytokine profiles of CD39^− and CD39^+CD4^+ T cells from IL-10^+/+ and IL-10^−/− mice after IL-35 or PBS treatment. Naive mice were given PBS or IL-35 for 7 d, and then splenic and LN CD4^+ T cells were sorted for CD39^− and CD39^+CD4^+ T cell subsets (>95% purity). Each (10^6 cells/ml) was stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs for 4 d. Mean concentration of cytokine in supernatant ± SEM from triplicate cultures is shown. A, CD39^−CD4^+ T cells’ cytokine profile. *p < 0.001; **p < 0.005; ***p < 0.01. B, CD39^+CD4^+ T cells’ cytokine profile. *p < 0.001; **p < 0.01; ***p < 0.05 between IL-10^+/+ and IL-10^−/− mice.
Evaluation of CD39⁺CD4⁺ T cells from C57BL/6 and IL-10⁻/⁻ mice revealed minimal to no IFN-γ responses (Fig. 6B). IL-35–treated C57BL/6 mice showed marked IL-17 suppression by CD39⁺CD4⁺ T cells, unlike IL-10⁻/⁻ CD39⁺CD4⁺ T cells that produced elevated levels of IL-17 and modestly enhanced IL-9 (Fig. 6B). CD39⁺CD4⁺ T cells from IL-35–treated C57BL/6 mice produced >10-fold more IL-10, but IL-4, IL-9, and IL-13 remained unaffected. Thus, these studies reveal in vivo IL-35 treatment stimulates IL-10 production.

IL-35–induced CD39⁺CD4⁺ T cells protect against CIA

To ascertain whether IL-35–induced CD39⁺CD4⁺ T cells are protective against CIA, adoptive transfer studies were performed. CIA was induced in C57BL/6 mice, and separate groups of C57BL/6 and IL-10⁻/⁻ mice were treated with IL-35 daily for CD39⁺CD4⁺ T cell induction (Fig. 7A). On day 15, CII-challenged mice received sorted CD39⁻CD4⁺ or CD39⁺CD4⁺ T cells from IL-35–treated mice (Fig. 7A). CD39⁺CD4⁺ T cells from IL-35–treated C57BL/6 mice significantly reduced incidence and clinical scores by recipients (Fig. 7C), mimicking protective efficacy obtained with IL-35 (Fig. 2A). Notably, CD39⁺CD4⁺ T cells from IL-35–treated IL-10⁻/⁻ mice failed to confer protection against CIA, much like PBS-treated C57BL/6 mice (Fig. 7C), supporting the notion that IL-10 plays an important effector role in IL-35–mediated immune suppression. Upon termination of study, CD4⁺ T cell cytokine analysis revealed significant IFN-γ and IL-17 reductions in recipients of C57BL/6 CD39⁺CD4⁺ T cells (Fig. 7E), unlike recipients given IL-10⁻/⁻ CD39⁺CD4⁺ T cells, in which IFN-γ and IL-17 levels were similar to recipients given PBS-treated C57BL/6 CD39⁺CD4⁺ T cells (Fig. 7E). IL-10 was produced only by protected mice, the recipients of

**FIGURE 7.** IL-35–induced CD39⁺CD4⁺ T cells from IL-10⁺⁺, not IL-10⁻/⁻ mice, confer protection to CIA. A, Scheme for adoptive transfer experiment. CIA was induced in recipient groups on day 0. On days 7–14, IL-35 was given to donor mice. On day 15 postinduction of CIA, recipient mice were adoptively transferred with 10⁶ of cell-sorted CD39⁻ or CD39⁺ CD4⁺ T cells (>93% purity) from IL-10⁺⁺ or IL-10⁻/⁻ mice. B, Severity of arthritis and incidence of the disease in recipients of CD39⁺ CD4⁺ T cells are shown. C, Severity of arthritis and incidence of the disease in recipients of CD39⁺CD4⁺ T cells are shown. Data represent mean clinical score per group of 10 mice ± SEM. *p < 0.001; **p < 0.005; ***p < 0.05. One of three experiments is depicted. CD4⁺ T cell cytokine production by recipients given CD39⁺CD4⁺ T cells (D) and by recipients given CD39⁺CD4⁺ T cells (E) is shown. LN cells were restimulated with CII for 4 d. as previously described. *p < 0.001; **p < 0.005; ***p < 0.05.
CD39⁺CD4⁺ from IL-35–treated C57BL/6 mice (Fig. 7E). Recipients of IL-35–treated C57BL/6 CD39⁺CD4⁺ T cells produced less IFN-γ, although no significant changes in IL-17 production were observed (Fig. 7D).

IL-35–induced CD25⁺CD39⁺CD4⁺ T cells are potent regulatory cells for CIA

Based on the finding that regulatory CD39⁺CD4⁺ T cells include both CD25⁻ and CD25⁺ phenotypic subsets, additional analyses were conducted to test the protective properties of these T cell subsets. As described above, CIA was induced in C57BL/6 mice on day 0, and on day 7, IL-35 treatments were initiated in a separate group of C57BL/6 mice. On day 15, CIA-challenged mice were adoptively transferred with cell-sorted CD25⁺CD39⁺CD4⁺ or CD25⁻CD39⁺CD4⁺ T cells from the IL-35–treated mice, and mice were monitored for development of clinical disease until day 39. Recipients of CD25⁺CD39⁺CD4⁺ T cells did not show any clinical symptoms during the observation period. In contrast, 40% of the CD25⁻CD39⁺CD4⁺ T cell recipients developed delayed clinical disease ~9 d later than in the PBS-treated control group, although these recipient mice did exhibit significantly lessened clinical disease (p = 0.006; Table I). The maximum score for the PBS-treated mice was 10, whereas the CD25⁺CD39⁺CD4⁺ T cell recipients showed a clinical score of 6. These results indicate that both regulatory CD39⁺CD4⁺T cells are capable of suppressing CIA, but it is evident that CD25⁺CD39⁺CD4⁺ T cells show the greatest potency in suppressing CIA.

Discussion

The IL-12 family members EBI3 and IL-12p35 together form a novel anti-inflammatory cytokine, IL-35 (2, 7, 8). Characterization of EBI3 revealed that it has a dual role, having either inflammatory or anti-inflammatory properties. As a heterodimer with IL-27p28 produced by APCs, EBI3 promotes Th1 cell differentiation from naïve T cells and, hence, inflammation (1, 3, 4). It has been shown that EBI3 mRNA and protein expression are restricted to Treg cells, but not effector T cells (Teff cells). EBI3⁻/⁻ Treg cells fail to suppress Teff cell proliferation in vitro, and these are unable to cease inflammatory bowel disease (7). Similarly, EBI3-p35-Fc fusion protein promotes development of Treg cells and suppresses Th17 cells (8). To further elucidate IL-35's protective mechanisms, rIL-35 was made as a single-chain polypeptide of IL-12p35 with EBI3. Consistent with previous findings, our IL-35 administered parenterally to OVA-challenged DO11.10 CD4⁺ T cell recipients suppressed IFN-γ, IL-6, and IL-17 production and proliferation of CD4⁺ T cells following in vitro OVA₃₂₃–₃₉₀ restimulation. Although it has been reported that IL-35-Fc could stimulate a modest IFN-γ response by CD25⁺CD4⁺ T cells following polyclonal anti-CD3 plus anti-CD28 stimulation (8), in the same study, the Ag-specific (more physiological) stimulation of DO11.10 CD25⁺CD4⁺ T cells resulted in suppressed proliferation and IFN-γ secretion (8), similar to our findings. IgG2a and IgG2b anti-OVA Ab responses were also lessened in comparison with PBS-treated mice. Concomitant with the reduced Th1- and Th17-type responses, reductions in Th2-type cytokines were observed, except for IL-9 and IL-10. IL-9, earlier described as Th2-type cytokine downregulating TNF-α, IL-12, and IFN-γ, and capable of stimulating IL-10 in mice (20), was significantly induced by exogenous IL-35 treatment.

Studies have shown that IL-35-Fc fusion protein administered s.c. or i.p significantly diminishes severity of CIA in DBA/1 mice (8). We exploited a CIA model in C57BL/6 mice to further investigate rIL-35’s properties. To examine its therapeutic potential, in this study, IL-35 treatments began 21 d post CII challenge at disease onset. Using this regimen, only 10% of C57BL/6 mice developed CIA, evidenced by the lack of infiltration and cartilage destruction in joints accompanied by suppression of CIA-specific Th1 and Th17 cells. Typically, CIA induces elevated IFN-γ relative to naïve controls (15, 21).

Th17 cells have been shown to be essential for development of autoimmune inflammation (22, 23). In the arthritis models, IL-17 promotes synovial infiltration with polymorphonuclear leukocytes and production of proinflammatory cytokotyes, IL-1 and IL-6 (24–26). It can also directly stimulate bone destruction via induction of the receptor activator of NF-κB ligand expression on CD4⁺ T cells and osteoclastogenesis (27). Consistent with an earlier report (8), IL-17 and IL-6 production by CD4⁺ T cells are suppressed by the IL-35 treatments supporting the notion that IL-35 can regulate Th17 cells, as recently made evident by the enhanced expression of IL-17, IL-22, and transcription factor RORγt, by CD4⁺ T cells from EBI3⁻/⁻ mice (28).

The anti-inflammatory impact by IL-35 manifested as enhanced IL-10 production by CD4⁺ T cells from treated mice without impacting IL-9 production. It has been shown that IL-10⁻/⁻ mice are more susceptible to CIA characterized by earlier onset and more severe clinical disease than IL-10⁻/⁻/⁻ mice (29). Our results demonstrated the importance of IL-10, particularly IL-10–producing regulatory CD4⁺ T cells, in CIA suppression by IL-35. Our findings regarding IL-10’s relevance add to the recent in vitro findings that IL-35–mediated suppression of T cell proliferation is IL-10-dependent, and cell–cell contact between Treg and Teff cells is not required (30). Notably, IL-35 was capable of delaying

<table>
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<th>Treatment</th>
<th>CIA/Total</th>
<th>Day Onset</th>
<th>Maximum Score</th>
<th>Average Score</th>
<th>Cumulative Score</th>
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<tr>
<td>PBS</td>
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<td>10</td>
<td>6.8 ± 0.57</td>
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<td>CD25⁺CD39⁺CD4⁺ T cells</td>
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<td>0</td>
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<td>0***</td>
</tr>
<tr>
<td>CD25⁻CD39⁺CD4⁺ T cells</td>
<td>2/5</td>
<td>35 ± 2.02</td>
<td>6</td>
<td>2.0 ± 1.26**</td>
<td>5.2 ± 3.87****</td>
</tr>
</tbody>
</table>

*Exogenous IL-35–induced CD25⁺CD39⁺CD4⁺ and CD25⁻CD39⁺CD4⁺ T cells were adoptively transferred to mice (2 × 10⁶ cells/mouse) with induced CIA on day 15 post CII challenge.

†Number of mice with CIA/total in a group for 39 d post CII challenge.

‡Mean day ± SEM of first symptoms onset in diseased mice.

§Maximum individual mouse score in group in entire observation period.

∥Average clinical score per group on day 39 postchallenge calculated as sum of individual scores divided by the number of mice in group ± SEM.

*Significantly different from PBS group; **p = 0.01 as compared with PBS group; ***p = 0.001 as compared with PBS group; ****p = 0.006 as compared with PBS group.

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REGULATORY CD39+ T CELLS CONFER PROTECTION

progression of already established CIA if treatments were started on day 35 post CII challenge. This observation is consistent with the IL-10–dependent anti-inflammatory mechanism of suppression by IL-35. Recently, it was shown that IL-10–secreting Treg cells adoptively transferred on day 28 postinduction of CIA were protective against autoimmunity (31).

Importantly, our study showed that two Treg cell subsets are induced in vivo by exogenous IL-35. IL-35 treatment subsequent CIA development resulted in the stimulation of FoxP3 expression by splenic CD25+CD39+ and CD25−CD39+ CD4+ T cells, and CD39 was only significantly induced by LN FoxP3+CD4+ T cells. Purified CD39+CD4+ T cells from PBS-dosed mice with CIA responded to CII restimulation, evident by their proliferation, and CD39+CD4+ T cells were unresponsive to CII. Purified CD39+CD4+ T cells from naive C57BL/6 mice treated with IL-35 showed dramatic reductions in IL-17, but enhanced IL-10 production. When adoptively transferred, these CD39+CD4+ T cells conferred protection in mice already induced with CIA; however, adoptive transfer of CD39−CD4+ T cells from IL-35–treated IL-10−/− mice was incapable of inhibiting CIA, supporting the notion that suppression by exogenous IL-35 is facilitated by IL-10. Although adoptively transferred CD39+CD4+ T cells from IL-35–treated mice resulted in an apparently lessened clinical score in recipients with induced CIA, this difference was not significant, and disease incidence remained similar to controls. Although these CD39+CD4+ T cells produced IL-10, their regulatory capacity was insufficient against autoimmune arthritis. Thus, these studies suggest that IL-35 treatments can stimulate alternative regulatory subsets to dampen autoimmunity.

Acknowledgments
We thank Nancy Kommers for assistance in preparing this manuscript.

Disclosures
The authors have no financial conflicts of interest.

References

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