Cutting Edge: Human Regulatory T Cells Require IL-35 To Mediate Suppression and Infectious Tolerance

Vandana Chaturvedi, Lauren W. Collison, Clifford S. Guy, Creg J. Workman and Dario A. A. Vignali

*J Immunol* 2011; 186:6661-6666; Prepublished online 16 May 2011; doi: 10.4049/jimmunol.1100315

http://www.jimmunol.org/content/186/12/6661

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http://www.jimmunol.org/content/suppl/2011/05/16/jimmunol.1100315.DC1

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**Errata**

An erratum has been published regarding this article. Please see next page or:

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Human regulatory T cells (T<sub>reg</sub>) are essential for the maintenance of immune tolerance. However, the mechanisms they use to mediate suppression remain controversial. Although IL-35 has been shown to play an important role in T<sub>reg</sub>-mediated suppression in mice, recent studies have questioned its relevance in human T<sub>reg</sub>. In this study, we show that human T<sub>reg</sub> express and secrete IL-35 for maximal suppressive capacity. Substantial upregulation of EBI3 and IL12A, but not IL10 and TGF-β, was observed in activated human T<sub>reg</sub> compared with conventional T cells (T<sub>conv</sub>). Contact-independent T<sub>reg</sub>-mediated suppression was IL-35 dependent and did not require IL-10 or TGF-β. Lastly, human T<sub>reg</sub>-mediated suppression led to the conversion of the suppressed T<sub>conv</sub> into iTreg cells, an IL-35-induced T<sub>reg</sub> population, in an IL-35–dependent manner. Thus, IL-35 contributes to human T<sub>reg</sub>-mediated suppression, and its conversion of suppressed target T<sub>conv</sub> into IL-35–induced T<sub>reg</sub> may contribute to infectious tolerance. The Journal of Immunology, 2011, 186:6661-6666.

Interleukin-35 (EBI3–IL-12α [IL12A] heterodimer) is required for murine regulatory T cells (T<sub>reg</sub>) function (1) and has been shown to induce the conversion of murine and human conventional T cells (T<sub>conv</sub>) into IL-35–induced T<sub>reg</sub> (iTr35) (2). Furthermore, IL-35 is produced by human T<sub>conv</sub> exposed to rhinovirus-infected dendritic cells (3) and human peripheral blood CD4<sup>+</sup> T cells from chronic hepatitis B virus-infected patients (4). However, two studies have suggested that human T<sub>reg</sub> neither express nor produce IL-35, increasing the controversy surrounding the physiological importance of IL-35 in human T<sub>reg</sub> (5, 6). Several studies have shown that both murine and human T<sub>reg</sub> can mediate infectious tolerance, the contagious spread of suppressive capacity from T<sub>reg</sub> to the suppressed target cell. The mechanisms used to mediate this induction and the subsequent mechanisms used by this induced regulatory population to mediate suppression remain obscure (7–9). However, studies with human T<sub>reg</sub> have suggested that IL-10 and TGF-β may contribute to these events (10, 11). Nevertheless, mechanistic insight into the regulatory preferences of human T<sub>reg</sub> is lacking.

Materials and Methods

Cell isolation, expansion, and labeling

CD4<sup>+</sup> T cells were obtained and purified from human cord blood or apheresis products, as previously described (2, 12). Purity was verified by intracellular staining of FOXP3 (eBioscience, San Diego, CA). T<sub>reg</sub> and T<sub>conv</sub> were expanded in X-VIVO medium containing beads coated with anti-CD3 and anti-CD28 (bead/cell ratio 1:1), 20% (v/v) human sera (Lonza, Conshohocken, PA), and either 500 IU/ml human IL-2 for T<sub>reg</sub> or 100 IU/ml for T<sub>conv</sub> (Life Technologies, Carlsbad, CA). For CFSE or eFluor670 labeling, freshly purified naïve T<sub>conv</sub> or T<sub>reg</sub> were resuspended in PBS (0.1% BSA), at 2 × 10<sup>5</sup> cells/ml, incubated with CFSE or eFluor670 (1 μM) for 10 min at 37°C, stopped with ice-cold PBS, and washed three times in culture media. All experiments using expanded T<sub>reg</sub> were performed at least 9 d postactivation.

RNA isolation and real-time PCR analysis

Analyses were performed as previously described (1, 2). Sequences are detailed in Supplemental Table I.

Intracellular staining and immunofluorescence

Analyses were performed as previously described (1, 2). PE-conjugated anti–IL-12A (clone 27357) and IgG1 (isotype control; clone 25711) were used for immunofluorescence and intracellular staining (R&D Systems, Minneapolis, MN). The 8E fix/perm buffer used for intracellular staining of IL-12A was kindly provided by Dario Campana (St. Jude Children’s Research Hospital).

<i>T<sub>reg</sub></i> suppression assay

Assay was performed as previously described (2, 12). Briefly, 96-well round-bottom microtiter plates were used to perform standard suppression assays. Freshly purified 5 × 10<sup>3</sup> naïve T<sub>conv</sub> were activated with anti-CD3/anti-CD28-coated latex beads and IL-2 (10 IU/ml) and used as target cells with varying concentrations of naïve T<sub>reg</sub>, in vitro-activated T<sub>reg</sub> or CFSE-labeled suppressed T<sub>conv</sub>. The cultures were pulsed with 1 μCi [3]H-thymidine for the final 8 h of the 5-d assay and harvested with a Packard harvester (PerkinElmer). Counts per minute were determined using a Packard Matrix 96 direct counter (PerkinElmer, Waltham, MA).

Transwell experiments were performed in 96-well Transwell plates with a 0.4 μM pore size (Millipore, Billerica, MA). Freshly purified naïve T<sub>conv</sub> were activated as described above and used as target cells in the bottom chamber of the 96-well plate. The suppressor populations in the top chamber of the Transwell were activated T<sub>reg</sub> or iTr35 cultured with naïve T<sub>conv</sub> or suppressed T<sub>conv</sub>. In some experiments, cocultured naïve T<sub>conv</sub> were fixed with 4% formaldehyde for 10 min at room temperature and washed twice prior to assay. The suppressor population was activated with anti-CD3/anti-
CD28–coated latex beads and IL-2 (40 IU/ml). Where indicated, neutralizing anti-Ebi3 (clone V1.4F5.25) (2) anti–IL-10 (JES9D7; BioLegend, San Diego, CA), anti–TGF-β (1D11; R&D Systems), or isotype controls were added. A combination of both anti-Ebi3 (clone V1.4F5.25) and anti–IL-12A (clone 27537) was used at indicated concentrations when anti–IL-35 Ab was used in neutralization studies. After 112 h, the top chambers were removed and [3H]thymidine was added to the bottom chambers for the final 8 h of the 5-d assay. Cultures were harvested with a Packard harvester (PerkinElmer), and counts per minute were determined using a Packard Matrix 96 direct counter (PerkinElmer).

Results and Discussion

Human umbilical cord blood is an ideal source of naive T conv and naive T reg due to their lack of previous antigenic exposure and thus the ease with which they can be reliably purified based on CD4 and CD25 expression (data not shown). Naive cord blood T reg expressed low levels of mRNA encoding both the EBI3 and IL12A subunits of IL-35 compared with T conv (data not shown). However, following activation with anti-CD3/anti-CD28–coated beads, EBI3 and IL12A were substantially upregulated in T reg (>35-fold over T conv) (Fig. 1A). Although a similar observation was made with T reg isolated from adult PBMC, the fold upregulation was less, perhaps due to the difficulty of generating pure populations from adult cells (Supplemental Fig. 1A). EBI3 and IL12A expression was not induced or influenced by IL-2, as the inclusion of reduced concentrations with T reg or its inclusion with T conv (100 IU/ml) had no effect (note that >100 IU/ml IL-2 induced T conv death). Surprisingly, expression of IL10 and TGFβ mRNA in cord blood T reg was modest in comparison with EBI3 and IL12A. Expression of mRNA encoding the other IL-12 family members, IL23 (p19), IL27 (p28), and IL12B (p40), was not upregulated in T reg isolated from either cords or PBMC, inferring that IL-35 may be the only IL-12 family cytokine human T reg to have the capacity to upregulate upon activation compared with T conv (Fig. 1A, Supplemental Fig. 1A). Expression of EBI3 and IL12A following activation remained low until day 3 postactivation, when there was a steep increase compared with similarly activated T conv (>100-fold), which was maintained through day 9 (Fig. 1B, 1C). We note that previous studies suggesting that human T reg do not express EBI3 and IL12A only analyzed expression in resting or activated T reg up to day 2 poststimulation (10, 11).

**FIGURE 1.** Human T reg express IL-35. CD4+CD25− (Tconv) and CD4+CD25+ (Treg) were purified by FACS from cord blood. A, Relative mRNA expression in T reg was determined. B and C, Cell types noted were analyzed for EBI3 or IL12A expression at the indicated days postactivation. Naive T conv were used for normalization (arbitrarily set to 1). D, At indicated time points, T conv (blue) and T reg (red) were stained with anti–IL-12A or isotype control. A representative histogram (left panels) and the mean percentage of IL-12A high cells (right panels) are depicted. E, Activated cells were restimulated with PMA plus ionomycin for 6 h and then stained with an isotype control or anti–IL-12A (yellow) plus phalloidin (actin, gray) and DAPI (nucleus, blue). Original magnification ×63 Data represent the mean ± SEM of 4 to 5 (A), 5 (B, C, E), and 8, 13, and 11 (D) independent experiments at the three time points indicated. *p < 0.05, **p < 0.005, ***p < 0.001.
Comparable, minimal intracellular expression of IL12A (p35) was seen in resting human Treg and CD4+ and CD8+ Tconv (data not shown). However, IL-12A expression increases ~10-fold following activation of human Treg but not Tconv, as determined by flow cytometry (Fig. 1D) and immunofluorescence (Fig. 1E). Although expression was bimodal (>30%) 9 d postactivation, subsequent restimulation and analysis 3 d later resulted in ~100% expression of intracellular IL-12A and further increases in IL-12A mean fluorescence (~30-fold) and EBI3 and IL12A mRNA expression (Fig. 1D, Supplemental Fig. 2A). It is possible that this bimodal IL-12A expression is related to the activation state of the Treg. It is important to note that neither activation nor reactivation substantially altered the low-level intracellular expression of IL-12A in CD4+ and CD8+ Tconv (Fig. 1D and data not shown). The basal amount of IL-35 expression detected in Tconv could be attributed to their activation state, as it has been shown that activated human Tconv also express FOXP3, TGF-β, and IL-10 and thus may express small amounts of IL-12A and EBI3, or this could be due to some low-level background due to the staining procedure (5). The relationship between FOXP3 and IL-35 expression following Tconv and Treg activation was then assessed. Whereas all the activated Tconv and Treg populations examined expressed comparable FOXP3 IL-12A expression differed (Supplemental Fig. 1B). Restimulated Treg exhibit the highest IL-12A expression, whereas a high percentage of Treg-suppressed Tconv express IL-12A and thus may be iTreg. Although activated Tconv express low levels of IL-12A, despite high FOXP3 expression, they are unlikely to secrete IL-35 given the absence of Ebi3 mRNA. Taken together, these data demonstrate that human Treg express IL-35 to a significantly greater extent than Tconv. Furthermore, expression of FOXP3 does not necessarily endow T cells with the ability to express IL-12A/EBI3 and secrete IL-35 (6).

We next assessed whether IL-35 secretion by human Treg contributed to their function. Naïve human cord blood Treg possess minimal suppressive capacity in vitro, whereas activated Treg which exhibit high levels of EBI3 and IL12A mRNA expression, are potent suppressive (data not shown). Our initial analysis suggested that IL-35 neutralization in a conventional in vitro Treg assay had a minimal effect on their suppressive capacity, likely due to the multiple contact-dependent and contact-independent mechanisms at their disposal (data not shown) (16, 17). However, activated human Treg have also been shown to mediate potent suppression when separated from their Tconv targets by a permeable Transwell membrane, emphasizing the importance of soluble factors, such as inhibitory cytokines, in mediating suppression (10, 11). Consequently, we assessed the relative contribution of IL-35 as well as IL-10 and TGF-β, two inhibitory cytokines implicated in mediating contact-independent suppression by human Treg (10, 11). Surprisingly, neutralizing anti-IL-10 and anti-TGF-β had no effect on Treg-mediated suppression (Fig. 2A). In contrast, neutralizing anti-IL-35 completely blocked suppression. Subsequent analysis demonstrated dose-dependent inhibition of Treg-mediated suppression by neutralizing anti-IL-35 (Fig. 2B). Similar observations were also made with adult PBL-derived CD4+CD25−CD45RA− Treg (data not shown). These data suggest that IL-35, but not IL-10 and TGF-β, is required to mediate contact-independent human Treg-mediated suppression.

Infectious tolerance is thought to play a substantial role in propagating Treg-mediated immune control, but the mechanisms used to convert suppressed Tconv into an induced regulatory population and the mechanisms by which they in turn suppress third-party Tconv remain contentious. Previous studies have suggested that human Treg mediate the conversion of cocultured Tconv into IL-10- or TGF-β–induced Treg populations (10, 11). More recently, we showed that IL-35 production by murine Treg mediates the conversion of suppressed target Tconv into an induced Treg population, termed iTreg, that mediate suppression via IL-35, but not IL-10 or TGF-β (2, 18). However, it is not known if human Treg can generate iTreg cells and if they contribute to immune regulation. Thus, we first assessed whether IL-35 expression and production by human Treg was modulated following contact with Tconv and whether the latter were induced to express IL-35. Modest increases in EBI3 and IL12A mRNA and intracellular IL-12A expression were observed in functionally suppressive, cocultured Treg, compared with activated Treg (Fig. 3A, 3B). However, suppressed, cocultured Tconv substantially upregulated EBI3 and IL12A mRNA and intracellular IL-12A expression to a level indistinguishable from maximally activated human Treg (Fig. 3A, 3B). Contrary to recent studies, we saw modest expression of IL10 and TGFβ mRNA in activated human Treg and no evidence for increased
expression in the functionally suppressive T_{reg} or suppressed T_{conv} isolated from cocultures (Supplemental Fig. 2B).

To determine if the increased EBI3 and IL12A expression observed was driven by IL-35, IL-10, and/or TGF-β, T_{reg}/T_{conv} cocultures were established in the presence of neutralizing Abs. EBI3 and IL12A mRNA expression was not significantly affected by neutralization of TGF-β or IL-10 (Fig. 3A). Conversely, in the presence of neutralizing anti–IL-35, EBI3 and IL12A expression was substantially reduced in cocultured T_{reg} and essentially prevented in cocultured T_{conv} (Fig. 3A). These data suggest that IL-35 generation is induced and expression maintained by IL-35 in an autocrine (in T_{reg}) and paracrine (in suppressed T_{conv}) fashion following cell contact between human T_{reg} and T_{conv}.

We have previously shown that activation of human T_{conv} in the presence of IL-35 mediates the generation of an induced T_{reg} population, iT\(\)R35 (2). The substantial expression of EBI3 and IL12A mRNA and intracellular IL-12A expression in suppressed T_{conv}, and the requirement for IL-35 to mediate this induction, infer the generation of iT\(\)R35 (2). This prompted us to investigate whether these suppressed T_{conv} gained regulatory activity and determine the mechanism of conversion and suppression. CFSE-labeled suppressed T_{conv} were purified from T_{reg}/T_{conv} cocultures after 3 d and their regulatory capacity determined in a secondary, standard in vitro suppression assay. These T_{reg}-suppressed T_{conv} exhibited potent, dose-dependent suppressive capacity, as previously reported (Fig. 3C). To determine the cytokines responsible for induction of this regulatory capacity, T_{reg}/T_{conv} cocultures were performed in the presence or absence neutralizing anti–IL-35, TGF-β, or IL-10 prior to purification and secondary suppression assay. When suppressed T_{conv} were isolated from cocultures established in the presence of neutralizing IL-35, IL-10, TGF-β, or an isotype control (10 μg/ml) was determined as in C. E. Assay performed as in D except that the neutralizing mAbs were added during the secondary suppression assay. Counts per minute of activated T_{conv} alone, in the absence of any suppression, were 70,000–125,000. Data represent the mean ± SEM of 8–14 (A) and 3 (B–E) independent experiments. *p < 0.05, **p < 0.005, ***p < 0.001.
However, if fresh, naive T conv were added to the top well activated T reg stimulated alone or activated T reg stimulated with naive T conv in 6 150,000. Data represent the mean top chamber were at 1:256 ratio with the target T conv. B the top chamber. 

by IL-35 and not TGF-β mAbs confirmed that the suppression observed was mediated by IL-35 and not TGF-β or IL-10 (Fig. 4B, 4C). These data suggest that human T reg-generated iTr35 might contribute to global suppression and mediate infectious tolerance during T reg insufficiency.

Our results demonstrate for the first time, to our knowledge, that activated cord blood- and PBMC-derived human T reg express and secrete IL-35, which contributes significantly to their suppressive capacity (19). Surprisingly, there appeared to be a minimal role of IL-10 and TGF-β. These data are in contrast with previous reports suggesting that IL-35 is not expressed by T reg isolated from PBMCs (5) and that IL-35 does not play a role in suppression mediated by FOXP3-transduced T cells (6). These discrepancies may be due to the timing of analysis, purification techniques, and/or reagents used and the populations under analysis. In addition, our data suggest that human T reg-derived IL-35 mediates the conversion of suppressed T conv into iTr35 that subsequently suppress via IL-35 in a manner analogous to our observations in the mouse (2). These parallels raise the possibility that iTr35 may constitute a mechanism of infectious tolerance in humans. These findings suggest that IL-35 neutralization may represent a valid immunotherapeutic strategy for the treatment of cancer and conditions in which excessive regulatory control might exist.

REFERENCES


Letter of Retraction

We wish to retract the article titled “Human Regulatory T Cells Require IL-35 To Mediate Suppression and Infectious Tolerance” by Vandana Chaturvedi, Lauren W. Collison, Clifford S. Guy, Creg J. Workman, and Dario A. A. Vignali, The Journal of Immunology, 2011, 186: 6661–6666.

A recent review by our research team found the approaches used by the first author, Vandana Chaturvedi, to calculate data in several suppression assays (Fig. 3C–E) to be flawed. The authors feel there is sufficient concern over these data in the article that retraction is warranted. All the authors concur with this retraction and sincerely regret any inconvenience this may have caused.

Vandana Chaturvedi
Department of Pediatrics and Infectious Diseases
Cincinnati Children’s Hospital
Cincinnati, OH

Lauren W. Collison
Opexa Therapeutics
The Woodlands, TX

Clifford S. Guy
Creg J. Workman
Dario A. A. Vignali
Department of Immunology
St. Jude Children’s Research Hospital
Memphis, TN
**SUPPLEMENTAL TABLE 1**: Primers used in this study.

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<th>Gene Name</th>
<th>Forward Primer</th>
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<td>5’ CCATGGGAGAACGCTGGACAT 3’</td>
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<tr>
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**SUPPLEMENTAL FIGURE S1**: (A) T<sub>conv</sub> and T<sub>reg</sub> cells were isolated from PBMCs based on CD4<sup>+</sup>CD25<sup>−</sup>CD45RA<sup>+</sup> (T<sub>conv</sub>) [open bars] and CD4<sup>+</sup>CD25<sup>−</sup>CD45RA<sup>−</sup> (T<sub>reg</sub>) [closed bars] expression and expanded for 9d using anti-CD3/anti-CD28-coated latex beads and IL-2 (500 IU/ml) for T<sub>reg</sub> and IL-2 (100 IU/ml) for T<sub>conv</sub>. After 9d, RNA was isolated, cDNA generated and qPCR analysis performed. Relative expression of mRNA encoding IL10, TGFβ, IL23, IL27, IL12B, IL12A and EBI3 was determined. Data represent the mean ± SEM of 6 independent experiments [* p < 0.05, ** p < 0.005 and *** p < 0.001]. Results are presented relative to those of naïve T<sub>conv</sub> cells. (B) Indicated cell populations were stained for FoxP3 and IL-12A expression. Cells were fixed and permeabilized using 8E buffer and then stained for IL-12A. This was followed by FoxP3 staining (eBioscience, San Diego, CA). Representative dot plots are shown. Naive and Activated T<sub>conv</sub> were used as control. Foxp3 and IL-12 A expression in CD4<sup>+</sup> gated cells. Stimulated T<sub>reg</sub> refer to 9 day activated T<sub>reg</sub>. Restimulated T<sub>reg</sub> refer to 9+3 day activated T<sub>reg</sub>. Suppressed T<sub>conv</sub> are the T<sub>conv</sub> isolated for cocultures.
**SUPPLEMENTAL FIGURE S2:** (A) Restimulation of T<sub>reg</sub> cells after 9d leads to increased EBI3 and IL12A expression. Human T<sub>conv</sub> and T<sub>reg</sub> cells were purified from umbilical cord blood by FACS based on cell surface expression of CD4 and CD25. Purified cells were expanded for 9 d, using anti-CD3/anti-CD28-coated latex beads and IL-2 (500 IU/ml). The cells were restimulated for additional 3 d, with anti-CD3/anti-CD28-coated latex beads and IL-2 (100 IU/ml) after the initial 9 d stimulation. RNA was isolated, cDNA generated and qPCR analysis performed. Relative EBI3 and IL12A expression. Data represents the mean ± SEM of 5 independent experiments [**p < 0.005]. Results are presented relative to those of T<sub>conv</sub> cells. The T<sub>conv</sub> cells were expanded similarly to T<sub>reg</sub> cells except that IL-2 was used at a final concentration of 100 IU/ml for the initial 9 d expansion. (B) Co-culturing T<sub>reg</sub> and T<sub>conv</sub> cells does not lead to substantial up-regulation of either IL-10 or TGFβ in either population. Activated T<sub>reg</sub> cells (prepared as described in Fig. 1) were labeled with eFluor®670 and cultured with CFSE-labeled naïve T<sub>conv</sub> cells in the presence or absence of IL-2, anti-CD3/CD28-coated latex beads. At the end of 3 d, cells were purified by FACS. RNA was extracted and cDNA was generated from the indicated populations. Relative IL10 (upper panel) and TGFβ expression (lower panel) was determined by qPCR. Data represents the mean ± SEM of 5 independent experiments [*p < 0.05, **p < 0.005, and *** p < 0.001]. Results are presented relative to those of naïve T<sub>conv</sub> cells.