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

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Human regulatory T cells (Treg) 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 Treg-mediated suppression in mice, recent studies have questioned its relevance in human Treg. In this study, we show that human Treg express and require IL-35 for maximal suppressive capacity. Substantial upregulation of EBI3 and IL12A, but not IL10 and TGFB, was observed in activated human Treg compared with conventional T cells (Tconv). Contact-independent Treg-mediated suppression was IL-35 dependent and did not require IL-10 or TGF-β. Lastly, human Treg-mediated suppression led to the conversion of the suppressed Tconv into iTr35 cells, an IL-35-induced Treg population, in an IL-35-dependent manner. Thus, IL-35 contributes to human Treg-mediated suppression, and its conversion of suppressed target Tconv into IL-35-induced Treg may contribute to infectious tolerance.

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

Materials and Methods

Cell isolation, expansion, and labeling

CD4+ T cells were obtained and purified from human cord blood or apheresis rings, as previously described (2, 12). Purity was verified by intracellular staining of FOXP3 (Bioscience, San Diego, CA). Treg and Tconv 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 Treg or 100 IU/ml for Tconv (13–15). For CFSE or eFluor670 labeling, freshly purified naïve Tconv or Treg were resuspended in PBS (0.1% BSA) at 2 × 10^6 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 Treg were performed at least 9 d postactivation.

RNA isolation and real-time PCR analysis

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

Intracellular staining and immunofluorescence

Analysis was performed as previously described (1, 2). PE-conjugated anti-IL-12A (clone 27537) and IgG1 (isotype control; clone 25711) were used for immunofluorescence and intracellular staining (R&D Systems, Minneapolis, MN). The 8 μM/perm buffer used for intracellular staining of IL-12A was kindly provided by Dario Campana (St. Jude Children’s Research Hospital).

Treg 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 × 10^5 naive Tconv 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 Tconv, in vitro-activated Treg or CFSE-labeled suppressed Tconv. The cultures were pulsed with 1 μCi [3H]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 Tconv 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 Treg, activated Treg cultured with naive Tconv, or suppressed Tconv. In some experiments, cocultured naive Tconv 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-

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CD28–coated latex beads and IL-2 (40 IU/ml). Where indicated, neutralizing anti-Ebi3 (clone V1.4F5.25) (2), anti–IL-10 (JES39D7; 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 Tconv and naive Treg 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 Treg expressed low levels of mRNA encoding both the EBI3 and IL12A subunits of IL-35 compared with Tconv (data not shown). However, following activation with anti-CD3/anti-CD28–coated beads, EBI3 and IL12A were substantially upregulated in Treg (>35-fold over Tconv) (Fig. 1A). Although a similar observation was made with Treg 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 Treg or its inclusion with Tconv (100 IU/ml) had no affect (note that >100 IU/ml IL-2 induced Tconv death). Surprisingly, expression of IL10 and TGFβ mRNA in cord blood Treg 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 Treg isolated from either cords or PBMC, inferring that IL-35 may be the only IL-12 family cytokine human Treg to have the capacity to upregulate upon activation compared with Tconv (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 Tconv (>100-fold), which was maintained through day 9 (Fig. 1B, 1C). We note that previous studies suggesting that human Treg do not express EBI3 and IL12A only analyzed expression in resting or activated Treg up to day 2 poststimulation (10, 11).

FIGURE 1. Human Treg express IL-35. CD4+CD25− (Tconv) and CD4+CD25+ (Treg) were purified by FACS from cord blood. A, Relative mRNA expression in Treg was determined. B and C, Cell types noted were analyzed for EBI3 or IL12A expression at the indicated days postactivation. Naive Tconv were used for normalization (arbitrarily set to 1). D, At indicated time points, Tconv (blue) and Treg (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.
Comparative, minimal intracellular expression of IL12A (p35) was seen in resting human T\textsubscript{reg} and CD4\textsuperscript{+} and CD8\textsuperscript{+} T\textsubscript{conv} (data not shown). However, IL-12A expression increases 10-fold following activation of human T\textsubscript{reg} but not T\textsubscript{conv}, 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 T\textsubscript{reg}. It is important to note that neither activation nor reactivation substantially altered the low-level intracellular expression of IL-12A in CD4\textsuperscript{+} and CD8\textsuperscript{+} T\textsubscript{conv} (Fig. 1D and data not shown). The basal amount of IL-35 expression detected in T\textsubscript{conv} could be attributed to their activation state, as it has been shown that activated human T\textsubscript{conv} also express FOXP3, TGF-\textbeta, 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 T\textsubscript{conv} and T\textsubscript{reg} activation was then assessed. Whereas all the activated T\textsubscript{conv} and T\textsubscript{reg} populations examined expressed comparable FOXP3 IL-12A expression differed (Supplemental Fig. 1B). Restimulated T\textsubscript{reg} exhibit the highest IL-12A expression, whereas a high percentage of T\textsubscript{reg}-suppressed T\textsubscript{conv} express IL-12A and thus may be iTr35. Although activated T\textsubscript{conv} 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 T\textsubscript{reg} express IL-35 to a significantly greater extent than T\textsubscript{conv}. 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 T\textsubscript{reg} contributed to their function. Naive human cord blood T\textsubscript{reg} possess minimal suppressive capacity in vitro, whereas activated T\textsubscript{reg} which exhibit high levels of EBI3 and IL12A mRNA expression, are potently suppressive (data not shown). Our initial analysis suggested that IL-35 neutralization in a conventional in vitro T\textsubscript{reg} 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 T\textsubscript{reg} have also been shown to mediate potent suppression when separated from their T\textsubscript{conv} 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-\textbeta, two inhibitory cytokines implicated in mediating contact-independent suppression by human T\textsubscript{reg} (10, 11). Surprisingly, neutralizing anti-IL-10 and anti-TGF-\textbeta had no effect on T\textsubscript{reg}-mediated suppression (Fig. 2A). In contrast, neutralizing anti-IL-35 completely blocked suppression. Subsequent analysis demonstrated dose-dependent inhibition of T\textsubscript{reg}-mediated suppression by neutralizing anti-IL-35 (Fig. 2B). Similar observations were also made with adult PBL-derived CD4\textsuperscript{+}CD25\textsuperscript{+}CD45RA\textsuperscript{+} T\textsubscript{reg} (data not shown). These data suggest that IL-35, but not IL-10 and TGF-\textbeta, is required to mediate contact-independent human T\textsubscript{reg}-mediated suppression.

Infectious tolerance is thought to play a substantial role in propagating T\textsubscript{reg}-mediated immune control, but the mechanisms used to convert suppressed T\textsubscript{conv} into an induced regulatory population and the mechanisms by which they in turn suppress third-party T\textsubscript{conv} remain contentious. Previous studies have suggested that human T\textsubscript{reg} mediate the conversion of cocultured T\textsubscript{conv} into IL-10– or TGF-\textbeta–induced T\textsubscript{reg} populations (10, 11). More recently, we showed that IL-35 production by murine T\textsubscript{reg} mediates the conversion of suppressed target T\textsubscript{conv} into an induced T\textsubscript{reg} population, termed iTr35, that mediate suppression via IL-35, but not IL-10 or TGF-\textbeta (2, 18). However, it is not known if human T\textsubscript{reg} can generate iTr35 cells and if they contribute to immune regulation. Thus, we first assessed whether IL-35 expression and production by human T\textsubscript{reg} was modulated following contact with T\textsubscript{conv} 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 T\textsubscript{reg} compared with activated T\textsubscript{reg} (Fig. 3A, 3B). However, suppressed, cocultured T\textsubscript{conv} substantially upregulated EBI3 and IL12A mRNA and intracellular IL-12A expression to a level indistinguishable from maximally activated human T\textsubscript{reg} (Fig. 3A, 3B). Contrary to recent studies, we saw modest expression of IL10 and TGF\beta mRNA in activated human T\textsubscript{reg} and no evidence for increased

![Figure 2](https://www.jimmunol.org/)
expression in the functionally suppressive T<sub>reg</sub> or suppressed T<sub>conv</sub> 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<sub>reg</sub>/T<sub>conv</sub> 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<sub>reg</sub> and essentially prevented in cocultured T<sub>conv</sub> (Fig. 3A). These data suggest that IL-35 generation is induced and expression maintained by IL-35 in an autocrine (in T<sub>reg</sub>) and paracrine (in suppressed T<sub>conv</sub>) fashion following cell contact between human T<sub>reg</sub> and T<sub>conv</sub>.

We have previously shown that activation of human T<sub>conv</sub> in the presence of IL-35 mediates the generation of an induced T<sub>reg</sub>, iTiTr35 (2). The substantial expression of EBI3 and IL12A mRNA and intracellular IL-12A expression in suppressed T<sub>conv</sub> and the requirement for IL-35 to mediate this induction, infers the generation of iTiTr35 (2). This prompted us to investigate whether these suppressed T<sub>conv</sub> gained regulatory activity and determine the mechanism of conversion and suppression. CFSE-labeled suppressed T<sub>conv</sub> were purified from T<sub>reg</sub>/T<sub>conv</sub> cocultures after 3 d and their regulatory capacity determined in a secondary, standard in vitro suppression assay. These T<sub>reg</sub>-suppressed T<sub>conv</sub> exhibited potent, dose-dependent suppressive capacity, as previously reported (Fig. 3C). To determine the cytokines responsible for induction of this regulatory capacity, T<sub>reg</sub>/T<sub>conv</sub> 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<sub>conv</sub> 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<sub>conv</sub> 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.

![FIGURE 3. T<sub>reg</sub>-mediated induction of iTiTr35. A and B. Activated T<sub>reg</sub> were labeled with eFluor670 and cultured with CFSE-labeled naive T<sub>conv</sub> at a ratio of 1:4 in the presence anti-CD3/CD28-coated beads and IL-2 (10 IU/ml) with or without neutralizing mAbs against IL-35, TGF-β, or IL-10 (10 μg/ml) for 72 h. Cells were purified by FACS on day 3 and analyzed for relative expression of EBI3 (A, upper panel) and IL12A (A, lower panel) and intracellular expression of IL-12A (B). C. Regulatory capacity of the sorted suppressed T<sub>conv</sub> was determined using naive T<sub>conv</sub> as targets. D. Regulatory capacity of suppressed T<sub>conv</sub> generated 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<sub>conv</sub> 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.](http://www.jimmunol.org/)}
regulatory capacity of T<sub>reg</sub> is insufficient to mediate effective suppression, a scenario that might occur in vivo (Fig. 4A). However, if fresh, naive T<sub>conv</sub> were added to the top well under these conditions, substantial cell contact-independent suppression across a Transwell membrane was observed (≈60%), raising the possibility that iTreg generated by the human T<sub>reg</sub> could compensate for this T<sub>reg</sub> insufficiency. Indeed, this suppression was lost when the naive T<sub>conv</sub> added to the top well were fixed (and thus could not be converted to iTreg), confirming that iTreg generated in the presence of very low numbers of human T<sub>reg</sub> can mediate contact-independent suppression (Fig. 4B). Addition of neutralizing 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<sub>reg</sub>-generated iTreg might contribute to global suppression and mediate infectious tolerance during T<sub>reg</sub> insufficiency.

Our results demonstrate for the first time, to our knowledge, that activated cord blood- and PBMC-derived human T<sub>reg</sub> 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<sub>reg</sub> 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<sub>reg</sub>-derived IL-35 mediates the conversion of suppressed T<sub>conv</sub> into iTreg that subsequently suppress via IL-35 in a manner analogous to our observations in the mouse (2). These parallels raise the possibility that iTreg 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.

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**Disclosures**

D.A.A.V., L.W.C., and C.J.W. have submitted patents that are pending and are entitled to a share in net income generated from licensing of these patent rights for commercial development.

**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.

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