Regulatory T Cell Suppression Is Potentiated by Target T Cells in a Cell Contact, IL-35- and IL-10-Dependent Manner[1]

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Regulatory T cells (T_{reg}) are believed to suppress conventional T cell (T_{conv}) proliferation in vitro in a contact-dependent, cytokine-independent manner, based in part on experiments in which T_{reg} and T_{conv} are separated by a permeable membrane. We show that the production of IL-35, a novel inhibitory cytokine expressed by natural T_{reg}, increases substantially following contact with T_{conv}. Surprisingly, T_{reg} were able to mediate potent suppression of T_{conv} across a permeable membrane when placed in direct contact with T_{conv} in the upper chamber of a Transwell plate. Suppression was IL-35 and IL-10 dependent, and T_{conv} activation was required for maximal potentiation of T_{reg} suppression. These data suggest that it is the induction of suppression, rather than the function of T_{reg} that is obligatorily contact dependent. *The Journal of Immunology,* **2009, 182: 6121–6128.**

Although several studies have assessed the mechanism of regulatory T cell (T_{reg})[3]-mediated suppression, many details remain unknown. It is thought that T_{reg} can suppress through direct contact with target cells or APCs (1–3) and by using secreted cytokines (4–6). The conventional assay for T_{reg} function in vitro tests the ability of T_{reg} to suppress conventional T cell (T_{conv}) proliferation when cultured together in a tissue culture plate. Experiments using Transwell inserts, in which T_{reg} and T_{conv} are separated by a permeable membrane, have demonstrated that T_{reg} are essentially unable to suppress T_{conv} proliferation when T_{conv} and T_{reg} are in separate compartments (1, 3). These data led to the notion that T_{reg}-mediated suppression is contact dependent. The addition of IL-10- and TGF-β-neutralizing Abs to conventional in vitro T_{reg} assays does not inhibit suppression by T_{reg}, suggesting that these cytokines are not required for T_{reg}-mediated suppression in vitro (1, 3, 7, 8). However, secreted cytokines are an important means of T_{reg}-mediated suppression in vivo (4, 9–17), making it difficult to reconcile these differential requirements.

We recently identified a novel inhibitory cytokine, IL-35, which is a member of the IL-12 family and a heterodimer comprised of Ebi3 (IL-27β) and Il12a/p35 (IL-12β) (18). It is secreted by T_{reg}, but not T_{conv}, and is required for maximal T_{reg} function in vitro and in vivo. We also showed that ectopic expression of IL-35 by T_{conv} is sufficient to confer regulatory activity and that rIL-35 can suppress the proliferation of anti-CD3-stimulated T_{conv}. Interestingly, preliminary data suggested that T_{reg} recovered from an in vitro T_{reg} assay, and thus in the process of active suppression, dramatically up-regulated *Ebi3* and *Il12a* mRNA expression. This indicated that the interaction between T_{conv} and T_{reg} might potentiate IL-35 secretion and led to the hypothesis that T_{conv}:T_{reg} coculture might also enhance T_{reg} function. Therefore, it is possible that in the Transwell experiments reported to date, T_{reg} were unable to suppress T_{conv} proliferation because optimal suppression, including the secretion of inhibitory cytokines, is potentiated by signals derived from the T_{conv} that are being suppressed. In this study, we tested this possibility using Transwell culture experiments to determine the requirements for, and necessity of, IL-35 and IL-10 in T_{reg}-mediated suppression. We also determined the activation requirements necessary for T_{conv}-induced T_{reg} suppression.

Materials and Methods

Mice

Ebi3$^{-/-}$ (C57BL/6: F11, now >98.83% B6 by microsatellite analysis (Charles River Laboratories)) were initially provided by R. Blumberg and T. Kuo (Brigham and Women's Hospital, Boston, MA), and subsequently obtained from our own breeding colony, which was rederived at Charles River Breeding Laboratories and housed at St. Jude Children's Research Hospital. *Foxp3*^{gfp} mice (C57BL/6: F7, now >95.32% B6 by microsatellite analysis (Charles River Laboratories)) were provided by A. Rudensky (Howard Hughes Medical Institute, University of Washington, Seattle, WA). *Il10*$^{-/-}$ mice (N10F33) were provided by T. Geiger (St. Jude Children's Research Hospital, Memphis, TN). *Il12a*$^{-/-}$ (N11F31), C57BL/6, and Thy1.1 C57BL/6 mice were purchased from The Jackson Laboratory. All animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific pathogen-free, helicobacter-free facilities in the St. Jude Animal Resource Center following national, state, and institutional guidelines. Animal protocols were approved by the St. Jude Animal Care and Use Committee.

Flow cytometric analysis and cell sorting

T_{conv} ($CD4^+CD25^-CD45RB^{high}$) and T_{reg} ($CD4^+CD25^+CD45RB^{low}$) from the spleens and lymph nodes of C57BL/6 or knockout age-matched mice were positively sorted by FACS. Purity of sorted T_{conv} and T_{reg} was verified by Foxp3 staining and FACS analysis. Where indicated, T_{conv} ($CD4^+Foxp3^-CD45RB^{high}$) and T_{reg} ($CD4^+$ $Foxp3^+CD45RB^{low}$) from the spleens and lymph nodes of *Foxp3*^{gfp} mice were positively sorted by FACS. Following RBC lysis with Gey's solution, cells were stained with Abs against CD4, CD25, and CD45RB (eBioscience) for T cell isolation and sorted on a MoFlo (DakoCytomation) or Reflection (i-Cyt). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences).

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[3] Abbreviations used in this paper: T_{reg}, regulatory T cell; CT, cycle threshold; T_{conv}, conventional effector T cell; T_{sup}, suppressed target T cell.

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Anti-CD3/CD28-coated latex beads

Sulfate latex beads (4 μM; Molecular Probes) were incubated overnight at room temperature with rotation in a 1/4 dilution of anti-CD3 plus anti-CD28 Ab mix (13.3 μg/ml anti-CD3 Ab (eBioscience) and 26.6 μg/ml anti-CD28 (eBioscience)). Beads were washed three times with 5 mM phosphate buffer (pH 6.5) and resuspended at 5×10^7/ml in sterile phosphate buffer with 2 mM BSA.

Polarized cell generation

Murine T cells were purified by MACS separation using biotinylated CD8, CD11b, CD11c, CD25, Ter^{119}, B220, pan-NK, Mac-1, and Gr-1 Abs to deplete non-T cells. MACS-purified T cells were seeded at 2×10^6/ml in a 24-well plate coated with anti-murine CD3ε (4 μg/ml) and soluble anti-CD28 (2 μg/ml). Recombinant cytokines and neutralizing Abs were added as indicated for polarization. Th1 conditions, 35 ng/ml rIL-12 and 10 μg/ml αIL-4; Th2 conditions, 50 ng/ml rIL-4 and 10 μg/ml anti-IFN-γ. Cells were split on day 3 and expanded in medium containing IL-2 (10 U/ml). On day 6, cells were collected, washed, and used in functional assays. Polarization was verified by intracellular cytokine staining, cytokine secretion using Luminex technology, and Tbet (Th1)/Gata3 (Th2) quantitative PCR.

RNA, cDNA, and quantitative real-time PCR

Purified T_{conv} and T_{reg} were cultured with anti-CD3/CD28-coated latex beads or in the absence of stimuli. Where indicated, T_{reg} were activated in the presence of T_{conv} at a 4:1 (T_{conv}:T_{reg}) ratio. T_{conv} were used at 2×10^6 cells/ml, and T_{reg} at 5×10^5 cells/ml in 100 μl were used in a total volume of 1 ml, with or without anti-CD3/CD28-coated latex beads. After 48 h, T_{conv} and T_{reg} were resorted based on Foxp3 expression or based on the expression of congenic markers Thy1.1 for T_{conv} and Thy1.2 for T_{reg} for analysis. T cell RNA was isolated from purified cells using the Qiagen micro RNA extraction kit or by using the TRIzol reagent. RNA was quantitated spectrophotometrically, and cDNA was generated using the Applied Biosystems high capacity cDNA reverse-transcription kit. The cDNA samples were subjected to 40 cycles of amplification in an ABI Prism 7900 Sequence Detection System instrument using TaqMan or SYBR Green PCR master mix (Applied Biosystems) (primer sequences listed in supplemental Table I).[4] Quantitation of relative mRNA expression was determined by the comparative cycle threshold (CT) method (Applied Biosystems User Bulletin no. 2, pg. 11, http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf), whereby the amount of target mRNA, normalized to endogenous β actin or cyclophillin expression, was determined by the following formula: $2^{-\Delta\Delta CT}$.

Immunoprecipitation and Western blotting

Immunoprecipitation and immunoblotting were performed, as previously described (6–8). Cellular supernatants were diluted in lysis buffer containing 0.1% Tween 20, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, and 1 complete protease inhibitor tablet (Roche) per 50 ml lysis buffer. Supernatants were immunoprecipitated with anti-mouse IL-12a (p35) Ab (clone C18.2; eBioscience) (precoupled to protein G-Sepharose beads). Immunoprecipitates were resolved by SDS-PAGE (Invitrogen Life Technologies), and blots were probed with a biotinylated anti-mouse Ebi3 mAb (30A1; eBioscience). Blots were developed using ECL (Amersham Biosciences), and bands were quantitated by densitometry using a Bio-Rad Gel Documentation System.

Cytokine quantitation

Cytokine secretion in cell culture supernatants was measured by Luminex technology. T_{conv} and T_{reg} were used at 2×10^6 cells/ml and 5×10^5 cells/ml, respectively, in a 100 μl vol in a 96-well round-bottom plate with or without anti-CD3/CD28-coated latex beads. After 72 h, the supernatants from these in vitro coculture assays and Transwell experiments were collected and analyzed for IL-10, IL-2, and TGF-β using Millipore multiplex kits from Linco Research. Cytokine concentrations were determined, in duplicate, using standard curves of reference proteins supplied by the manufacturer.

Proliferation and T_{reg} suppression assays

For standard proliferation assays, 5×10^4 T_{conv} were activated with anti-CD3/anti-CD28- or anti-Vβ8-coated latex beads. Cultures were pulsed with 1 μCi of [^{3}H]thymidine for the final 8 h of the 72-h assay and harvested with a Packard harvester. The cpm were determined using a Packard Matrix 96 direct counter (Packard Biosciences). In vitro T_{reg} function was measured by culturing 5×10^4 T_{conv} with anti-CD3/anti-CD28-coated latex beads, and titrations of T_{reg}. T_{conv} were activated with anti-CD3/anti-CD28-coated latex beads for 72 h. For direct comparison of T_{reg} suppressive capacity with and without T_{conv} contact, T_{reg} were also cultured in direct contact with responder T_{conv} in the bottom chamber of the Transwell plate. In indicated assays, rIL-10 (eBioscience) or neutralizing anti-IL-10 Ab (clone JES5-2A5; BD Biosciences) were added to standard T_{reg} assays and Transwell experiments. Cultures were pulsed and harvested, as described for proliferation assays.

Cell activation, fixation, and Transwell experiments

Where indicated, T_{conv} were fixed at a 1/5 dilution of 20% formaldehyde in culture medium, incubated at room temperature for 20 min, and washed three times with medium before culture. "Not preactivated" cells were cultured immediately following purification. Where indicated, some cells were preactivated before fixation and culture. For these experiments, T_{reg} were activated for 24 h at 5×10^5/ml in 96-well round-bottom plates containing anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml). Following activation, T_{reg} were washed thoroughly, counted, and fixed, where appropriate. Transwell experiments were performed in 96-well plates with pore size 0.4 μM (Millipore). Freshly purified responder T_{conv} (5×10^4) were cultured in the bottom chamber of the 96-well plates in medium containing anti-CD3/anti-CD28- or anti-Vβ8-coated latex beads, where indicated. Cells assayed for regulatory capacity, in medium with or without anti-CD3/anti-CD28- or anti-Vβ8-coated latex beads, were cultured in the top chamber. T_{conv} and T_{reg} were either cultured alone at 1.25×10^4/well or cocultured at a ratio of 4:1 with a total of 2.5×10^4 cells in the top chamber. After 64 h in culture, top chambers were removed and [^{3}H]thymidine was added directly to the responder T_{conv} in the bottom chambers of the original Transwell plate for the final 8 h of the 72-h assay. Cultures were harvested with a Packard harvester. The cpm were determined using a Packard Matrix 96 direct counter (Packard Biosciences).

Results

IL-35, and to a lesser degree, IL-10 production by T_{reg} is potentiated by T_{conv} contact

We have previously shown that among $CD4^+$ T cells, IL-35 expression and secretion are restricted to $Foxp3^+$ T_{reg} (18). Reports indicate that T_{reg} require stimulation to mediate suppression, suggesting that T_{reg} activation may induce the production of T_{reg}-specific cytokines. To test this hypothesis, T_{reg} from *Foxp3*^{gfp} mice were assayed by real-time quantitative PCR for expression of IL-10 and IL-35 (*Ebi3* and *Il12a*), inhibitory cytokines made by natural T_{reg}, either in the presence or absence of T_{conv}. Upon activation of either T_{conv} or T_{reg}, IL-10 mRNA levels increased when compared with expression in resting cells (Fig. 1*A*). When T_{reg} from Thy1.1 mice were cultured in the presence of T_{conv} from Thy1.2 mice, either in the absence or presence of stimulation, and subsequently resorted using Thy1 congenic markers, IL-10 mRNA was reduced in T_{reg} and absent in T_{conv}. It is noteworthy that the 3′ untranslated region of *Il10* mRNA contains AU-rich regions that can affect its stability, raising the possibility that contact with T_{conv} may induce this *Il10* mRNA reduction (19). We also measured IL-10 secretion from individual T_{reg} or T_{conv} populations or T_{reg}:T_{conv} coculture to determine the relative amount of IL-10 derived from each cell population. Consistent with mRNA expression, IL-10 secretion by both T_{conv} and T_{reg} was enhanced following activation (Fig. 1*B*). In contrast, there was no IL-10 secreted by either T_{reg} or T_{conv} in the absence of stimulation. Total IL-10 secretion by the T_{reg}:T_{conv} coculture was greater than the sum of that generated by the individual cell populations, indicating that cumulative IL-10 secretion was enhanced by T_{reg}:T_{conv} coculture. Our results suggest that in the coculture, IL-10 was derived primarily from T_{reg} because using *Il10*$^{-/-}$ T_{conv} had no impact on mRNA expression in T_{reg} (Fig. 1*A*) nor on total IL-10 secretion (Fig. 1*B*). However, using

[4] The online version of this article contains supplemental material.

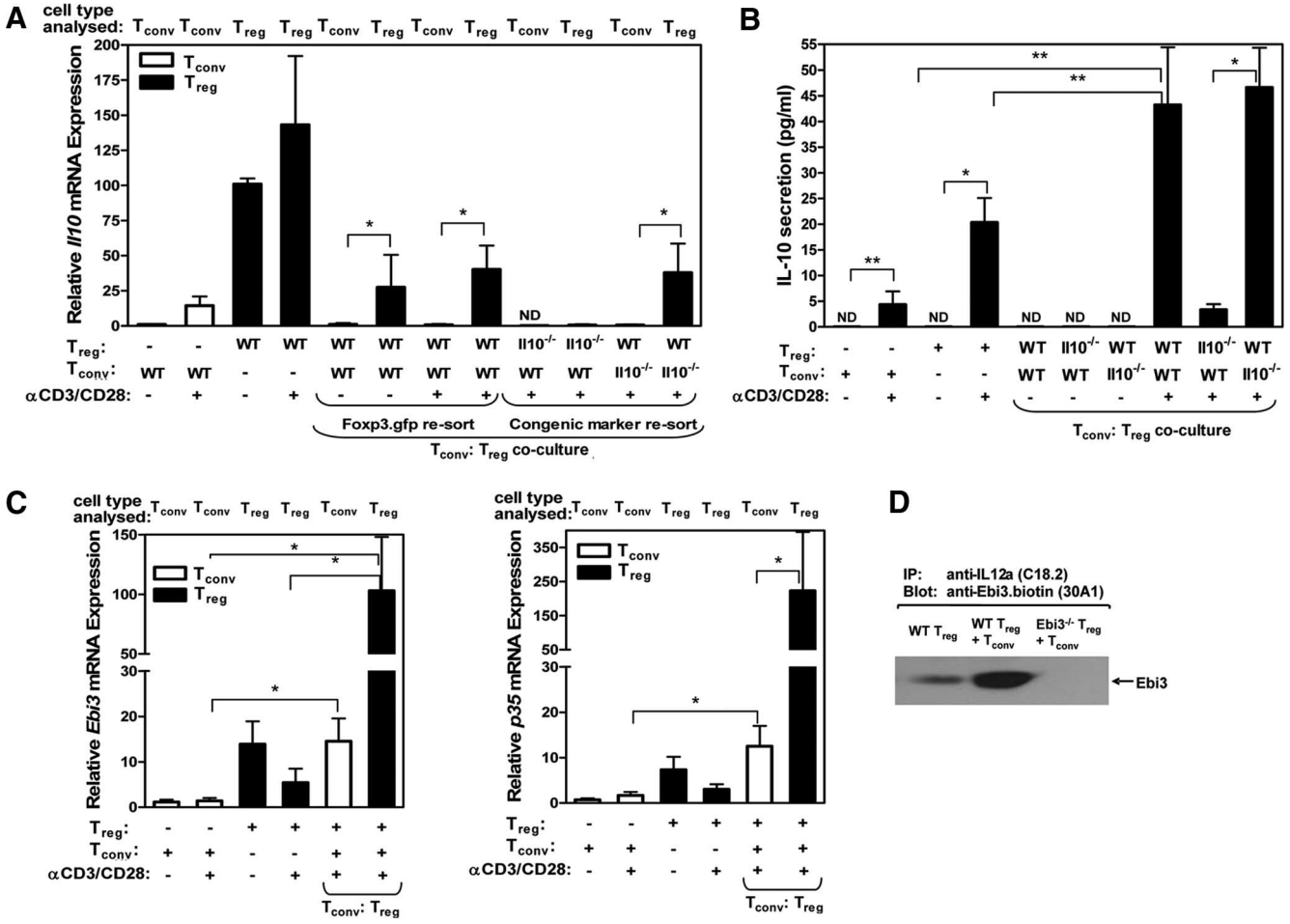

FIGURE 1. IL-35 and, to a lesser extent, IL-10 expression is potentiated by contact with T_{conv}. T_{conv} or T_{reg} from the spleens and lymph nodes of *Foxp3*^{gfp}, C57BL/6, *Ebi3*$^{-/-}$, or *Il10*$^{-/-}$ mice were purified by FACS. *A*, RNA was extracted and cDNA was generated from *Foxp3*^{gfp} T_{conv} and T_{reg}, alone or from cocultures (resorted based on GFP expression), and from T_{conv}:T_{reg} cocultures resorted based on differential Thy1 markers. Relative mRNA expression was determined by quantitative real-time PCR from the populations and conditions indicated: unstimulated, stimulated for 48 h with anti-CD3/CD28, and/or cultures containing both T_{conv} and T_{reg}. *B*, Supernatants were collected from purified T_{conv} or T_{reg} under indicated conditions, and IL-10 secretion was measured using Luminex technology. *C*, Relative *Ebi3* (*left panel*) and *Il12a* (*right panel*) mRNA expression of the T_{conv} and T_{reg} populations indicated using the same experimental approach described in *A*. *D*, Purified T_{reg} in the absence or presence of T_{conv} were cultured for 72 h with anti-CD3/CD28. Supernatant was collected for overnight immunoprecipitation with an anti-IL-12a (p35) mAb, and eluted proteins were resolved on an SDS-PAGE gel and blotted with anti-Ebi3 mAb. Data represent the mean ± SEM of three to eight (*A*), six (*B*), and four (*D*) independent experiments. Statistical analysis: *, $p < 0.05$; **, $p < 0.01$.

Il10$^{-/-}$ T_{reg} substantially reduced the amount of IL-10 secreted. Whether this is reflective of the small amount of IL-10 secreted by T_{conv} or a role for T_{reg}-derived IL-10 in inducing production of IL-10 by T_{conv} remains to be determined. The mechanism that underlies the apparent discordance between *Il10* mRNA and protein expression remains unclear and appears complex (20). However, consistent with our observations, several studies in monocytes have suggested that autocrine and perhaps paracrine IL-10 production and STAT3-dependent IL-10R signaling can enhance *Il10* mRNA instability and degradation (21).

We have previously shown that in contrast to *Il10*, *Ebi3* and *Il12a* mRNA levels are essentially unaltered upon TCR ligation (18). However, *Ebi3* and *Il12a* mRNA expression was dramatically up-regulated in T_{reg} purified from a coculture with T_{conv} (Fig. 1*C*). We now show that this increase in *Ebi3* and *Il12a* mRNA translates into a substantial increase in the total amount of IL-35 secreted (Fig. 1*D*). Interestingly, expression of both *Ebi3* and *Il12a* mRNA was up-regulated in the suppressed T_{conv} purified from this coculture (Fig. 1*C*).

T_{conv}-potentiated T_{reg} suppress across a permeable membrane in an IL-35- and IL-10-dependent manner

Previous studies using Transwell culture plates suggested that T_{reg} were unable to suppress responder T_{conv} proliferation when the two cell populations were separated by a permeable membrane (1, 3, 7, 8). It is important to note that in these studies, resting or activated T_{reg} (in the absence of T_{conv}) were cultured in the top chamber of a Transwell plate, and their ability to suppress activated T_{conv} in the bottom chamber was determined by [^{3}H]thymidine incorporation. However, our data indicate that T_{reg} required direct contact with T_{conv} for maximal secretion of IL-35, suggesting that cytokine dependence or independence of in vitro T_{reg} function should be re-examined under these conditions. Thus, different cell populations in the upper chamber of a Transwell plate were assayed for their ability to suppress proliferation of purified responder T_{conv} that were cultured with anti-CD3 (clone 145-2C11)/anti-CD28 (clone 37.51)-coated latex beads in the bottom chamber. As other groups have shown, minimal suppression of responder T_{conv} proliferation in the lower chamber was observed with resting

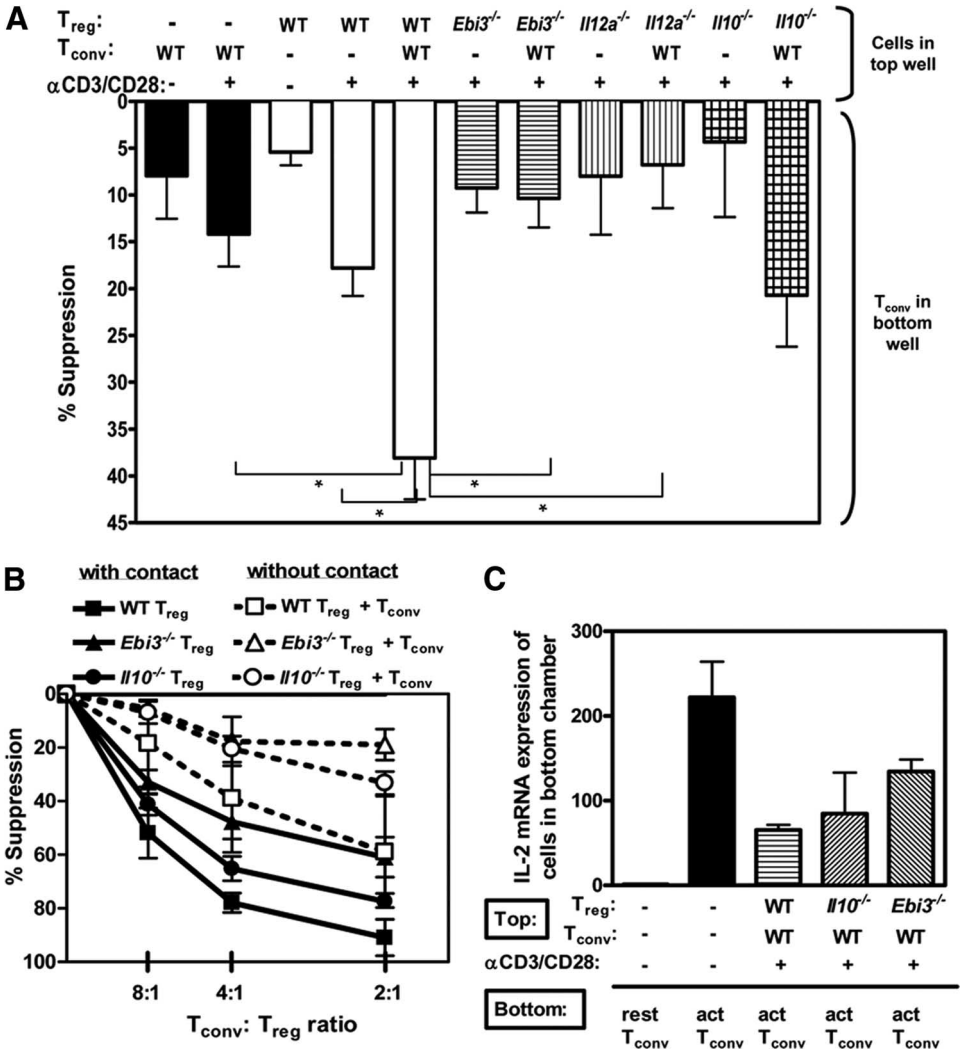

FIGURE 2. T_{reg}-mediated suppression is potentiated by T_{conv} contact in an IL-10- and IL-35-dependent, but not IL-2, manner. T_{conv} or T_{reg} from the spleens and lymph nodes of C57BL/6, *Ebi3*$^{-/-}$, *Il12a*$^{-/-}$, and *Il10*$^{-/-}$ mice were purified by FACS. *A*, Cells assayed for regulatory capacity (T_{conv} or T_{reg} alone or in combination at a 4:1 T_{conv}:T_{reg} ratio) were cultured in the top chambers of a Transwell culture plate, as indicated. Freshly purified wild-type responder T_{conv} were cultured in the bottom chamber of the 96-well flat-bottom plates in medium containing anti-CD3/anti-CD28-coated latex beads. After 64 h in culture, top chambers were removed and [^{3}H]thymidine was added directly to the responder T_{conv} in the bottom chambers of the original Transwell plate for the final 8 h of the 72-h assay. Cultures were harvested and cpm was determined. *B*, Purified T_{reg} from wild-type, *Ebi3*$^{-/-}$, and *Il10*$^{-/-}$ mice were cultured at the indicated ratios with purified T_{conv} and assayed for regulatory capacity in the top wells of the Transwell plate. In parallel, T_{reg} were assayed for regulatory capacity when T_{reg} were in direct contact with responder T_{conv} in the bottom chamber of the Transwell plate. Suppression of purified responder T_{conv} was measured by [^{3}H]thymidine incorporation. *C*, RNA was extracted from cells in the bottom chamber of the Transwell plate. Groups analyzed were those cultured with no cells in the top chamber or wild-type T_{conv} cocultured with T_{reg} (WT, *Il10*$^{-/-}$). IL-2 expression was determined by real-time quantitative PCR. Data represent the mean ± SEM of four (*A*), two (*B*), two (*C*), and independent experiments. Statistical analysis: ∗, $p < 0.05$. The cpm of T_{conv} activated alone, in the absence of any suppressors, were 25,000–60,000.

or activated T_{conv} or T_{reg} in the upper chamber (Fig. 2*A*). Remarkably, wild-type T_{reg} in the presence of T_{conv} (at a 4:1 T_{conv}:T_{reg} ratio in the upper chamber) suppressed responder T_{conv} proliferation (in the lower chamber) by 40% in the absence of cell contact (Fig. 2*A*). Control experiments, in which the total number of cells in the top chamber of the Transwell plate was kept the same, demonstrate that this was not merely a result of combined suppression of individual T_{conv} and T_{reg}, but rather T_{conv} potentiation of T_{reg} suppression (supplemental Fig. 1).[4] Because this suppression occurred across a permeable membrane, this inferred a role for soluble factors. Thus, we next assessed whether the inhibitory cytokines IL-35 and IL-10 contributed to the T_{conv}-potentiated T_{reg} suppression. Strikingly, IL-35-deficient T_{reg} from either *Ebi3*$^{-/-}$ or *Il12a*$^{-/-}$ mice fail to increase suppression across the permeable membrane in the presence of T_{conv} in the upper chamber (Fig. 2*A*). This suggests that IL-35 is required for this cell contact-independent suppression by T_{reg} in vitro, which correlates with the 40-fold increase in IL-35 secretion observed upon contact with T_{conv} (Fig. 1*C*). Previous studies have suggested that IL-10 does not contribute to T_{reg} function in vitro, even though there are substantial data to support its importance in vivo. To our surprise, in the Transwell system, *Il10*$^{-/-}$ T_{reg} in the presence of T_{conv} have reduced suppressive capacity when compared with wild-type T_{reg}; however, this was greater than in the absence of T_{conv} in the upper chamber (Fig. 2*A*). This raises the possibility that IL-10 and IL-35 act together or synergize for maximal suppression mediated by T_{reg} and that their function is potentiated by T_{conv}.

T_{conv} potentiation of T_{reg} suppression is robust and not controlled by strength of activation

The ability of T_{conv} to potentiate T_{reg}-mediated suppression across the Transwell was not dependent upon the strength of proliferation of the responder T_{conv} in the bottom chamber of the Transwell. Transwell T_{reg} suppression was seen when the proliferation of responder T_{conv} alone ranged from 5,000 to 75,000 cpm (data not shown). It should be noted that all cell populations were sorted based on cell surface marker expression, T_{conv} ($CD4^+CD45RB^{high}CD25^-$) and T_{reg} ($CD4^+CD45RB^{low}CD25^+$), and were both pure and expressed comparable levels of Foxp3, as determined by intracellular staining (supplemental Fig. 2).[4] Thus, any differences in their suppressive capacities were not due to differences in purity or Foxp3 expression. To illustrate the robustness of the suppression seen across the Transwell, parallel suppression assays were performed in which T_{reg} were placed in contact with T_{conv} in the lower chamber. Our results show that Transwell suppression mediated by T_{conv}:T_{reg} coculture was approximately half what was seen in conventional T_{reg} assays in which T_{conv} and T_{reg} are in direct contact (2:1 T_{conv}:T_{reg} ratio, 91 vs 59%; 4:1 T_{conv}:T_{reg} ratio, 78 vs 39%) (Fig. 2*B*). This is surprisingly robust given that the IL-35 and IL-10 secreted by the T_{reg} in the upper chamber have to diffuse across the entire well to achieve suppressive concentrations. This contrasts with the substantially higher concentration of cytokine that would be present in close proximity to targets with which T_{reg} are in contact (22).

Reduced suppression in the absence of IL-10 and IL-35 is not due to differential consumption of IL-2

Recent data suggest that the suppressive effects of T_{reg} are mediated in part by consumption of IL-2 (23). To determine whether differences seen in the ability of wild-type and cytokine-deficient T_{reg} to suppress across the Transwell were due to differential consumption of IL-2, we measured IL-2 concentration in the Transwell T_{reg} assay. Total IL-2 secretion was not reduced in the Transwell culture when responder cell proliferation was suppressed (supplemental Fig. 3*a*).[4] These data suggest that the Transwell suppression observed was not due to IL-2 deprivation-mediated apoptosis.

Because there are dual sources of IL-2 in this assay (from T_{conv} in the top and bottom chambers of the Transwell), IL-2 secretion may in fact be reduced on a per cell basis. Indeed, if one adjusts for T_{conv} number, IL-2 secretion per 10^4 cells in the assay was reduced (supplemental Fig. 3*b*).[4] To directly assess whether IL-2 expression was reduced by Transwell suppression, we measured IL-2 mRNA expression in the target responder T_{conv} in the bottom chamber following suppression by T_{conv}:T_{reg} cocultured in the top chamber. We found that the responder T_{conv} IL-2 expression was reduced by 30–50%, depending upon the genotype of the T_{reg} in the top chamber (Fig. 2*C*). This suggests that IL-2 expression is reduced by Transwell suppression in the absence of direct contact with target T_{conv} in the bottom chamber.

The role of preactivation in mediating Transwell suppression

Recent studies using preactivated and fixed T_{reg} have contributed to our understanding of the characteristics and conditions required for T_{reg} to suppress T_{conv} proliferation (1, 24). Reports indicate that previously activated T_{reg} do not require restimulation through their TCR to suppress T_{conv} proliferation. Moreover, once preactivated, T_{reg} can be fixed and still retain their suppressive capacity. To determine whether *Il10*$^{-/-}$ and *Ebi3*$^{-/-}$ T_{reg} were able to suppress under these conditions, wild-type, *Il10*$^{-/-}$, and *Ebi3*$^{-/-}$ T_{reg} were assayed for proliferative capacity following preactivation, with and without fixation, in both conventional and Transwell T_{reg} assays. Like wild-type T_{reg}, freshly isolated, fixed *Il10*$^{-/-}$ and *Ebi3*$^{-/-}$ T_{reg} were unable to suppress T_{conv} proliferation in a conventional T_{reg} assay. Not surprisingly, if T_{reg} were preactivated before fixation, all T_{reg} regardless of genotype were able to mediate suppression, albeit to a slightly lesser degree than freshly isolated T_{reg} (Fig. 3*A*). In a Transwell assay, preactivation did not enhance the suppressive capacity of the T_{reg}, and preactivated and subsequently fixed T_{reg}, regardless of genotype, were unable to suppress responder T_{conv} proliferation in the bottom well (Fig. 3*B*).

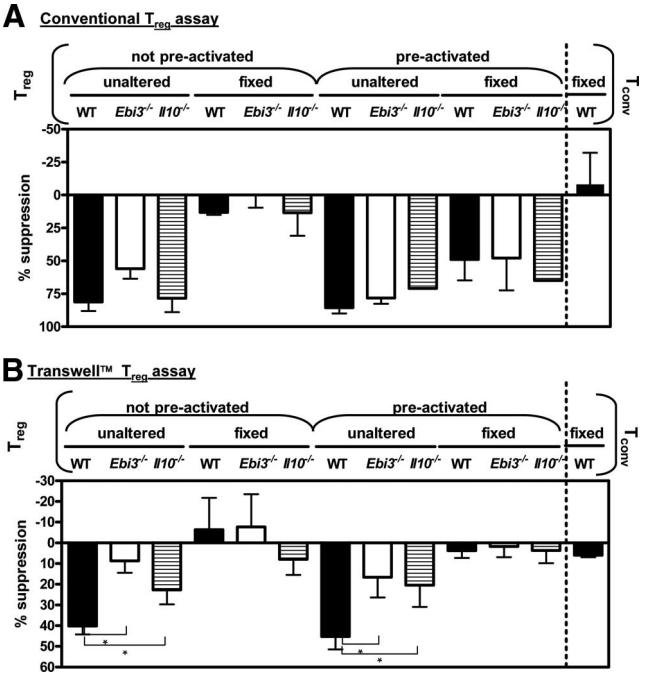

FIGURE 3. Regulatory capacity of fresh and preactivated fixed T_{reg}. T_{conv} or T_{reg} from the spleens and lymph nodes of C57BL/6, *Ebi3*$^{-/-}$, and *Il10*$^{-/-}$ mice were purified by FACS. Cells were cultured fresh or preactivated for 24 h before culture, with or without fixation with 20% formaldehyde. Cells assayed for regulatory capacity (T_{conv} or T_{reg} alone or in combination at a 4:1 T_{conv}:T_{reg} ratio) were cultured in *A*, direct contact with responder T_{conv} in a 96-well round-bottom plate. Statistical analysis indicates fixation does not affect the ability of preactivated T_{reg} to suppress while in direct contact with responder T_{conv} (*B*) top chambers of a Transwell culture plate, as indicated. Freshly purified wild-type responder T_{conv} were activated in the bottom chamber of the 96-well plates. After 64 h in culture, top chambers were removed and [^{3}H]thymidine was added directly to the responder T_{conv} in the bottom chambers of the original Transwell plate for the final 8 h of the 72-h assay. Cultures were harvested and cpm was determined. Statistical analysis: *, $p < 0.05$.

The role of IL-10 in T_{reg} suppression in vitro

It is well known that IL-10 is essential for T_{reg} suppression in vivo (4, 9–17). However, previous studies using IL-10-neutralizing Abs suggested that IL-10 is not required for T_{reg}-mediated suppression in vitro (1, 3, 8). We sought to expand upon these results by using an IL-10-neutralizing Ab in both conventional and Transwell T_{reg} assays. We show that IL-10 neutralization has no effect on suppression in a conventional T_{reg} assay, as previously reported; however, in the Transwell system, neutralizing IL-10 reduces the suppression mediated by wild-type T_{reg} from 40 to 15% and further reduces the suppression by *Il10*$^{-/-}$ T_{reg} from 25 to 5% (Fig. 4). Hence, IL-10 secretion from both the T_{reg} and suppressed T_{conv} populations appears important to maintaining the fidelity of T_{reg}

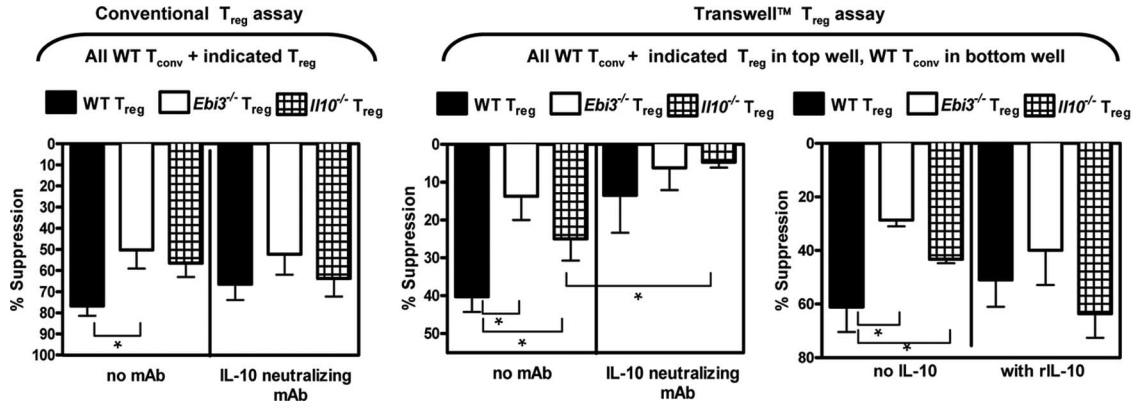

FIGURE 4. T_{reg}-mediated suppression across the Transwell is IL-10 dependent. Purified T_{reg} were assayed for regulatory capacity in conventional T_{reg} and Transwell T_{reg} assays in the presence of an IL-10-neutralizing Ab (10 μg/ml) or with rIL-10 (100 ng/ml), as indicated. Percentage of suppression was calculated in reference to the activated T_{conv} control under the conditions indicated. All percentage of suppression to the *left* of the line was in reference to T_{conv} without anti-IL-10 or rIL-10, and the reference for percentage of suppression to the *right* of the line was T_{conv} with anti-IL-10 or rIL-10, respectively. Data represent the mean ± SEM of three independent experiments. Statistical analysis: *, $p < 0.05$. The cpm of T_{conv} activated alone, in the absence of any suppressors, were 25,000–60,000.

suppression across the Transwell. This is surprising given that the amount of IL-10 that the suppressed target T cells (T_{sup}) produce appears small when *Il10*$^{-/-}$ T_{reg} were cultured with T_{conv} (Fig. 1, *A* and *B*). However, it is possible that the actual IL-10 contribution is much higher because T_{reg}-derived IL-10 may potentiate IL-10 production by the T_{sup}. Importantly, Transwell suppression by *Il10*$^{-/-}$ T_{reg} was restored by addition of rIL-10 (Fig. 4). Thus, the differential and potentially synergistic use of IL-10 and IL-35 by T_{reg} may help explain and unify in vitro and in vivo results regarding the necessity of IL-10 for T_{reg} suppression. In a Transwell T_{reg} assay, ~300 pg/ml TGF-β was detected in the culture supernatant (data not shown). Given the modest amount of TGF-β present in the Transwell T_{reg} assay, it is unlikely that suppression of T_{conv} proliferation is influenced by TGF-β; however, this will need to be formally tested.

T_{conv} activation is required for maximal potentiation of T_{reg} function

We have shown that T_{reg} must be activated in the presence of T_{conv} to mediate suppression in the absence of cell contact (i.e., across the permeable membrane). Next, we wanted to determine the characteristics required by T_{conv} to facilitate this suppression. We first showed that polarized Th1 or Th2 CD4$^+$ effector T cells in the upper chamber could potentiate T_{reg}-mediated suppression across the membrane (supplemental Fig. 4).[4] We then asked whether the T_{conv} that were cocultured with T_{reg} required activation to potentiate suppression across the permeable membrane. To determine this, we used two experimental approaches that gave comparable results. First, we cultured T_{reg} in the presence of T_{conv} that were fixed with formaldehyde and fail to proliferate (Fig. 5*A*). We show that they have a significantly reduced capacity to potentiate T_{reg}-mediated suppression across the membrane (reduced by 55%) (Fig. 5*B*). Second, we used an anti-TCR Vβ8 mAb (F23.1) to stimulate, rather than anti-CD3ε, so that the activation of T_{conv} in the upper chamber could be controlled by the Vβ expression of the T_{conv} used (Vβ8$^+$ T_{reg} in the presence of Vβ8$^+$ or Vβ8$^-$ T_{conv}). As seen with the fixed T_{conv}, Vβ8$^-$ T_{conv} fail to proliferate (Fig. 5*C*), and the suppressive capacity of Vβ8$^+$ T_{reg} stimulated with anti-Vβ8 mAb-coated beads could be potentiated by Vβ8$^+$ T_{conv}, but only partially by Vβ8$^-$ T_{conv} (45% reduction) (Fig. 5*D*). Thus, in both systems, the ability of T_{conv} to augment T_{reg} activity was reduced by ~50%. Thus, potentiation of T_{reg} suppression across a permeable membrane can be mediated by different CD4$^+$ T cell subsets, with which T_{reg} require direct contact, and was maximal when the T_{conv} were activated.

Although IL-10 production was primarily derived from T_{reg}, T_{conv} also secrete a small amount of IL-10 (Fig. 1, *A* and *B*). Therefore, IL-10 production by these suppressed T cells (T_{sup}) may contribute to the suppression observed in Transwell experiments. To further elucidate the importance of T_{sup}-derived cytokines in Transwell suppression, we cultured wild-type, *Ebi3*$^{-/-}$,

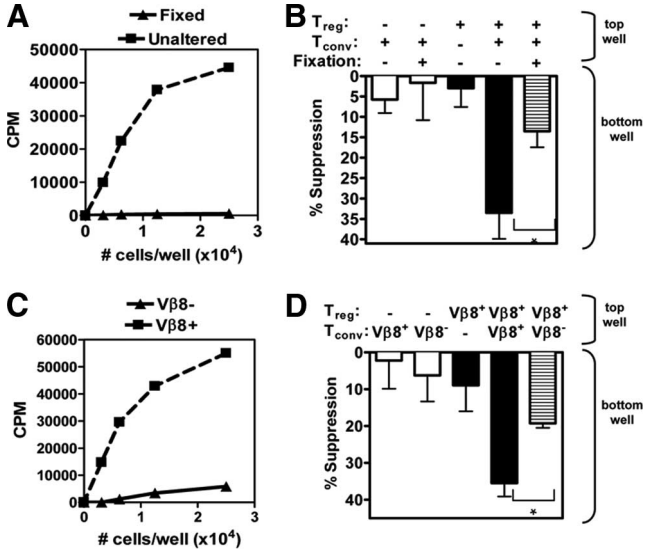

FIGURE 5. T_{reg}-mediated suppression is potentiated, in part, by TCR signals derived from T_{conv} contact. *A*, Purified T_{conv} with or without fixation with 20% formaldehyde were stimulated for 72 h with anti-CD3/CD28-coated beads and assayed for their proliferative capacity by [^{3}H]thymidine incorporation. *C*, Purified Vβ8$^+$ or Vβ8$^-$ T_{conv} were stimulated for 72 h with anti-Vβ8-coated beads and assayed for their proliferative capacity by [^{3}H]thymidine incorporation. *B* and *D*, Purified T_{conv} or T_{reg} were cultured under indicated conditions: unstimulated, stimulated for 72 h with anti-CD3/CD28-coated beads with or without fixation with 20% formaldehyde (*B*), or anti-Vβ8-coated beads (*D*), and assayed for their ability to suppress responder T_{conv} proliferation across the Transwell membrane. Data represent the mean ± SEM of four to six independent experiments. Statistical analysis: *, $p < 0.05$. The cpm of T_{conv} activated alone, in the absence of any suppressors, were 25,000–60,000.

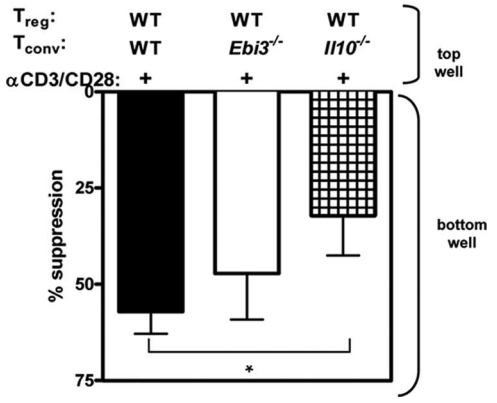

FIGURE 6. T_{sup}-derived IL-10 may contribute to the regulatory milieu. Wild-type T_{reg} were cultured with T_{conv} from wild-type, *Ebi3*$^{-/-}$, and *Il10*$^{-/-}$ mice in the top chambers of the Transwell plate. The ability of these cocultures to suppress fresh responder T_{conv} proliferation in the bottom chamber was measured by [^{3}H]thymidine incorporation. Data represent the mean ± SEM of five independent experiments. Statistical analysis: *, $p < 0.05$. The cpm of T_{conv} activated alone, in the absence of any suppressors, were 25,000–60,000.

and *Il10*$^{-/-}$ T_{conv} with wild-type T_{reg} in the top chamber of a Transwell experiment in which wild type T_{conv} were in the bottom chamber. Cocultures with wild-type and *Ebi3*$^{-/-}$ T_{conv} mediate Transwell suppression equally well; however, cocultures with *Il10*$^{-/-}$ T_{conv} exhibit reduced suppression, suggesting that in addition to the T_{reg}-derived IL-10, T_{sup}-derived IL-10 may also contribute to the regulatory milieu (Fig. 6).

Discussion

These observations present a model for the relative contribution of IL-10 and IL-35 to the suppressive activity of T_{reg}. There is the general belief that T_{reg} mediate suppression in a largely cytokine-independent, cell contact-dependent manner, primarily based on studies using Transwell culture plates. However, our results indicate that whereas T_{reg} activation alone does not maximally induce IL-35 expression or mediate long-distance/distal suppression, as examined across a permeable membrane, both activities are mediated and potentiated by contact with T_{conv}.

Many interactions between cells of the immune system are mediated by bidirectional signals. Although the mechanism whereby T_{conv} boost T_{reg} function is currently unknown, we hypothesize that receptor-ligand interactions between cocultured T_{conv} and T_{reg} occur to initiate distinct signaling pathways within each cell type. In the T_{reg}, a signaling pathway that leads to enhanced IL-35 secretion is most likely coupled with enhanced expression of many other regulatory proteins. Although the identity of this receptor: ligand interaction is unknown, our data suggest that it is most likely mediated by a cell surface receptor on T_{conv} because fixed cells could mediate T_{reg} potentiation. Furthermore, although resting cells could mediate potentiation, this was clearly maximal following T_{conv} stimulation, suggesting that this is an activation-induced ligand.

The observation that T_{reg} function is potentiated by contact with T_{conv} suggests an important shift in the concept of cell contact dependency of suppression. Our data suggest that it is not the function of T_{reg} that is solely contact dependent, but rather the induction of suppression that is mediated by T_{conv} contact. This does not preclude a role for contact-dependent suppression, but does suggest that this contact may also play a key role in potentiating T_{reg} function. These data also suggest that the bystander suppression that characterizes T_{reg} function could be facilitated by cytokine-mediated distal suppression. Given that T_{reg} represent a minor population, this may be particularly important in allowing T_{reg} to create a local suppressive milieu within defined/confined anatomical locations, such as lymph nodes. The data presented in this study also help to reconcile conflicting assessments on the role of IL-10 in T_{reg}-mediated suppression from in vitro and in vivo studies. Given the multitude of regulatory mechanisms available to T_{reg} (25), loss of a single mechanism may have little effect in a conventional in vitro T_{reg} assay. It is likely that this does not adequately reflect the challenges faced by T_{reg} in vivo. Our study shows that IL-10 is indeed a significant contributor to T_{reg} function in vitro when the T_{reg} arsenal is restricted. As such, the T_{reg}:T_{conv} Transwell assay we have described in this study may be a useful in vitro correlate for in vivo function that may be of particular value in assessing the regulatory capacity of different human T_{reg} populations.

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Disclosures

The authors have no financial conflict of interest.

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