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Notch1 Signaling and Regulatory T Cell Function

Naoki Asano,* Tomohiro Watanabe,*† Atsushi Kitani,* Ivan J. Fuss,* and Warren Strober1*

Previous studies have shown that the Notch1 and TGF-β signaling pathways are mutually re-enforcing. Given recent evidence that regulatory T cell (Treg) effector function is mediated by TGF-β signaling, we investigated whether Notch1 signaling also participated in Treg effector function. Initial studies showed that Notch1 ligands, particularly Jagged1, are present on Tregs and that, indeed, blockade of Notch1 signaling with an anti-Jagged1 or a blocking anti-Notch1 Ab inhibits Treg suppressor function in vitro. We then showed that a signaling component generated by Notch1 activation (Notch1 intracellular domain) of dendritic cells physically interacts with a signaling component generated by TGF-β signaling (pSmad3). Furthermore, this interaction has functional downstream effects because over-expression of Notch1 intracellular domain facilitates pSmad3 translocation to the nucleus and enhances pSmad3 transcriptional activity of a Smad-sensitive promoter linked to a luciferase reporter. Finally, we showed that blockade of TGF-β signaling and Notch signaling did not have additive inhibitory effects on Treg suppressor function. These results are consistent with the conclusion that Notch1 signaling facilitates TGF-β-mediated effector function of Tregs. The Journal of Immunology, 2008, 180: 2796–2804.

The CD4+CD25+ regulatory T cells (Tregs) play an important role in regulating the immune system by suppressing self-reactive T cells that have escaped negative selection in the thymus as well as hyperactive T cells that are induced during excessive immune responses in peripheral lymphoid tissues (1–3). Over the past several years, strong evidence has accumulated supporting the idea that the suppressive function of Tregs is mediated by TGF-β, an evolutionarily conserved cytokine that is known to have a suppressive function as one of its numerous properties (4). Perhaps the most important of these with respect to Treg function is the fact that Tregs secrete TGF-β and express the latter on the cell surface, as well as the fact that TGF-β inhibits APC function, the main focus of Treg suppression (5–9). Thus, when TGF-β binds to its receptor, the receptor phosphorylates the signaling molecule Smad3 that then forms a heterodimeric or heterotrimeric complex with Smad4; this complex then translocates to the nucleus where it plays a negative role in the transcription of various genes (10). It should be noted, however, that TGF-β can also initiate positive signaling pathways including one pathway that is important to Treg function. This involves the ability of TGF-β to induce naïve CD4+ cells to differentiate into Tregs and, thus, to facilitate the expansion of peripheral Treg populations (8, 11, 12). The molecular mechanisms underlying this positive function, however, are poorly understood.

The Notch signaling pathway is another pathway that may be involved in Treg suppressor function. This possibility arises from several reports that overexpression of Notch ligand can induce regulatory cells (13–15); in addition, it has recently been shown that Tregs with membrane-bound TGF-β stimulate the Notch pathway and that such stimulation is necessary for the induction of tolerance to respiratory Ags in the airway (16). Notch is a phylogenetically old molecule that was first identified as a signaling component essential for the differentiation of the embryonic nervous system of Drosophila (17, 18). Later studies showed that Notch was also involved in mammalian differentiation processes including those governing the development of the immune system (19). The latter included evidence for a role of Notch signaling in the intrathymic differentiation of T cells (20–22) as well as Th cell development in the periphery (23–27). Notch is, like TGF-β, an evolutionarily conserved molecule but, unlike TGF-β, is a type I transmembrane receptor rather than a receptor ligand. Upon binding with its ligands (Jagged1 and 2 and Delta-like1, 3, and 4), it releases its intracellular domain into the cytoplasm which then translocates to the nucleus (19). Recently, it has been shown that the released signaling models arising from Notch1 activation enhance Smad signaling function in fibroblast and other mesenchymal cells (28), suggesting that Notch1 signaling could interact with the TGF-β signaling occurring in Treg function.

In this study, we show that Tregs preferentially express cell membrane Notch ligands and that blocking Notch signaling initiated by such ligands inhibits Treg suppressor function. We then showed that phosphorylated Smad3 arising from TGF-β signaling binds to Notch1 intracellular domain (NICD) arising from Notch signaling and that the complex formed facilitates the translocation of phosphorylated Smad3 to the nucleus. These findings suggest that Treg suppressor function does involve Notch signaling, but the effect of the latter is channeled through the suppressor signal mediated by TGF-β.

Materials and Methods

Mice

A group of 5–7-wk-old BALB/c female mice were purchased from The Jackson Laboratory and maintained in the animal facility of the National Institutes of Health.
Cells

HT29 cells, THP-1 cells, and Jurkat cells were purchased from American Type Cell Culture (ATCC) and maintained according to the ATCC protocol. MLEC-PAI cells (mink lung epithelial cells stably expressing plasminogen activation inhibitor-1 (PAI-1) luciferase reporter construct originally developed by Abe et al. (29)) was obtained from Dr. L. van de Water (Massachusetts General Hospital, Boston, MA) and maintained as previously described (30).

Abs and reagents

Anti-mouse CD3e Ab was purchased from BD Pharmingen. Notch-1 Ab-1 (Clone A6) was purchased from Neomarkers; this Ab has been shown in previous studies to be a blocking Ab that inhibits Notch1 signaling (16, 31). Abs for Notch ligands, multispecies TGF-β1, 2, and 3 mAb (Clone 1D11), mouse IgG1, mouse IgG2b isotype control, and recombinant TGF-β1 were purchased from R&D Systems. pSmad3 Ab and anti-cleaved Notch1 Ab was purchased from Cell Signaling Technology. Affinity purified agarose immobilized V5 Ab was purchased from Bethyl Laboratories, and recombinant human GM-CSF and IL-4 were purchased from PeproTech. Collagenase D was from Roche.

Suppressor assays

CD4+ CD25+ T cells and CD4+ CD25− T cells were isolated from the spleen of the BALB/c mice using a mouse CD4+ T cell enrichment column (R&D Systems) and a mouse CD4+ CD25+ Treg kit purchased from Miltenyi Biotec. A total of 5 × 10^6 CD4+ CD25+ T cells were cultured with 3000 rad irradiated splenocytes (as APCs), various ratios of CD4+ CD25+ T cells, and 1 µg/ml anti-CD3e in 96-well round-bottom plates for 72 h. A total of 1 µCi/well [3H]thymidine was added to the wells during the last 8 h of culture. The cells were then harvested and assessed for thymidine incorporation in a liquid scintillation counter. In the studies in which suppression was blocked with Abs to TGF-β, Notch1, Jagged1, or Delta-like4, the Abs were added to the culture at a final concentration of 50 µg/ml. Blocking studies were controlled with the use of isotype-control (mouse IgG1 and mouse IgG2b) at a final concentration of 50 µg/ml.

Mouse splenic dendritic cells (DCs)

Spleens obtained from 5–7-wk-old BALB/c female mice were injected with Collagenase D solution (containing 2 mg/ml Collagenase D in HBSS with calcium chloride, magnesium chloride, and magnesium sulfate) and then cut into small pieces with a carbon steel surgical blade (Millex). The spleen pieces were then incubated in Collagenase D solution for 30 min at 37°C and passed through a 70 µm cell strainer (BD Falcon). The cells were then subjected to magnetic selection with CD11c microbeads (Miltenyi Biotec). Isolated cells were then stained with appropriate Ab and analyzed by flow cytometry.

Human monocyte-derived DCs

Monocytes were elutriated from the peripheral blood of healthy donors and were cultured in 6-well plates (1 × 10^6/ml) in 5 ml of complete medium (RPMI 1640 medium supplemented with 2 mM l-glutamine and 10% FCS) supplemented with rGM-CSF (20 ng/ml) and rIL-4 (20 ng/ml). After 3 days of culture, half of the medium in each well was exchanged. After 6 days of culture, at the time of their use, >90% of the cells expressed characteristic DC-specific markers (CD1a and HLA-DR), as determined by flow cytometry.

Generation of Jagged1 expressing Jurkat cell line

Jurkat cells were transfected with Jagged1 expressing pcDNA3.1/Hygro plasmid (gift from Dr. Doreen Kacer, Maine Medical Center Research Institute; Ref. 32), and positive clones were selected by limiting dilution using 1000 µg/ml hygromycin. The obtained Jagged1 expressing Jurkat cells will be referred to as Jag Jurkat cells.

Immunoprecipitation and Western blotting

A total of 1 × 10^6 THP1 cells were cultured in 6-well plates filled with 2.5 ml complete RPMI 1640 medium (10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). The cells were transfected with either cleaved Notch1 expression vector pN1ICD-V5 or empty pcDNA4.0-V5/his version A vector (gifts from Dr. Doreen Kacer, Ref. 32). A total of 48 h after transfection, the cells were cultured with or without 10 ng/ml rTGF-β for 30 min, washed twice with PBS, and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, and 1% NP40) containing a protease inhibitor mixture (Roche). The cell lysates were then collected and mixed with agarose-immobilized anti-V5 on a rotary shaker at 4°C overnight, after which the agarose beads were washed three times with RIPA buffer. The eluted protein was then subjected to SDS-PAGE followed by transfer to nitrocellulose membrane (Amersham Biosciences). In experiments using human monocyte-derived DCs, 6 × 10^6 human monocyte-derived DCs were cultured in 6-well plates filled with 3 ml of complete medium with or without 10 ng/ml rTGF-β and with or without either 2 × 10^5 Jurkat or Jag Jurkat cells. A total of 4 h after transfection, the cells were washed twice with PBS and lysed with RIPA buffer containing a protease inhibitor mixture (Roche) and phosphatase

FIGURE 1. Notch ligands are expressed on CD4+ CD25+ T cells. Naive CD4+ CD25− T cells and CD4+ CD25+ T cells were isolated from spleens of BALB/c mice. The cells were stained for Notch ligands, Notch1 and Foxp3, and their expression was assessed by flow cytometry.
inhibitor mixture (Roche). The cell lysates were then collected and mixed with anti-cleaved Notch1 on a rotary shaker at 4°C overnight. Protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz Technology) was then added to the mixture and the samples were mixed on a rotary shaker at 4°C for another 2 h, after which the samples were centrifuged and supernatants were collected. The agarose beads were then washed three times with RIPA buffer and the eluted protein was subjected to SDS-PAGE followed by transfer to nitrocellulose membrane (Amersham Biosciences). After blocking with 5% skim milk, the membrane was incubated with appropriate primary Abs at 4°C overnight and then washed and incubated with appropriate secondary Abs for 1 h at room temperature. Finally, after incubation, the membrane was washed and the proteins were visualized by chemiluminescence according to the manufacturer’s protocol (Pierce). In the experiments with human DCs, the collected supernatant was also subjected to SDS-PAGE and assessed for pSmad3 and actin.

Luciferase assay

A total of 2.5 \times 10^5 MLEC-PAI cells were transfected with either empty pcDNA4.0-V5/his vector or Notch1 expression vector pN1ICD-V5 (gifts from Dr. Doreen Kacer; Ref. 32) together with pSV-\beta-galactosidase control vector (Promega) in a 96-well flat-bottom plate. A total of 48 h after transfection, the cells were cultured with various concentrations of recombinant TGF-\beta for 8 h, after which cell lysates were prepared using a Glo-Lysis (Promega) or \beta-gal kit (Applied Biosystems). Luciferase activity in the lysates was measured by Wallac Victor3 luminometer (PerkinElmer) and relative luciferase activity was calculated by dividing luciferase units with \beta-gal units.

Nuclear extraction and Western blotting

A total of 2.5 \times 10^5 HT29 cells were cultured in 6-well plates filled with 2.5 ml complete DMEM/F-12 medium (10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 \mu g/ml streptomycin). The cells were transfected with either pN1ICD-V5 or empty pcDNA4.0-V5/his version A vector using TransIT-LT1 transfection reagent (Mirus Bio Corporation) according to the manufacturer’s protocol. A total of 36 h after transfection, the medium was changed to serum-free DMEM/F-12 containing 1% BSA, and 48 h after transfection, the cells were cultured with or without 1 ng/ml rTGF-\beta for 30 min, after which nuclear extraction was performed using a transfactor extraction kit (Clontech Laboratories). The nuclear extracts were subjected to SDS-PAGE, transfer to the nitrocellulose membrane, and chemiluminescence as indicated above.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was valued by Student’s t test.

Results

**CD4^+ CD25^+ T cells, but not CD4^+ CD25^- T cells, express cell surface Notch ligand Jagged1**

In initial studies, we assessed the expression of Notch ligands on freshly isolated splenic CD4^+ CD25^+ T cells and CD4^+ CD25^- T cells.
cells by two-color flow cytometry. As shown in Fig. 1, ~90% of isolated CD4^+CD25^+ cells expressed Foxp3, indicating that this cell population was indeed composed largely of Tregs (and can be referred to as such). As also shown in Fig. 1, Jagged1 and, to a lesser extent, Delta-like4 was expressed on Tregs whereas such expression was minimal on CD4^+CD25^- T cells. Jagged2 or Delta-like1 was not expressed on either cell type. Both Tregs and CD4^+CD25^- T cells expressed Notch1.

**DCs express Notch1 on their surface**

In parallel studies, we assessed the expression of Notch1 or Notch1 ligands on splenic CD11c DCs by flow cytometry. As shown in Fig. 2a, unstimulated murine splenic DCs express Notch1, but not Notch ligands, on their surface. As shown in Fig. 2b, similar results were obtained with human monocyte-derived DCs.

**Blocking Notch1 signaling partially inhibits the regulatory function of CD4^+CD25^+ T cells**

Because the Notch ligands, especially Jagged1, are preferentially expressed on Tregs and Notch1 is expressed on DCs, it seemed possible that Notch signaling was involved in the regulatory activity exerted by these cells. To test this possibility, we performed an in vitro suppression assay in which we determined the ability of a blocking anti-Notch Ab to inhibit suppressor activity at various CD25^-CD25^+ cell ratios. In this assay, irradiated splenocytes served as APCs. As shown in Fig. 3a, blocking anti-Notch1 (50 μg/ml) did in fact inhibit suppression, but only in cultures that contained a 4:1 and greater ratio of CD25^- T cells to CD25^+ T cells. Similar, albeit weaker, inhibitory effects were noted in suppression assays in which blocking anti-Jagged1 Ab was added to the cultures (Fig. 3b). In an additional study, we also determined whether a greater inhibition of suppression could be obtained with...
a mixture of anti-Jagged1 and anti-Delta-like4 present in equal concentration. As shown in Fig. 3c, the presence of both Abs was only marginally better than anti-Jagged1 Ab alone in the reversal of suppression. In control studies shown in Fig. 3d, addition of isotype control IgG did not inhibit suppression in the suppressor assay.

We also attempted to evaluate the ability of an inhibitor of Notch signaling, γ-secretase inhibitor (GSI), to influence Treg function in a suppressor assay. This proved to be infeasible because GSI itself had a profound anti-proliferative effect on CD25- cells in the absence of Tregs (data not shown), possibly because GSI affects many proteins that are cleaved by γ-secretase (33).

An intracellular Notch1 component released upon Notch1 activation binds to phosphorylated Smad3

Previous studies have shown that Treg suppressor activity is mediated by membrane-bound TGF-β (30). We therefore hypothesized that the above participation of Notch signaling in Treg effector function may be due to its ability to enhance TGF-β signaling in APCs. To investigate this possibility, we initially determined whether a major TGF-β signaling intermediate (Smad3) physically interacts with a Notch signaling intermediate, NICD, in TGF-β-stimulated cells. To this end, we transfected the human macrophage cell line, THP1 cells, with either empty vector or with V5-tagged NICD and stimulated the transfected cells with TGF-β. We then obtained whole cell lysates of the stimulated cells at 30 min after stimulation, the time of peak expression of phosphorylated Smad3. Similar results were obtained in studies of NICD-transfected HT29 cells (data not shown).

In further studies, we determined whether the Smad/NICD interaction also occurs in untransfected primary APCs. To this end, we developed a Jurkat cell line stably transfected with Jagged1 (Jag-Jurkat) (see Materials and Methods) and, as shown in Fig. 4b, expressing Jagged1 on its surface to about the same extent as Tregs; untransfected Jurkat cells, however, exhibited only weak Jagged1 expression. Next, using the Jag-Jurkat cells to simulate Tregs, we cocultured the Jag-Jurkat cells (or Jurkat cells) with human monocyte-derived DCs in the presence of TGF-β and after 4 h lysed the cells to obtain whole cell lysates for immunoprecipitation studies of Notch-Smad interactions. As shown in Fig. 4c, immunoprecipitation of the lysates with anti-V5-coated agarose beads followed by SDS-PAGE and immunoblotting with anti-pSmad3 Ab yield a strong pSmad3 band in TGF-β-stimulated NICD-transfected cells as well as a weak pSmad3 band in nonstimulated NICD-transfected cells, the latter probably due to the TGF-β present in the cell culture medium. Similar results were obtained in studies of NICD-transfected HT29 cells (data not shown).

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FIGURE 4. Activated Notch1 binds to pSmad3. a, THP1 cells were transfected with either empty vector or NICD-expressing vector and then stimulated with TGF-β. Cell lysates were collected and subjected to immunoprecipitation with anti-V5 followed by immunoblotting anti-pSmad3. b, Jurkat cells or Jurkat cells stably transfected with a Jagged1-expression plasmid (Jag-Jurkat) were stained with anti-Jagged1 and then analyzed for surface Jagged1 expression by flow cytometry. c, Human monocyte-derived DCs were cultured alone or cocultured with Jurkat or Jag-Jurkat cells with or without TGF-β. After 4 h of culture, cells were lysed and the whole cell lysates obtained were subjected to immunoprecipitation with anti-NICD Ab followed by immunoblotting anti-pSmad3.
Activated Notch1 enhances pSmad3 translocation into the nucleus

We next determined whether the above physical interaction between NICD and pSmad3 had functional consequences. Because activated Notch1 (NICD) translocates into the nucleus, one such consequence could be the facilitation of pSmad3 nuclear translocation. To test this possibility, we transfected an increasing amount of NICD vector into HT29 cells and stimulated the cells with TGF-β/H9252. We then isolated nuclear extracts from the cells and performed Western blots with anti-pSmad3. As shown in Fig. 5, transfection of NICD led to a dose-dependent increase in intranuclear pSmad3. Thus, another mechanism of enhancement of TGF-β signaling by Notch activation is facilitation of intranuclear transport.

Activated Notch1 enhances TGF-β-induced PAI-1 luciferase activity

In a second approach to the question of whether NICD/pSmad3 binding has a functional consequence, we determined the influence of NICD on TGF-β signaling in MLEC-PAl cells stably transfected with a Smad-sensitive PAI-1 promoter linked to a luciferase reporter cassette. In particular, we transfected the MLEC-PAl cells with either empty vector or NICD vector, together with a control pSV-β-galactosidase vector, and then stimulated the transfected cells with TGF-β. As shown in Fig. 6, transfection of the NICD vector enhanced TGF-β-induced PAI-1 luciferase activity over a range of TGF-β concentrations and especially at 500 pg/ml, the highest concentration tested. These data suggest that Notch signaling and generation of NICD leads to augmentation of TGF-β transactivation.

Notch1 suppresses the proliferation of CD4+CD25+ cells through TGF-β

Taken together, the above findings relating to TGF-β/Notch1 signaling are consistent with the idea that Notch1 signaling affects Treg effector function via its effect on TGF-β signaling rather than via a TGF-β-independent mechanism. To test this hypothesis, we reasoned that if the TGF-β and Notch1 signaling effects on Treg effector function were independent then blockade of suppression by blocking Abs in an in vitro suppressor assay would be additive, whereas if the effects were dependent then blockade of suppression would not be additive. As shown in Fig. 7, under conditions in which blockade achieved with anti-TGF-β is suboptimal (to be able to monitor the possibility of an additive effect of anti-Notch1) an additive effect was not seen at any effector:suppressor cell ratio. Thus, it is likely that the two pathways are dependent. In addition, because blockade of either pathway has an equally inhibitory effect on suppression, it is likely that Notch signaling is a necessary accompaniment of TGF-β signaling in Treg effector function.
**Discussion**

In previous studies, we and other investigators established that either cell surface TGF-β (in the form of TGF-β linked to latency associated protein, i.e., latent TGF-β or latency associated protein) or secreted TGF-β is a key component of the effector mechanism underlying the suppressor activity of naturally occurring Tregs. These studies show, for instance, that Tregs have TGF-β on their cell surface and that by blocking such TGF-β the in vitro suppressive ability of Tregs is greatly diminished (30, 34). In addition, they show that cells lacking the ability to respond to TGF-β signaling cannot be suppressed by Tregs, either in vitro or in vivo (30, 35). Finally, they show that T cell populations depleted of cells bearing membrane-bound TGF-β or T cells from mice with T cell-specific inability to produce TGF-β cannot mediate Treg function in vivo (36). These and other findings settled the question of the involvement of TGF-β in Treg effector function and, at the same time, opened the door to other questions relating to how TGF-β signaling actually leads to suppression of immune function.

With regard to these latter questions, recently it was shown that cells bearing surface TGF-β (but not cells that lacked this property) could activate the Notch1 signaling pathway in potential target cells (16). More importantly, the activity of regulatory cells that prevent the development of experimental asthma could be blocked in the whole animal by the administration of a blocking anti-Notch1 Ab (16). These results thus suggested that Notch signaling was also involved in Treg effector function, albeit in the context of TGF-β because only cells bearing TGF-β could activate the Notch pathway. It should be noted, however, that these studies of the possible role of Notch signaling in regulatory T cell function were grounded in earlier studies seeking to establish an interrelationship between TGF-β signaling and Notch signaling at the biochemical level. The first hint of such an interrelationship came from studies that showed that TGF-β signaling leads to rapid up-regulation of Hes-1, a major target of Notch signaling in keratinocytes (37). Later, more definite evidence of “cross-talk” between the two pathways has come from studies showing TGF-β signaling in neural stem cells in vivo and in myoblasts in vitro led to Notch-dependent Hes-1 expression (28). In addition, intracellular signaling molecules intrinsic to the TGF-β (Smad3) and Notch1 (NICD) signaling pathways synergize in the activation of Notch- and TGF-β-specific synthetic promoters. Finally, it was shown that Smad3 and NICD physically interact and that such interaction led to recruitment of Smad3 to Notch target genes via binding to CSL, a key DNA-binding protein of the Notch pathway.

These previous findings led us to determine whether similar TGF-β/Notch interactions governed the TGF-β signaling underlying Treg function. We found that, indeed, the machinery for activation of the Notch pathway in Treg function was in place in that Tregs expressed surface Jagged1 (and lesser amounts of Delta-like4) and potential target cells expressed Notch; moreover, we found that this machinery “worked” in relation to Treg regulatory function in that blockade of Notch1 and to a lesser extent, of Jagged1 impaired Treg suppressor function in an in vitro suppression assay. In further studies to understand the basis of these findings, we determined that Smad3 generated by TGF-β signaling of human monocyte-derived DCs (or THP1 cells (a human macrophage cell line)) associates with NICD, the Notch fragment derived from activated cell surface Notch1. In addition, we showed that transfection of NICD leads to enhanced nuclear translocation of pSmad3 and that NICD transfection of reporter cells responding to TGF-β signaling with a luciferase response led to enhanced TGF-β-induced responses, indicating that Notch activation augments Smad-mediated transcription at Smad-sensitive promoters. These studies make it apparent that Notch1 activation can enhance TGF-β signaling both at the level of Smad translocation into the nucleus and at the level of Smad transactivation at promoter sites. In a final set of studies, we showed that blockade of TGF-β signaling and Notch signaling in a suppression assay do not have additive effects. Taken together, this set of findings is consistent with the conclusion that while TGF-β signaling is essential for Treg effector function, Notch signaling plays an important and perhaps essential role in facilitating TGF-β signaling in such effector function. Furthermore, it is highly likely that this conclusion applies to TGF-β signaling mediated by membrane-bound TGF-β on Tregs in as much as we have previously shown that such TGF-β induces robust and rapid Smad activation (5, 34).

Although the conclusion above is the most likely, the data at hand could still support the notion that TGF-β signaling is enhancing Notch-mediated effector function, rather than the other way around. This arises from the fact that while in these studies we showed that Notch signaling enhances TGF-β function, in other studies cited above the enhancement was mutual (28). This latter possibility would be supported by evidence that Notch signaling has an inherently negative effect on T cell and/or APC function; however, this does not seem to be the case. Thus, while a number of studies of the effect of Notch signaling on T cell proliferation have disclosed that such signaling has a negative effect on cell proliferation (38–40), a number of other studies have indicated a positive effect (41, 42). In addition, it has been shown that Notch signaling may selectively influence T cell differentiation depending, in some studies, on the type of Notch ligand inducing Notch activation (24–27); this type of Notch signaling function could be considered positive or at least neutral because T cell differentiation occurs within a positive proliferative milieu. Finally, with respect to the effect of Notch signaling on APCs, there is clear evidence that stimulation of APCs (DCs) via Jagged1 enhances APC function from the point of view of maturation markers, cytokine production, and differentiating effects on T cells (43). Overall, therefore, whether one considers the T cell or the APC the main target of Treg suppression, there is no consistent evidence that Notch signaling has the required negative effects that one would expect of a mediator of suppression. This, taken together with the fact TGF-β does have an inherently negative effect on cell function and the fact mentioned above that cells lacking the ability to be signaled by TGF-β cannot be suppressed, puts the TGF-β pathway firmly in the primary position with respect to Treg effector function.

Yet another consideration is whether TGF-β can act in the absence of Notch1 signaling to mediate Treg suppression. In the study shown in Fig. 7, the capacities of anti-TGF-β and anti-Notch1 to inhibit suppression were equivalent; however, in several other studies, including several in which the inhibitory capacities of these Abs were compared by CFSE dilution measurement, the anti-TGF-β was able to inhibit at a higher CD25+ to CD25− ratio (data not shown); it is therefore likely that the TGF-β signaling pathway in Treg effector function is the more primary pathway, but additional studies will be necessary to firmly establish this conclusion.

Finally, the data reported here showing that Notch ligands are found on Tregs and Notch is found on APCs implies that the Notch signaling pathway in relation to suppression occurs mainly as a result of regulatory T cells acting on and inhibiting the function of APCs. There is now ample evidence for this mode of regulatory T cell activity (44–47) including one report in which two-photon
laser-scanning microscopy was used to show that Tregs form stable associations with DCs, but not with helper T cells (44). In addition, it has been shown that Tregs cocultured with DCs enhance IL-10 and suppress IL-6 production by the DCs, whereas naive T cells have the opposite effect at least under noninflammatory conditions (45). This said, it remains possible that Treg function involving Notch signaling also involves direct effects on T cells as suggested by previous studies showing Hes-1 activation in CD4+ T cells under in vivo tolerogenic conditions (16). It is also important to consider the possibility that the Notch signaling pathway may be involved in negative signals delivered by DCs to T cells rather than vice versa as considered so far. This is possible because while Notch ligands were not detected on resting DCs in the studies reported here, there is ample evidence that activated DCs express such ligands (48–50). In addition, in a recent study it was shown that interactions between DCs and T cells are bi-directional under conditions of cell activation (48). It is not clear, however, whether the Notch signal in the DC to T cell direction is suppressive; while in one study it was shown that APCs that overexpress Notch ligand have suppressive qualities, in another study it was shown under more physiologic conditions that inhibition of Notch signaling led to decreased cytokine production (IFN-γ) by interacting T cells (48, 51). Thus, while we recognize that mature DCs can be directly tolerogenic, there is no convincing evidence that the latter involves the Notch pathway.

**Disclosures**

The authors have no financial conflict of interest.

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