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**Notch activation on effector T cells increases their sensitivity to Treg cell-mediated suppression through upregulation of TGF-βRII expression.**

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**Abstract**

Notch proteins play an important role in embryonic development and cell-fate decisions. Notch influences also the activation and differentiation of peripheral T cells. Here, we investigated whether Notch signalling modulates the response of effector T cells to Treg cells. Pre-exposure of $CD4^{+}CD25^{-}$ effector T cells to the Notch ligands Delta-4 and Jagged-1, but not Delta-1, increases significantly effector T-cell sensitivity to Treg cell-mediated suppression through upregulation of TGF-βRII expression and increased levels of the phosphorylated form of the Smad 3 protein. This effect is relieved by anti-TGF-β Abs. We demonstrate that HES, the main transcription factor downstream of Notch, induces strong transactivation of *TGF-ßRII* by binding the TGF-βRII promoter through its DNA-binding domain. Thus, the crosstalk between Notch and the TGF-β pathway leads to potentiation of the suppressive effect of Treg cells.

## 1. Introduction

The Notch signalling system is conserved from Drosophila to humans and regulates cell differentiation, proliferation and survival. Notch pathways play an important role in embryonic development, T-cell development and function, and in disease processes including carcinogenesis and autoimmunity. In mammals there are four Notch receptors (1 to 4) and five Notch ligands (Jagged-1, Jagged-2, Delta-like 1 (DL-1), DL-3 and DL-4) [1].

Notch proteins exert their pleiotropic effects through the regulation of expression of various downstream genes, many of which require the interaction of Notch proteins with the DNA binding transcription factor CSL in order to form a short-lived nuclear transcription complex [2]. After engagement with its ligands, successive proteolytic events cause clipping of the Notch protein. The first is mediated by ADAM proteases and the second by the γ-secretase complex, in which presenilins (PS1 and PS2) constitute the active center of the enzyme complex. These proteolytic events ultimately release the intracellular domain of Notch (NICD). The formation of a complex of activated intracellular Notch protein and CSL converts CSL from a transcriptional repressor to a transcriptional transactivator. The genes encoding the HES (hairy and enhancer of split) family of basic helix-loop-helix proteins are Notch targets that are known to be essential for T-cell development and signalling.

In addition to influencing Th1- and Th2-cell differentiation [3], Notch signalling has also been involved in the differentiation and expansion of regulatory T (Treg) cells [4]. Several reports have shown that the presence of Notch ligands, mostly of the Jagged family, can enhance Treg-cell differentiation and function in vitro [5]. For example, exposure of Treg cells to Jagged-2 expressed by hematopoietic progenitor cells has been shown to modulate peripheral Treg-cell expansion and prevents the development of diabetes in an autoimmune disease mice model [6]. Transgenic mice over expressing the active intra-cellular form of Notch3 exhibit an increased percentage of Treg cells and are refractory to the induction of

experimental autoimmune diabetes when treated with streptozotocin [7]. Moreover, there is emerging data suggesting that Notch can crosstalk or cooperate with other signalling pathways and thereby broaden the spectrum of target genes that are influenced by Notch signalling. For example, the interaction of Notch and TGF-β signal pathways plays a role in Treg-cell effector function through a modulation of FoxP3 expression or by facilitating TGF-β mediated suppressive function of Treg cells[8, 9]. For example, Asano et al showed that Treg cells express Notch ligands and also that Notch signaling plays an important role on effector function of TGF-β and its signaling through an interaction of NICD and Smad 3 [9]. Therefore, most of the reports focused on the influence of Notch signalling on Treg cells.

Here, we hypothesized that Notch signalling modulates the response of effector T cells to Treg cell-mediated suppressive effects. We show that Notch activation through its ligands increases exquisitely the sensitivity of effector $CD4^+$ T cells to the suppressive effect of Treg cells even at low frequency. This effect is mediated through an upregulation of TGF-βRII and the phosphorylated form of Smad 3 protein on effectors T cells. We show that HES-1 transactivates the TGF-βRII promoter.

## 2. Results

### Notch1 signaling is involved in Treg cell-mediated suppression

In order to investigate the involvement of Notch signalling in Treg cell-mediated suppression, we performed co-culture experiments of purified $CD4^{+}CD25^{high}$ and autologous $CD4^{+}CD25^{-}$ T cells stimulated with anti-CD3 mAbs in the presence or absence of a γ-secretase inhibitor (GSI). While GSI did not affect the proliferation of $CD4^{+}CD25^{-}$ T cells cultured alone, addition of this compound at the begining of the coculture relieved the Treg cell-mediated suppression (Fig1A). We found that $CD4^{+}CD25^{-}$ T cell proliferation was inhibited (mean +/- SD) by 79.2% +/- 12.2 and 60.2 % +/- 24.8 when cultured with Treg cells at a ratio ($T_{REG}/T_{EFF}$) 1:4 and 1:8, respectively (mean of 4 experiments) (Fig 1B). In the presence of GSI, Treg-cell mediated suppression became 46.9% +/- 20.5 ($P<0.05$ for comparison with coculture performed with GSI vehicle) and 49.3 % +/- 21 at the corresponding ratios.

In a second step, the same experiments were performed in the presence of immobilized Notch ligands DL-1, DL-4 and Jagged-1 or IgG control at 5μg/ml. As shown in Fig1C, the presence of Notch ligands potentiates the inhibitory effects of Treg cells on the proliferation of $CD4^{+}CD25^{-}$ T cells. Percentages of inhibition in cultures performed at ratio 1:8 ($T_{REG}/T_{EFF}$) were 58, 73 and 72% in the presence of DL-1, DL-4 and Jagged-1, respectively as compared to 43% in the presence of Ig control ($P<0.05$ for DL-4 and Jagged-1 as compared to IgG control). At a higher ratio (1:16) these percentages are 39%, 54% and 64% as compared to 20% in control conditions ($P<0.05$ for DL4 and Jagged-1 as compared to IgG control). Altogether, these results suggest that notch ligands potentate Treg cell-mediated suppression. However, they could not discriminate between direct effects of Notch ligands on Treg cells and/or effector T cells.

### Notch signalling sensitizes effector T cells to Treg cell-mediated suppression.

In order to discriminate between an effect of Notch signalling on Treg cells or effector T cells, we performed coculture experiments in which either Treg or $CD4^+CD25^-$ T cells were first preincubated or not with immobilized recombinant Notch ligands or IgG control overnight. Exposure of Treg cells to DL-1, DL-4 or Jagged-1 did not modify the Treg-cell capacity to suppress $CD4^+CD25^-$ proliferation in the presence of anti-CD3 mAbs (Fig 2A). In contrast, initiation of Notch signalling in $CD4^+CD25^-$ following exposure to DL-1, DL-4 or Jagged-1 increased significantly their sensitivity to Treg cell-mediated suppression in 5-days coculture experiments in the presence of anti-CD3 (Fig 2B). As shown in figure 2C, percentages of Treg-cell inhibition of cell proliferation were 64.8, 64.2, 65 % (mean of 4 experiments at a ratio 1:8) in cocultures performed with $CD4^+CD25^-$ T cells pre-exposed to DL-1, DL-4 and Jagged-1, respectively as compared to pre-incubation with IgG control (29.2 %) ($P<0.05$ for all comparisons to IgG control). These results show that Notch signalling acts on effector T cells and that Notch ligands potentiate Treg cell-mediated suppression.

**Notch suppresses the proliferation of $CD4^+CD25^-$ T cells through TGF-β signalling.**

Although the mechanisms of Treg-cell suppression are not fully elucidated, the TGF-β pathway plays a key role in the regulation of Treg-cell function. Moreover, previous results have shown an interaction between Notch and TGF-β pathways [8-10]. We surmise that Notch1 affects effector T cell responses in the presence of Treg cells through a TGF-β signalling mechanism. To test this, and to increase the sensitivity of the system, we performed coculture experiments of effector T cells and Treg cells at a low ratio (1:16) in the presence of immobilized Notch ligands DL1, DL4 and Jagged-1 or IgG control (5μg/ml). Anti-TGF-β (2.5μg/ml) or isotype control mAbs were added at the beginning of the culture. Coculture experiments performed in the presence of either DL-4 or Jagged-1 led to a mean inhibition of T cell proliferation of 47% and 65%, respectively as compared to 12% when

cells were pre-treated with IgG control. As shown in figure 3A, these percentages became 6.3% and 19.3 % in the presence of anti-TGF-β mAbs ($P<0.05$) while no changes were noted in the presence of isotype control. Finally, anti-TGF-β did not affect coculture performed with Treg cells and effector T cells pre-treated with DL-1 ligands.

Active TGF-β mediates its biological functions by binding to TGF-β type I and type II receptors (TGF-βRII). Therefore, we first investigated the effects of Notch activation on TGF-βRII expression of effector T cells. As shown in figure 3B, $CD4^{+}CD25^{-}$ effector T cells stimulated with coated anti-CD3 and Notch ligands (DL-4 or Jagged-1) for 3 hours, exhibited a marked increase in TGF-βRII RNA expression as compared to pre-exposure to IgG control (4.4 and 2.8 fold increase for DL-4 and Jagged-1, respectively). This effect is abrogated in the presence of GSI ($P<0.05$ for comparison with culture performed without GSI vehicle). According to the results presented above, pre-exposure of effector T cells to DL-1 alone, or in the presence of GSI, did not significantly modify TGF-βRII RNA expression.

We next assessed TGF-βRII protein expression by flow cytometry. In these experiments, effector $CD4^{+}CD25^{-}$ T cells were isolated and incubated for 48 hours in the presence of Notch ligands or IgG control. Flow analysis showed that DL-4 and Jagged-1 increased expression of TGF-βRII (MFI: 166 and 130 respectively) as compared to IgG (MFI: 53) or DL-1 (MFI: 85) (Figure 3B-C). Together these results suggest that engagement of DL-4 and Jagged-1 increases TGF-βRII expression on $CD4^{+}CD25^{-}$ T cells.

**DL-4 and Jagged-1 sustain TGF-βRII function through HES interaction with the TGF-βRII promoter**

Given the above observations showing an upregulation of TGF-βRII following Notch activation, we hypothesized that Jagged-1 and DL-4 led to an activation of TGF-β signalling

in effector T cells. $CD4^+CD25^-$ T cells were isolated and cultured for 48 hours in wells coated with Notch ligands or IgG control in the presence of TGF-β (5ng/ml). We then performed immunoblot to assess the expression of pSmad3, a major TGF-β signalling intermediate [11]. As shown in figure 4A, DL-4 and Jagged-1 exposure induced pSmad3 expression, while only a weak pSmad3 band was detected in IgG control and DL-1 stimulated cells. Western blot analysis showed that TGF-β induced a significant increase of pSmad3 expression in $CD4^+CD25^-$ T cells after 30 minutes (D0) of stimulation (ratio pSmad/actin: 2.5). This expression decreased dramatically at 48 hours when cells were incubated in the presence of IgG (ratio pSmad3/actin: 0.26) but not in the presence of DL-4 (ratio pSmad3/actin: 2.7) or Jagged-1 (ratio pSmad3/actin: 1.25). By contrast and in accordance with data presented above, DL-1 stimulated $CD4^+CD25^-$ T cells showed very low amount of pSmad3 (ratio pSmad3/actin: 0.26) after 48 hours of TGF-β treatment (Figure 4B). These results suggest that DL-4 and Jagged-1 sustain TGF-βRII function on $CD4^+CD25^-$T cells.

To explore the mechanism by which Notch may regulate TGF-βRII expression, we have performed transactivation experiments. Since these experiments cannot be performed easily on primary $CD4^+$ T cells, we used rat PC12, a cell line widely used to explore the role of HES-1 as a modulator of cell differentiation and proliferation [12]. We analyzed the transactivation of the TGF-βRII promoter by wtHes-1 (pCI-*HES-1*) or a DN mutant (*dnHES-1*). In three separate experiments, we found that *wtHES-1* induced a strong transactivation of the *TGF-ßRII* luciferase reporter promoter containing 5' sequences compared with transfection with the empty vector (figure 4C). No increase in luciferase activity was seen when *wtHES-1* and *dnHES-1* were cotransfected. Interestingly, when transfected alone, the *dnHES-1* plasmid decreased luciferase activity below that measured with the empty vector, since it neutralized endogeneous HES-1 activity on the *TGF-ßRII* reporter promoter. These results demonstrated that HES-1 activates the *TGF-ßRII* promoter through its DNA-binding activity.

## 3. Discussion

Although, Treg cells mediate their suppressive effects through different mechanisms, it is well established that Treg cells produce or express at their surface TGF-β, which participates to their suppressive effects [13-15]. In the present study, we focused our interest on the role of Notch pathway on the sensitivity of effectors T cells to Treg cell-mediated suppression. Our results extend the knowledge on the role of Notch in Treg/Effectors T cells cross talk showing that Notch activation may also significantly increase the sensitivity of effector cells to Treg cells even at a low ratio. We demonstrate that Notch ligands DL-4 and Jagged-1, but not DL-1, increase significantly the suppressive effect of Treg cells on effectors T cells through an upregulation of TGF-βRII expression and the phosphorylated form of Smad3 protein. This effect is relieved by anti-TGF-β Abs.

Therefore, the picture of the interaction between Treg cells and effectors T cells in the context of the presence of Notch in the cellular environment becomes more complex. Globally, interactions between Notch and TGF-β pathways impact on the activating or inhibitory functions of Notch. Therefore, in one hand, Notch activation of Treg cells increases TGF-β mediated effector function of these cells and upregulates Notch expression on target cells [4]. For example, it has been shown that cells bearing surface TGF-β (but not cells lacking this property) could activate the Notch signalling pathway leading to a tolerance state to respiratory Ags in the airway [4]. Notch signalling may also play a role in the differentiation of Treg cells as demonstrated in vitro or in vivo models [5-7] or in the modulation of TGF-β signalling [8, 9]. A recent report suggests that NICD interacts with pSmad3 enhancing its activity and TGF-β mediated effectors function of Treg cells. According to this, it has been recently shown that Notch1 activation enhances TGF-β signalling by increasing Smad translocation into the nucleus and its transactivation effect at promoter sites [9].

On the other hand, and as shown here, Notch activation of effectors T cells increases their

sensitivity to TGF-β. Finally, TGF-β is involved also on the generation and maintenance of adaptative Treg cells [8]. Collectively these data suggest that activated effectors T cells and Treg cells may be maintained at certain equilibrium by a regulatory loop induced by Notch activation.

Earlier studies established an interrelationship between TGF-β signalling and Notch signalling at the biochemical level. TGF-β signalling leads to a rapid upregulation of HES, a major target of Notch signalling in keratinocytes [16], in neural stem cells and in myoblasts. In addition, Smad3 and NICD signalling physically interact and such interaction lead to recruitment of Smad3 to Notch target genes via binding to CSL, a key DNA-binding protein of the Notch pathway. Here, we found that HES, the main transcription factor donwstream of Notch, induced a strong transactivation of the *TGF-ßRII* by binding the TGF-βRII promoter through its DNA-binding activity. Taken together, this set of findings is consistent with the conclusion that Notch signalling plays an important role in facilitating TGF-β signalling in effector function and, in turn, TGF-β signalling amplifies these effects by upregulating HES.

There is clear evidence that stimulation of APC (Dendritic Cells) via Jagged enhances APC function from the point of view of maturation markers, cytokine production and differentiation effects on T cells [17]. This raises the question of whether DCs are exposed to Notch ligands in the microenvironments where they reside. In skin, keratinocytes constitutively express high levels of Jagged-1 [18]. Therefore, to prevent constitutive maturation of DCs, Notch signalling must be tightly regulated. It is conceivable that TGF-β inhibit the DCs maturation in physiological conditions. In the context of inflammation or infection for example [19, 20], upregulation of Notch ligands on DCs, may modulate specific T cell responses in the presence of Treg cells.

As shown by our group and others in various culture systems, Notch ligands and engagement of different Notch family members may lead to distinct functional outcomes either in early or late stages of T cell development [21, 22]. Indeed, Notch proteins have been described as having a role in directing the differentiation of activated T cells to Th1 or Th2 T-cell lineages. Interactions between DL-1 and Notch3 were described to influence the differentiation of activated $CD4^+$ T cells, by promoting a Th1 cell phenotype [23, 24] while $CD4^+$ T cells incubated with APCs expressing Jagged-1 adopted a Th2 cell-cell fate, producing IL-4 and IL-5 [25]. Taken together, these data implicated Jagged and DL ligands in the development of Th2 and Th1 cell immune responses, respectively. However, the molecular mechanisms by which Delta-like and Jagged-1 drive differently these functions are currently unknown. Our data may contribute to the understanding mechanisms behind this observation since Notch signalling delivered through DL-4 and Jagged-1, but not DL-1, increases the sensitivity of effector T cells to TGF-β through upregulation of TGF-βRII and a sustained expression of phosphorylated smad3 protein expression.

Collectively these data show that cell environment may modulate T cell responses through Notch/Notch ligand interactions by increasing sensitivity of effectors T cells to Treg suppression. From a physiology stand point, our study suggest that, by increasing the susceptibility of effectors T cells to TGF-β, Notch may provide signals which converge to limit tissue/organ damage mediated by inflammation.

## 4. Materials and methods

### Cell culture

Peripheral blood mononuclear cells were separated by Ficoll-Hypaque centrifugation (Amersham Biosciences) from buffy coats obtained from healthy blood donors (EFS, Créteil). $CD4^+$ $CD25^-$ T cells and $CD4^+CD25^{high}$ T cells were isolated using a $CD4^+$ T cell enrichment column and a human $CD4^+CD25^+$ Treg isolation kit purchased from Miltenyi Biotec. $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells were enriched to greater than 95 % purity.

Cells were plated in media at a concentration of $1.25x10^5$ to $5x10^5$ cells per ml in a 96-well round-bottom plates coated with 1µg/ml of anti-CD3 (UCHT1) and 5µg/ml of Notch Ligand (DL-1-Fc, DL-4-Fc and Jagged-1-Fc are a generous Gift from Dr. Sakano).

### Suppression assay

A total of $2.5x10^4$ $CD4^+CD25^-$ T cells were cultured with various ratios of $CD4^+CD25^{high}$ cells and 1µg/ml of anti-CD3 for 5 days, in the presence or absence of 10µm of the gamma-secretase inhibitor Compound E. A total of 0.5 Ci [$^3$H] thymidine (Amersham Pharmacia) was added to the wells during the last 16 hours of culture. The cells were then harvested and assessed for [$^3$H] thymidine incorporation using a liquid scintillation counter. Results were expressed as mean cpm of quadriplicate culture wells.

Percentage of inhibition was calculated as follows: 1 - (mean cpm of co-culture wells divided by mean cpm of $CD4^+CD25^-$ cells cultured alone) x 100.

In sensitizing asssays $CD4^+CD25^-$ and $CD4^+CD25^{high}$ were pre-incubated during 16 hours on coated Notch Ligand. Cells were also collected, washed and incubated alone or with Treg cells and stimulated as described above. Some experiments were performed in the presence of anti-TGF-β or IgG control (2.5 µg/ml).

### Real-time PCR

$CD4^+CD25^-$ T cells were cultured for 3 hours in coated wells with anti-CD3 (200ng/ml) and

Notch ligands (5µg/ml) or IgG control (5µg/ml). Total RNA was extacted with Trizol (Invitrogen) and purified by chloroform extraction. RNA was then reverse transcribed using 1mM oligodT and the Superscript™ HH Rnase H-reverse transcriptase (Invitrogen Life technologies) according to the manufacturer's instructions. Quantitative PCR was performed in a LightCycler System (Roche diagnostics) using a SYBR Green PCR kit from Roche Diagnostics. The cDNA input for each population was normalized to obtain equivalent signals with *Splicing Factor 3A1 (SF3A1)* used as housekeeping gene. Primers used were:

S14: Forward: 5' GGCAGACCGAGATGAATCCTCA 3'

Reverse: 5' CAGGTCCAGGGGTCTTGGTCC 3'

TGF-βRII Forward: 5' CTGCAAGATACATGGCTCCA 3'

Reverse: 5' CTCGATCTCTCAACACGTTGT 3'

**Surface and intracellular staining**

For surface staining, the cells were harvested, washed twice and resuspended in 1%FCS buffer. The cells were stained with mAbs for CD4 (Beckman Coulter), TGF-βRII (R&D) as per manufacturer's instructions for 20 min at 4°C. Cells were then analyzed by flow cytometry using FACScalibur™ and CellQuest™ software (Becton Dickinson, San Jose, California, USA).

**Immuno-blot analysis**

$CD4^{+}CD25^{-}$ T cells were stimulated with Notch Ligand during 2 days. 1ng/ml of TGF-β was also added during 30 min to induce Smad3 phosphorylation. Cells were collected, washed and lysed with Tris HCL (20mM) 0,5 % SDS buffer in presence of DNase (benzonase), inhibitors of phosphatase and protease. The samples were centrifuged for 5min at 4°C at 15,000*g*. The resulting supernatants were boiled for 3min and frozen at 80°C or used immediately. Aliquots of the supernatants were used for protein determination (micro-bicinchoninic acid protein assay; Pierce). The 3X Laemmli buffer with 2-βME and bromophenol blue was added, and

cell lysate proteins (10μg/lane) were resolved by on precast polyacrylamide gels (gradient 4–12% gels, Invitrogen). Proteins were then electroblotted onto nitrocellulose filters with 0.45 μm pores, and the filters were blocked by incubation for 1h with 5% non fat milk powder in TBS, 0.1% Tween20. The filters were incubated for 1h at room temperature or overnight at 4°C with anti-Phospho Smad3 (Cell Signalling); anti- Smad3 or β-actin. Blots were washed three times for 10min each, in TBS, 0.1% Tween20, and were then incubated for 1h with peroxidase-labeled anti-mouse or anti- rabbit Ig. Blots were developed by enhanced chemiluminescence ($ECL^+$ from GE Healthcare) using a CCD camera (G:BOX, Syngene)

**Transient transfection and Luciferase assay.**

*Expression plasmids.* The *TGF-ßRII* promoter-luciferase constructs were described previously, *wtHES-1* and dominant negative (DN) *HES-1* were provided by R. Kageyama and ß-Galactosidase by I. Dusanter-Fourt (INSERM U 567, Paris). The DN HES-1 has three amino acid mutations only in the basic region (DNA-binding domain), so it cannot bind to the DNA or interact with promoters but can dimerize with endogenous wild-type HES-1 to form a non DNA-binding heterodimer complex.

*Transfection procedure.* Transfections reporter assays were carried out in six-well tissue culture dishes with the indicated plasmids by using the Lipofectamine plus reagent (Life technologies) as indicated by the manufacturer. PC12 cells were seeded the day prior to transfection at a concentration that will give 50% confluency. Transfection was carried out 18 hours in serum free optiMEM, and cells were then incubated another 48 hours in complete medium (RPMI, 10% HS, 5% FCS). For normalization of transfection efficiency, pCMV-ß Gal plasmid diluted one-fourth was added as an internal control.

**Statistical analysis**

Results were expressed as means ± SE. Statistical significance was determined by two-tailed *t* test with a significance level of 0.05, using GraphStat software (GraphPad, San Diego,CA).

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**Conflict of interest**

The authors declare no financial or commercial conflict of interest.

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**Figure legends**

**Figure 1: Notch signalling is involved in Treg cell-mediated inhibition of anti-CD3-stimulated $CD4^+CD25^-$ T-cell proliferation**

A, B) The indicated ratios of $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells ($T_{REG}/T_{EFF}$ ratio) were cultured for 5 days in wells coated with anti-CD3 (1µg/ml) in the presence or absence of inhibitor of γ-secretase (GSI 10µM). (B) Percentages of inhibition were calculated from (A) the raw data using the equation: 1- (mean cpm of co-culture wells divided by mean cpm of $CD4^+CD25^-$ cells cultured alone) x 100. Data are shown as the mean ± SEM of 3 replicates / samples pooled from 5 independent experiments.

C) The indicated ratios of $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells ($T_{REG}/T_{EFF}$ ratio) were cultured for 5 days in wells coated with anti-CD3 (1µg/ml) in the presence of Notch ligands (5 µg/ml) or IgG control at different ratios. Percentages of inhibition were calculated from the raw data using the equations: 1- (mean cpm of co-culture wells divided by mean cpm of $CD4^+CD25^-$ cells cultured alone) x 100. The data represent the mean ± SEM of 3 replicates/sample pooled from 5 independent experiments. * indicates $P<0.05$. ** indicates $p<0.01$ (two-tailed t test).

**Figure 2: Notch signalling sensitizes effector T cells to Treg cell-mediated suppression**

A) $CD4^+CD25^+$T cells were cultured for 18 hours in wells coated with anti-CD3 and Notch ligands (5µg/ml) or IgG control (5µg/ml). Then, the Treg cells were washed and incubated for 5 days in wells coated with anti-CD3 in the presence of $CD4^+CD25^-$T cells ($T_{EFF}$) at the indicated ratios. One representative experiment of 4 is shown.

B, C) $CD4^+CD25^-$T cells were cultured for 18 hours in wells coated with anti-CD3 and Notch ligands (5µg/ml) or IgG control (5µg/ml). Then, the T cells were washed and incubated for 5 days in wells coated with anti-CD3 in the absence or presence of Treg cells at a ratio 1:8.

The data are presented as (B)cpm or (C) inhibition of T-cell proliferation and are mean ± SEM of 3 replicates/samples pooled from 5 independent experiments. * indicates P<0.05, ** indicates p<0.01 (two-tailed t test).

**Figure 3: Notch suppresses the proliferation of $CD4^+CD25^-$ T cells through TGF-β**

A) $CD4^+CD25^-$T cells were cultured for 5 days in wells coated with anti-CD3 and Notch ligands (5µg/ml) or IgG control (5µg/ml) in the presence of Treg cells at a ratio 1:16. Anti-TGF-β or isotype control (2.5 µg/ml) were added at the beginning of the co-culture. The data represent the mean ± SEM of 3 replicates/samples pooled from 4 independent experiments.

B) $CD4^+CD25^-$T cells were purified using Miltenyi microbeads from PBMC and cultured for 3 hours in wells coated with anti-CD3 (200ng/ml) and Notch ligands (5µg/ml) or IgG control (5µg/ml) ) in the presence or absence of inhibitor of γ-secretase (GSI 10µM).. Quantitative real-time PCR was performed to measure the transcript levels of TGF-βRII. Data are mean ± SEM of 3 replicates/samples pooled from 4 independent experiments. * indicates P<0.05 (two-tailed t test).

C) Protein levels of TGF-βRII in $CD4^+CD25^-$T cells stimulated with DL-1, DL-4 and Jagged-1 as measured by flow cytometry. Thin line represents isotype control, thick line anti-TGF-βRII. Data are representative of 4 independent experiments.

D) Mean of fluorescence of TGF-βRII in $CD4^+CD25^-$T cells stimulated with DL-1, DL-4 and Jagged-1 as in (C) and are mean ± SEM of 4 independent experiments. * indicates P<0.05 (two-tailed t test).

**Figure 4: TGF-βRII function on effector T cells stimulated with Notch ligands**

A) Western blot analysis was performed on $CD4^{+}CD25^{-}$T cells cultured for 48 hours in wells coated with anti-CD3 (200 ng/ml) and Notch ligands (5µg/ml) in the presence of TGF-β (5ng/ml). The whole cell lysate was analysed by immunoblot for pSmad3 and β-actin was used as loading control. The experiment was repeated twice with similar results.

B) Quantification of the data in (A) showing the ratio of pSmad3/actin in one representative experiment of two performed on $CD4^{+}CD25^{-}$T cells.

C) Equal numbers of PC12 cells were cotransfected with 1 µg of *TßRII* promoter-luciferase constructs, 1 µg of ß-galactosidase and with 50 ng of either pCI-HES-1 or pCI-dnHES-1, or both, or empty vector. Cells (whole cell extracts) were analyzed for luciferase activity 48 hours later and data normalized for ß-galactosidase activity. Data are representative of three independent experiments.