

# Up-Regulation of Gene Related to Anergy in Lymphocytes Is Associated with Notch-Mediated Human T Cell Suppression<sup>1</sup>

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A growing body of literature indicates that the Notch pathway can influence the activation and differentiation of peripheral murine T cells, though comparatively little is known about the effects of Notch signaling in human T cells. In the present report we demonstrate that Jagged-1-induced Notch signaling (using immobilized Jagged-1 fusion protein) during stimulation of purified human CD4<sup>+</sup> and CD8<sup>+</sup> T cells potently inhibits T cell proliferation and effector function, including both Th1- and Th2-associated cytokines. Inhibition of T cell activation is not due to apoptosis or disruption of proximal TCR signaling, but is associated with up-regulation of GRAIL (gene related to anergy in lymphocytes) in CD4<sup>+</sup> T cells, with modest effects on other E3 ubiquitin ligases such as c-Cbl and Itch. When evaluated for its effects on CD4<sup>+</sup> T cell differentiation, Jagged-1-mediated signaling inhibits T cell cytokine secretion with no significant effect on proliferative responses. Collectively, these data demonstrate that Notch signaling in human T cells induced by Jagged-1 promotes a novel form of T cell hyporesponsiveness that differs from anergy, whereby primary T cell proliferation and cytokine secretion are potently inhibited, and effector function but not proliferative capacity are ameliorated upon secondary stimulation. *The Journal of Immunology*, 2007, 178: 6158–6163.

Notch signaling plays an essential role in developing tissues by influencing the specification of cell fates in diverse settings (1, 2). Mammals have genes encoding four Notch receptors (Notch 1–4) and five ligands, classified into two structurally related groups: Delta-like ligands (Dll-1, Dll-3, and Dll-4) and serrate-like ligands (Jagged-1 and Jagged-2). Notch receptors and ligands are widely expressed by both hemopoetic and nonhemopoetic cells, and a given cell can express multiple ligands and receptors. Notch signaling is initiated by receptor-ligand interaction, which leads to proteolytic cleavages that free the cytoplasmic portion of Notch (Notch-IC) from the membrane and allow it to enter the nucleus and bind to the transcription factor CSL (core binding factor-1, suppressor of hairless, Lag1). In turn, CSL displaces corepressors and induces expression of Hes (Hairy-Enhancer of Split) and HERP (Hes-related proteins) gene family members. Notch signaling, however, is regulated by a wide variety of mechanisms that include regulation of Notch ligand surface expression, glycosylation of Notch receptors, targeting cytoplasmic or nuclear Notch-IC for ubiquitination and proteosomal destruction, and regulating CSL transcriptional activity (3). As signals are dose- and context-dependent (cell type), the consequences of Notch signaling cannot reliably be predicted, and can variably promote cell proliferation or cell cycle arrest, differentiation or self-renewal, survival or apoptosis (4).

The role of Notch signaling during lymphocyte maturation and differentiation is well documented (5). In addition, it is becoming

increasingly clear that Notch signaling regulates peripheral T cell responses (5). There are several reports demonstrating that Notch signaling can inhibit T cell activation (6–8), influence Th cell differentiation (9, 10), or generate immunosuppressive regulatory T cell populations (11–13). The overwhelming majority of these data are derived from murine studies.

In the present study we evaluated the role of the Notch pathway in regulation of peripheral human T cell activation. Using immobilized recombinant Jagged-1 Fc protein to initiate Notch signaling, we demonstrate that Jagged-1-mediated Notch signaling inhibits the activation of naive and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Mechanistically, Jagged-1-mediated signaling does not induce apoptosis of T cells or disrupt proximal TCR signaling. Jagged-1-mediated signaling does, however, up-regulate expression of the gene related to anergy in lymphocytes (GRAIL),<sup>3</sup> although small interfering RNA (siRNA) experiments suggest that GRAIL is not solely responsible for the Jagged-1-associated T cell suppression. Finally, secondary stimulation experiments indicate that Jagged-1-mediated Notch signaling in human CD4<sup>+</sup> T cells induces a unique form of T cell suppression whereby T cells can proliferate normally upon secondary stimulation but secrete significantly reduced levels of cytokine.

## Materials and Methods

### Cell purification/isolation

In compliance with appropriate institutional review board protocols, PBMC were isolated from leukopaks by density gradient centrifugation. Total CD4<sup>+</sup>, total CD8<sup>+</sup>, and naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) CD4<sup>+</sup> T cells were isolated from PBMCs by negative selection using immunomagnetic beads and the MACS system (Miltenyi Biotec). Unless specified otherwise, cells were resuspended in serum-free Ex Vivo 15 medium (Cambrex/BioWhittaker).

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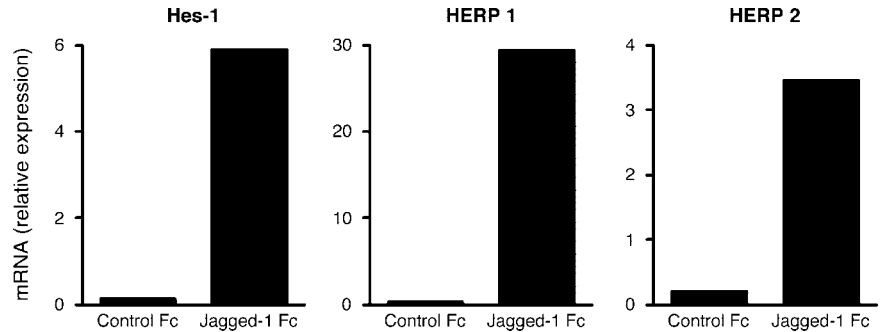
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<sup>3</sup> Abbreviations used in this paper: GRAIL, gene related to anergy in lymphocyte; siRNA, small interfering RNA; Ct, cycle threshold.

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**FIGURE 1.** Immobilized Jagged-1 Fc induces Notch signaling in human T cells. Anti-CD3/CD28 mAbs were immobilized on beads in the presence of Jagged-1 Fc fusion protein or control Fc protein. Purified human CD4<sup>+</sup> T cells were stimulated with the beads, and mRNA was isolated after 4 h. Quantitative PCR analysis demonstrates that Jagged-1 Fc induces the expression of mRNA for several genes associated with Notch signaling (Hes-1, HERP-1, HERP-2). Data were normalized to expression of GAPDH and experiments were run in triplicate.



### T cell functional assays

Recombinant rat Jagged-1/Fc chimera (R&D Systems) was used to deliver signals through Notch, with purified human IgG1 used as an Fc control (The Binding Site). The homology between the extracellular domains of rat and human Jagged-1 is 98%. Purified T cells ( $2.5 \times 10^4$ /well) were stimulated in triplicate with plate-bound anti-CD3 (OKT3) and anti-CD28 (3D10) mAbs (both at  $1 \mu\text{g/ml}$ ) with graded doses of plate-bound Jagged-1 Fc or control Fc. After 48 h at  $37^\circ\text{C}$ , assay supernatants were collected for cytokine analysis, and cells were pulsed with  $1 \mu\text{Ci}$ /well of [ $^3\text{H}$ ]thymidine for the last 24 h. Cells were harvested and [ $^3\text{H}$ ]thymidine uptake was determined. Paired capture or detection mAbs against IFN- $\gamma$ , IL-13, and IL-10 (BD Pharmingen) were used to detect cytokines by ELISA as we have previously described (14). In some experiments, a  $\gamma$ -secretase inhibitor (DAPT; Calbiochem) and vehicle control (DMSO) were added at graded doses from 0.25–25  $\mu\text{M}$ .

### T cell differentiation assay

Naive (CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells ( $10^6$ /ml/tube) were incubated at  $37^\circ\text{C}$  in 5-ml polypropylene round-bottom tubes (BD Biosciences) that were coated with anti-CD3 mAb alone, or with anti-CD3/CD28 mAbs (both at  $1 \mu\text{g/ml}$ ) in the presence of control Fc or Jagged-1 Fc ( $5 \mu\text{g/ml}$ ). Fresh medium was replaced as necessary over the course of 14 days, at which time T cells were washed and counted, and equivalent numbers were stimulated in the presence or absence of anti-CD3/CD28 mAbs. T cell proliferation and cytokine production were measured as described.

### mRNA expression analysis

Tosyl-activated beads (DynaL Biotech) were coated with Jagged-1 Fc or control Fc alone, as well as with anti-CD3/CD28 mAbs (both at  $1 \mu\text{g/ml}$ ; BD Biosciences) plus Jagged-1 Fc or control Fc (each at  $5 \mu\text{g/ml}$ ). Comparable amounts of mAbs and Jagged-1 Fc or human Ig were confirmed by flow cytometry (data not shown). CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were incubated at  $37^\circ\text{C}$  in 96-well optical reaction plates (Applied Biosystems) with either anti-CD3/CD28 Jagged-1 Fc or anti-CD3/CD28 control Fc beads at a 4:1 bead to T cell ratio. mRNA from CD4<sup>+</sup> T cells was obtained using RNeasy Mini columns (Qiagen), following the manufacturer's instructions. Samples were analyzed by quantitative RT-PCR (TaqMan) for HERP-1, Hes-1, HERP-2, GRAIL, cbl-b, and Itch (primer and probes from Applied Biosystems). mRNA levels reported are relative to GAPDH, normalized using the equation  $1/2^{\Delta\text{Ct}}$ . Optimal induction of HERP-1, Hes-1, and HERP-2 was observed at 4 h, whereas for GRAIL optimal induction was at 8 h.

### Assessment of T cell apoptosis

CD4<sup>+</sup> T cells ( $10^6$  cells/ml/tube) were incubated at  $37^\circ\text{C}$  for 4 or 20 h in 5-ml polypropylene round-bottom tubes (BD Biosciences) that were coated either with nothing (unstimulated condition) or with anti-CD3/CD28 mAbs (both at  $1 \mu\text{g/ml}$ ) plus Jagged-1 Fc or control Fc (each at  $5 \mu\text{g/ml}$ ). Cells were stained with annexin V-FITC in conjunction with propidium iodide, using an Apoptosis Detection kit (BD Biosciences). Data was collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

### Influence of Jagged-1-mediated signaling on proximal TCR signaling

Phosphorylation-state analysis was performed on human whole blood using BD PhosFlow technology, according to the manufacturer's instructions (BD Biosciences). Each 1 or 2 ml of whole blood was stimulated with soluble anti-CD3 (UCHT1) mAb plus Jagged-1 Fc or control Fc in 50-ml

polypropylene Falcon conical tubes. After induction of phosphorylation at  $37^\circ\text{C}$ , cells were fixed using 10 ml of BD PhosFlow Lyse/Fix buffer and permeabilized with 1 ml of BD Perm buffer III (both from BD Biosciences). For four-color cell surface and intracellular staining,  $2.5 \times 10^5$  cells were stained with CD4-PerCP, CD3-allophycocyanin, and two phosphorylated site-specific mAbs conjugated with Alexa Fluor 488 or PE (all from BD Biosciences): PE mouse anti-CD3 $\zeta$  (CD247, pY142), PE mouse anti-LAT (pY171), Alexa Fluor 488 mouse anti-Lck (pY505), PE mouse anti-Zap70 (pY292), and Alexa Fluor 488 mouse anti-SLP-76 (pY128). Data was analyzed using FlowJo software.

### siRNA-mediated reduction of GRAIL

siRNA gene silencing was performed using a nucleofector kit for unstimulated human T cells (Amaxa Biosystems) following the manufacturer's instructions. A total of six constructs were used for nucleofection: four different GRAIL siRNA constructs (Qiagen), one GFP-positive control vector (Amaxa Biosystems), and one silencer-negative control siRNA (Ambion), all at  $2 \mu\text{g}$  per reaction. Use of the GFP control vector demonstrated a 70% transfection efficiency. After nucleofection, cells were incubated overnight at  $37^\circ\text{C}$ , and then used for functional assays.

### Statistics

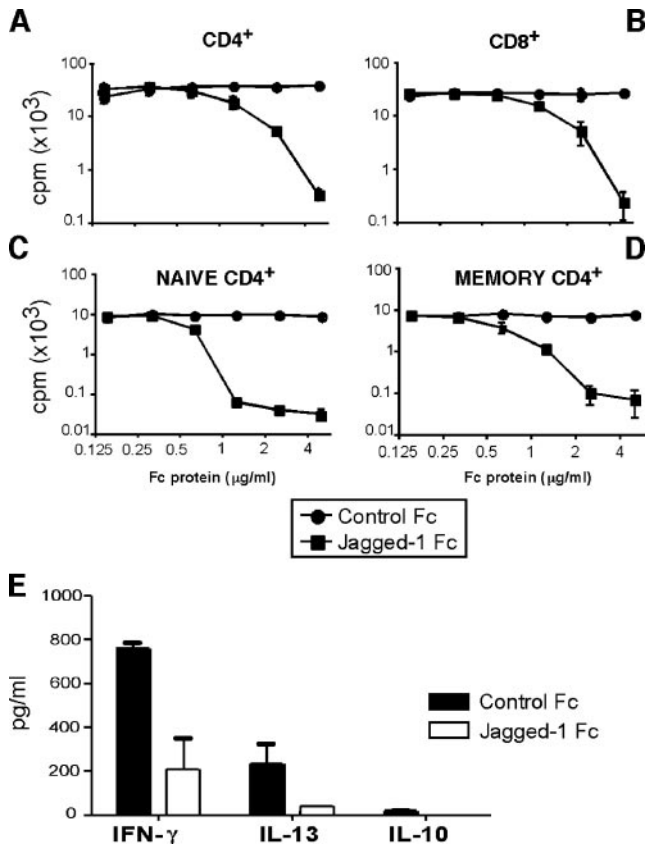
SD for experimental values was calculated using Prism 4.0 software (GraphPad).

## Results

### Jagged-1-mediated signaling inhibits human T cell activation

To evaluate the effect of Notch signaling during human T cell activation, we stimulated purified ex vivo human CD4<sup>+</sup> and CD8<sup>+</sup> T cells with immobilized Abs against CD3 and CD28 in the presence of immobilized Jagged-1 Fc fusion protein or control Fc. We first confirmed that use of immobilized Jagged-1 Fc would induce Notch signaling. Indeed, 4 h of stimulation of CD4<sup>+</sup> T cells with beads coated with anti-CD3/CD28 mAbs and Jagged-1 Fc up-regulated expression of Notch target genes Hes-1 (6-fold), HERP-1 (30-fold), and HERP-2 (4-fold), confirming that Jagged-1 Fc delivered an activating signal through a Notch receptor (Fig. 1).

Using plate-bound anti-CD3/CD28 mAbs and graded doses of immobilized Jagged-1 Fc, we then evaluated the effect of Jagged-1-mediated Notch signaling on human T cell activation. Total CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation was potently inhibited in a dose-dependent fashion after anti-CD3/CD28 stimulation in the presence of Jagged-1 Fc (Fig. 2, A and B). We further evaluated the effects of Jagged-1 engagement of naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) CD4<sup>+</sup> T cells (Fig. 2, C and D). As for total CD4<sup>+</sup> and CD8<sup>+</sup> T cells, Jagged-1 engagement suppressed proliferation of both naive and memory Th cells, with a more pronounced effect on naive T cells. T cell effector function was also inhibited after stimulation in the presence of Jagged-1, as evidenced by suppression of both Th1 (IFN- $\gamma$ ) and Th2 (IL-13) cytokines; there was no evidence for induction of the immunosuppressive cytokine IL-10 (Fig. 2E). Addition of a  $\gamma$ -secretase inhibitor to cultures did not

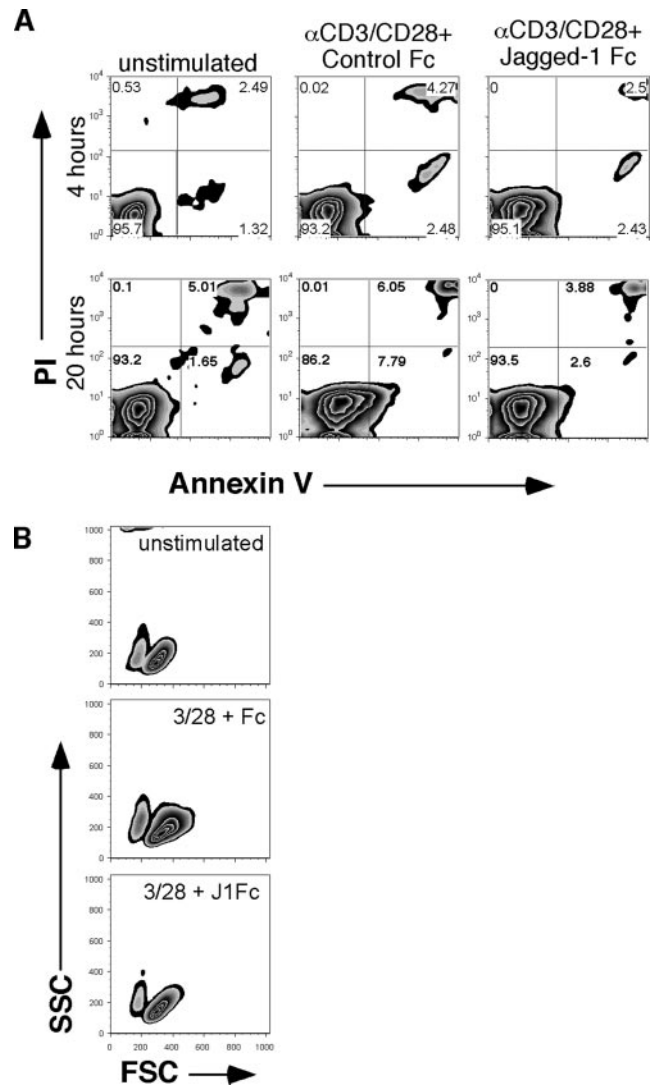


**FIGURE 2.** Jagged-1-mediated Notch signaling inhibits human T cell activation. Purified populations of total CD4<sup>+</sup> (A), CD8<sup>+</sup> (B), naive (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) CD4<sup>+</sup> (C), or memory (CD45RA<sup>-</sup>CD45RO<sup>+</sup>) CD4<sup>+</sup> (D) T cells were isolated from human peripheral blood by negative selection and stimulated with immobilized anti-CD3/CD28 mAbs in the presence of increasing doses of Jagged-1 Fc or control Fc. Jagged-1 Fc had no effect on T cells in the absence of TCR stimulation. Comparable results were obtained in two to three independent experiments for each cell population. Jagged-1 stimulation (5  $\mu$ g/ml) inhibited Th1-associated IFN- $\gamma$  and Th2-associated IL-13 and was unable to induce IL-10 (E) cytokine secretion from memory CD4<sup>+</sup> T cells.

reverse suppression associated with Jagged-1 Fc stimulation, indicating that  $\gamma$ -secretase-mediated cleavage of Notch is not required for Jagged-1-mediated suppression of peripheral human T cells (data not shown). Other recent reports have similarly observed that Notch-mediated inhibition of peripheral CD4<sup>+</sup> T cell function can occur independently of  $\gamma$ -secretase (8, 15).

#### Jagged-1-mediated signaling does not induce apoptosis in T cells

Several mechanisms could explain the ability of Jagged-1 signaling to suppress T cell proliferation and cytokine secretion. We first examined whether Jagged-1 signaling might induce apoptosis in T cells. To do so, we determined the frequency of apoptotic cells after anti-CD3/CD28 stimulation of CD4<sup>+</sup> T cells in the presence of Jagged-1 Fc or control Fc; unstimulated T cells served as a negative control. A certain proportion of T cells undergo apoptosis after stimulation (activation-induced cell death), evidenced by the increased frequency of annexin V-positive (early apoptotic) and annexin V-positive propidium iodide-positive (late apoptotic) cells in stimulated vs unstimulated cells. However, we failed to observe an increase in the frequency of apoptotic T cells that received Jagged-1 signaling during activation, relative to stimulated cells (Fig. 3A). On the contrary, Jagged-1 signaling appeared to inhibit



**FIGURE 3.** Jagged-1-mediated Notch signaling does not induce T cell apoptosis. A, Cells in the early stages of apoptosis (annexin V-positive propidium iodide (PI)-negative) appear in the lower right quadrants, whereas cells in the late stages of apoptosis (annexin V-positive PI-positive) appear in the upper right quadrants. Jagged-1 signaling consistently inhibited apoptosis induced during T cell activation in three independent experiments at both time points examined. B, Analysis of forward and side light scatter (FSC/SSC) properties of T cells stimulated in the presence of Jagged-1 Fc indicates a lack of T cell blasts (increased FSC/SSC profile) observed when T cells are stimulated in the presence of control Fc protein. The FSC/SSC profile of unstimulated cells is shown for comparative purposes. Data are derived from the 20-h time point of the experiment shown in A.

activation-induced cell death because the frequency of viable (non-apoptotic or necrotic T cells) in T cells stimulated in the presence of Jagged-1 Fc was comparable to unstimulated T cells. The effects of Jagged-1 signaling on the frequency of apoptotic cells were apparent at early and later time points examined (4 and 20 h). Thus, apoptosis does not explain the reduction in T cell activation in the presence of Jagged-1 signaling.

#### Jagged-1-mediated suppression does not abrogate proximal TCR signaling events

During the course of performing the described assays, we consistently observed under light microscopy that T cells stimulated in the presence of Jagged-1 Fc looked morphologically like unstimulated T cells (small and round) rather than T cells stimulated with

immobilized anti-CD3/CD28 mAbs (larger and irregularly shaped) This was confirmed by analyzing FSC/SSC properties of stimulated cells in the presence or absence of Jagged-1 Fc (Fig. 3*B*). This observation, in addition to the data described, led us to hypothesize that Jagged-1 signaling might abrogate TCR-mediated signaling events such that the T cells behaved as though they had received no signal through the TCR. Indeed, this hypothesis is bolstered by reports that Notch receptors colocalize with TCRs during T cell activation and that coligation of TCR, CD28, and Notch-1 inhibits AKT phosphorylation and activation of several TCR-induced transcription factors (6, 8, 16). To directly test this hypothesis, we used flow cytometry and phospho-specific Abs to a variety of molecules to determine whether Jagged-1 Fc signaling during T cell stimulation could abrogate or reduce proximal TCR signaling events. In contrast to our hypothesis, we found no evidence for a reduction in the activation (phosphorylation status) of any of the molecules examined, even when we used suboptimal doses of anti-CD3/CD28 mAb stimulation and a 10-fold excess of Jagged-1 Fc protein (Fig. 4*A*). This was true regardless of the time point examined or the amount of anti-CD3 mAb used to activate T cells. Control experiments confirmed that Jagged-1 Fc stimulation alone did not induce phosphorylation of molecules associated with early TCR signaling events (Fig. 4*B*). Although we were unable to observe any direct evidence for a role of Jagged-1 Fc engagement in abrogation of proximal events associated with TCR signaling, we have observed that Jagged-1 Fc-mediated suppression occurs only when delivered simultaneously with TCR stimulation (data not shown).

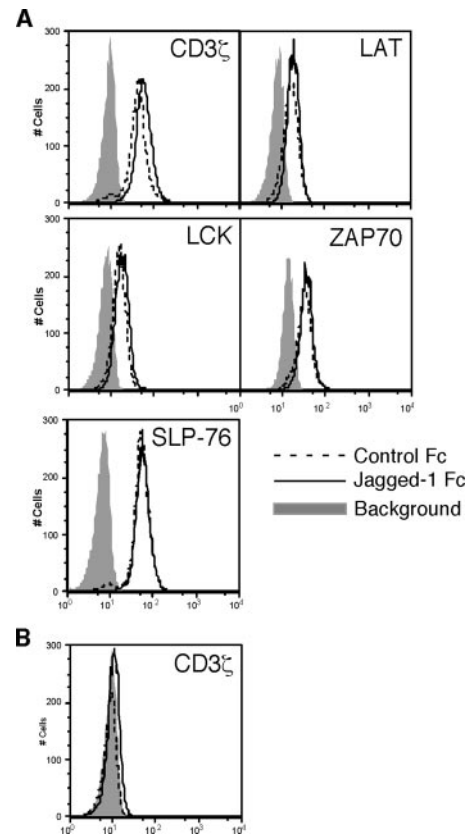
#### *Jagged-1-mediated signaling up-regulates the expression of GRAIL*

We reasoned that Jagged-1-mediated signaling might induce genes responsible for the suppressive effects on T cell activation. To elucidate which genes were associated with Jagged-1-mediated T cell suppression, we stimulated total CD4<sup>+</sup> T cells with beads coated with anti-CD3/CD28 mAbs and Jagged-1 Fc or control Fc, or with Jagged-1 Fc or control Fc alone. Messenger RNA was extracted for expression analysis by quantitative PCR. We consistently observed that Jagged-1-mediated signaling induced GRAIL expression in CD4<sup>+</sup> T cells (Fig. 5*A*). Among E3 ubiquitin ligases, up-regulation of GRAIL was unique, as Jagged-1-mediated signaling did not modulate *cbl-b* or *Itch*. Stimulation of CD4<sup>+</sup> T cells with Jagged-1 Fc alone failed to induce GRAIL, although addition of soluble anti-CD3/CD28 mAbs to T cells stimulated with Jagged-1 Fc beads did induce GRAIL (Fig. 5*B*). Thus, to observe Jagged-1-mediated GRAIL induction, simultaneous TCR signals must also be delivered.

Using siRNA technology, we suppressed the expression of GRAIL in CD4<sup>+</sup> T cells to determine whether expression of GRAIL was responsible for the unresponsive phenotype of Jagged-1-stimulated CD4<sup>+</sup> T cells. After confirming the efficiency of GRAIL silencing (Fig. 5*C*), we stimulated these CD4<sup>+</sup> T cells with anti-CD3/CD28 mAbs in the presence of Jagged-1 Fc or control Fc. We found that suppression of GRAIL expression did not rescue Jagged-1-treated T cells from their inability to proliferate (Fig. 5*D*). Thus, although GRAIL induction is associated with Jagged-1-mediated T cell suppression, it alone is not responsible for the suppression.

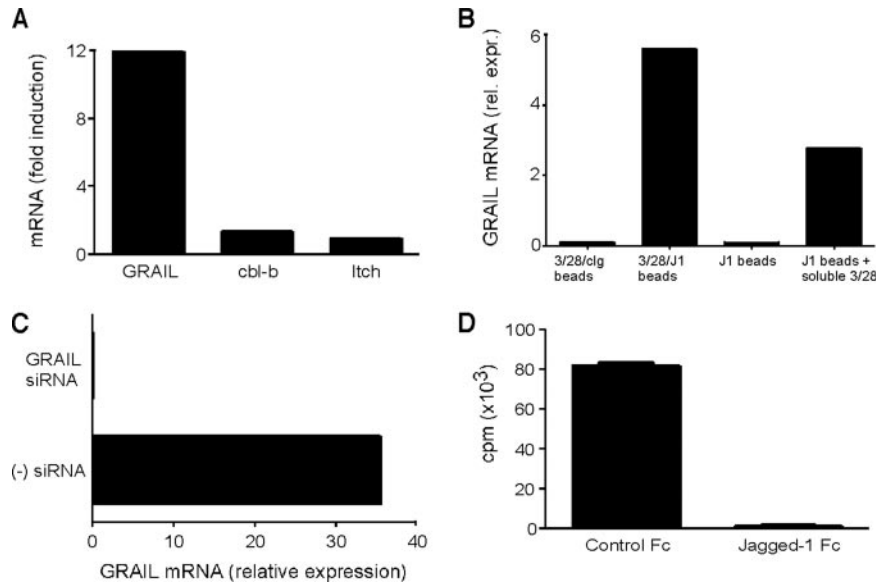
#### *Jagged-1-mediated signaling alters T cell differentiation*

Although Jagged-1 Fc stimulation potentially inhibited T cell activation, it did not kill the T cells (Fig. 3). These data, in light of reports that Notch signaling can influence Th cell differentiation, prompted us to evaluate the consequences of Jagged-1-mediated signaling during primary naive T cell activation. Accordingly, pu-



**FIGURE 4.** Jagged-mediated Notch signaling does not alter anti-CD3-induced TCR signaling in CD3<sup>+</sup>CD4<sup>+</sup> T cells and does not induce TCR signaling by itself. *A*, Fresh human whole blood was stimulated with anti-CD3 mAb (at 1  $\mu$ g/ml) plus Jagged-1 Fc or control Fc (each at 5  $\mu$ g/ml) for 3 min at 37°C. Comparable results were obtained in independent repeat experiments studying anti-CD3 concentrations ranging from 0.1 to 10  $\mu$ g/ml whole blood, times of stimulation for 3 and 5 min at 37°C, and Jagged-1 Fc or control Fc concentrations of 5 or 50  $\mu$ g/ml. Abs used were directed against phosphorylated (activated) forms of signaling molecules. *B*, There was no evidence of CD33 phosphorylation when cells were stimulated with Jagged-1 Fc or control Fc in the absence of anti-CD3 stimulation.

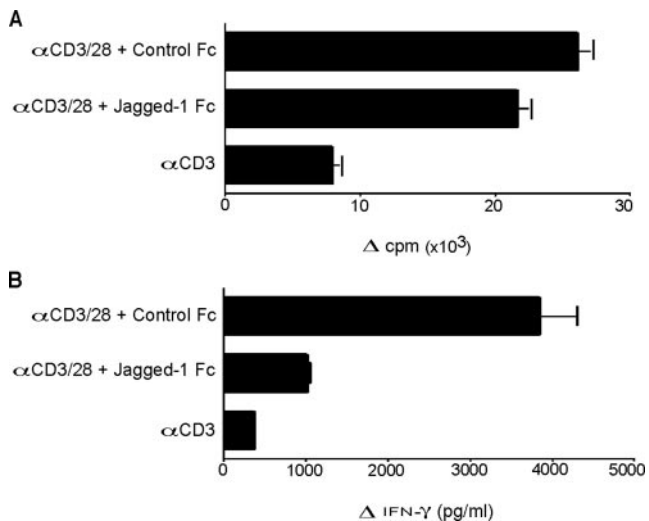
rified naive (CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells were stimulated with immobilized anti-CD3/CD28 mAbs in the presence of Jagged-1 Fc or control Fc and were expanded over the course of 14 days. After 14 days, cells were harvested and washed, and an equivalent number of T cells from each condition were restimulated with immobilized anti-CD3/CD28 mAbs. Stimulation of naive CD4<sup>+</sup> T cells with anti-CD3 mAb in the absence of a costimulatory signal was performed as a comparison for an anergic phenotype present in human CD4<sup>+</sup> T cells. As expected, naive CD4<sup>+</sup> T cells that were primarily stimulated with anti-CD3/CD28 mAbs in the presence of control Fc extensively proliferated after secondary stimulation (Fig. 6*A*) and secreted large amounts of IFN- $\gamma$  (Fig. 6*B*). Naive CD4<sup>+</sup> T cells stimulated with anti-CD3/CD28 mAbs in the presence of Jagged-1 Fc in the primary stimulation proliferated to an extent nearly comparable to that of cells primarily stimulated with anti-CD3/CD28 mAbs in the presence of control Fc, but secreted significantly lower amounts of IFN- $\gamma$ . In repeat experiments we observed no consistent, statistically significant reduction in the proliferative response of T cells stimulated initially in the presence of Jagged-1 Fc, but did consistently observe an ~5-fold reduction in the amount of secreted IFN- $\gamma$ . There was no evidence for Th2 deviation or increased secretion of IL-10 or TGF- $\beta$  in repeated experiments.



**FIGURE 5.** Jagged-1-mediated Notch signaling up-regulates the expression of GRAIL in human CD4<sup>+</sup> T cells. **A**, Total CD4<sup>+</sup> T cells were stimulated with beads bound with immobilized anti-CD3/CD28 mAbs and Jagged-1 Fc or control Fc. GRAIL mRNA expression was up-regulated (12-fold) after Jagged-1 Fc stimulation compared with control Fc stimulation. Similar results were observed in four independent experiments. There was minimal induction of other related E3 ubiquitin ligases such as cbl-b or Itch. **B**, GRAIL induction in response to stimulation of T cells with anti-CD3/CD28 mAbs occurs when Jagged-1 Fc but not control Fc is immobilized on the beads. Stimulation with Jagged-1 Fc beads alone does not induce GRAIL, but addition of soluble anti-CD3/CD28 mAbs to Jagged-1 Fc beads does induce GRAIL expression. **C**, GRAIL mRNA expression was effectively suppressed in CD4<sup>+</sup> T cells stimulated with anti-CD3/CD28 Jagged-1 Fc-containing beads by siRNA nucleoporation when using GRAIL-specific siRNA oligonucleotides but not irrelevant control siRNA oligonucleotides. Data have been normalized to expression of GAPDH. Comparable inhibition of GRAIL was observed using two different siRNA constructs. **D**, GRAIL-silenced CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 plus Jagged-1 Fc or control Fc, and [<sup>3</sup>H]thymidine incorporation was determined after 72 h. Reduction of GRAIL expression did not revert the unresponsive phenotype induced by Jagged-1 signaling. Similar results were observed in three experiments conducted.

As classically defined, anergic T cells are not killed or rendered apoptotic, rather, they are functionally unable to respond to optimal stimulation in secondary responses (17) and often proliferate

less during the initial/inductive phase. Thus, we compared the function of cells stimulated in the presence of Jagged-1 Fc to naive CD4<sup>+</sup> T cells that were only stimulated with anti-CD3 mAb (in the absence of a costimulatory signal). There was little proliferation or IFN- $\gamma$  secretion after secondary stimulation among naive CD4<sup>+</sup> T cells that were primed only with anti-CD3 mAb. Thus, stimulation of naive CD4<sup>+</sup> T cells in the presence of Jagged-1 Fc affected subsequent T cell function in a manner distinct from that of anergic cells, primarily influencing T cell effector function (cytokine secretion) but not proliferative capacity.



**FIGURE 6.** Jagged-1 signaling does not induce classical anergy in human T cells. **A**, Naive CD4<sup>+</sup> T cells stimulated with Jagged-1 Fc in addition to anti-CD3/CD28 mAbs in a primary stimulation proliferated after secondary stimulation as well as did cells that had been primarily stimulated with anti-CD3/CD28 and control Fc. In contrast, cells that only received anti-CD3 during primary stimulation (Signal 1 without Signal 2) failed to proliferate after secondary stimulation (anergic cells). **B**, Naive T cells that received Jagged-1 signals during primary stimulation secrete lower amounts of IFN- $\gamma$  than do T cells stimulated in the presence of control Fc. Similar results were obtained in four independent experiments.

## Discussion

Given that the effects of Notch pathway signaling vary so much depending on the responding cell type and the Notch ligand used to activate Notch receptors, in addition to the experimental approach used to study the effects of Notch signaling (18), we used a reductionist approach to study the effects of Notch signaling during human T cell activation. Rather than using transduced APCs, we used a chimeric protein that fuses the extracellular region of Jagged-1 with the constant region of human IgG1 to deliver signals through Notch receptors. Thus, we are able to ascribe our results to direct effects of Jagged-1-mediated signaling. Of the four Notch receptors, Jagged-1-mediated signals in our assays were likely directed through either Notch-2 or Notch-1, as the ex vivo CD4<sup>+</sup> T cells used in our studies expressed Notch-2 at highest levels followed by Notch-1, with little or no expression of Notch-3 or Notch-4 receptors (data not shown). Our observation that Jagged-1-mediated suppression of T cell function was independent of Notch cleavage mediated by  $\gamma$ -secretase is consistent with other reports in both murine and human T cells (8, 15).

Several groups have recently evaluated the effects of different Notch ligands on murine Th cell differentiation, with Jagged-1

signaling promoting Th2 differentiation and Delta-1 signaling promoting Th1 differentiation (7, 9, 10). We have evaluated the effects of Jagged-1 signaling on both primary human T cell activation and naive human CD4<sup>+</sup> T cell differentiation. Consistent with our data, one group has observed that although low doses of Delta-1 (signaling through Notch-3) could promote Th1 differentiation in murine T cells, higher doses completely inhibited T cell activation (7). A recent study on the effects of Notch signaling during murine T cell activation is largely consistent with our data using human T cells. Coligation of CD3/CD28 molecules and Notch-1 inhibited T cell activation, and both Delta-1 and Jagged-1 ligands similarly inhibited TCR-mediated T cell activation (6). Immobilized Delta-1 and Jagged-1, but not Delta-4, have also recently been shown to inhibit murine T cell activation (8).

Comparatively little is known about the effects of Notch signaling in human T cells. One study has reported that after transduction of a human B lymphoblastoid cell line with an adenoviral vector expressing Jagged-1, coculture with human T cells inhibited proliferation and cytotoxicity that was concomitant with elevated secretion of IL-10 (12). A murine study similarly observed that injection of mice with APCs transfected with Delta-1 promoted CD8<sup>+</sup> T cell secretion of IL-10 in vivo (13). We did not observe any modulation of IL-10 after Jagged-1-mediated stimulation of CD4<sup>+</sup> T cells. One explanation for the discrepancy in data may be that transduction of APCs with Notch ligands alters APC function, given that APCs undoubtedly express Notch receptors.

Our data extend the study of Notch signaling to human T cells. We report that human T cell activation is inhibited when T cells are stimulated in the presence of Jagged-1-mediated Notch signaling; T cell proliferation, as well as Th1 and Th2 cytokine production, is abrogated. There was no evidence for modulation of IL-10 or any other cytokine by Jagged-1-mediated signaling. Our data demonstrate that neither apoptosis nor abrogation of proximal TCR signaling is responsible for the inability of Jagged-1-stimulated T cells to proliferate.

We postulated that Notch signaling might induce genes associated with T cell nonresponsiveness. Indeed, we have found that Jagged-1-mediated signaling up-regulates expression of GRAIL in human CD4<sup>+</sup> T cells. GRAIL is a member of the E3 ubiquitin ligase family, and its expression, induced in anergic T cells, limits the transcription of both IL-2 and IL-4 genes (19). The 428-aa GRAIL molecule is a transmembrane protein associated with recycling endosomal compartments (18). Cbl-b and Itch, also members of the E3 ubiquitin ligases family, have also been linked to anergy (20, 21). However, Jagged-1-mediated signaling did not modulate their expression levels. Despite its up-regulation after Jagged-1-mediated signaling, GRAIL is not solely responsible for the observed T cell unresponsiveness, as this phenotype was not reversed after suppressing GRAIL expression induced during concomitant Jagged-1-signaling and T cell activation. It remains possible that GRAIL cooperates with other molecules uniquely induced by simultaneous Notch- and TCR-mediated signaling events to suppress peripheral human T cell activation.

In summary, our data indicates that Jagged-1-mediated Notch signaling induces an unresponsive phenotype in human T cells, a condition associated with GRAIL up-regulation. As human T cells stimulated in the presence of Jagged-1-mediated Notch signaling are not anergic, our data suggest that up-regulation of GRAIL in T cells is associated with lack of T cell proliferation and not exclusively with T cell anergy. There is a report demonstrating that Notch-1 signaling induces anergy in murine T cells (6); this difference may be related to differences between human and murine T cells, or to the Notch receptor that initiates the signaling cascade.

Nevertheless, although human T cells stimulated in the presence of Jagged-1 Fc are not rendered anergic in a classical sense, they do have a lasting deficit in their ability to secrete IFN- $\gamma$ . Given that Jagged-1, in addition to other Notch receptors, is constitutively expressed by both T cells and APCs, such as dendritic cells, it will be important in future studies to determine in what physiological context altered or heightened Notch signaling modulates murine and human T cell function.

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## Disclosures

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