γ-Secretase inhibitors repress thymocyte development

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Communicated by David M. Kipnis, Washington University School of Medicine, St. Louis, MO, April 25, 2001 (received for review March 20, 2001)

A major therapeutic target in the search for a cure to the devastating Alzheimer's disease is γ-secretase. This activity resides in a multiprotein enzyme complex responsible for the generation of Aβ42 peptides, precipitates of which are thought to cause the disease. γ-Secretase is also a critical component of the Notch signal transduction pathway; Notch signals regulate development and differentiation of adult self-renewing cells. This has led to the hypothesis that therapeutic inhibition of γ-secretase may interfere with Notch-related processes in adults, most alarmingly in hematopoiesis. Here, we show that application of γ-secretase inhibitors to fetal thymus organ cultures interferes with T cell development in a manner consistent with loss or reduction of Notch1 function. Progression from an immature $CD4^-/CD8^-$ state to an intermediate $CD4^+/CD8^+$ double-positive state was repressed. Furthermore, treatment beginning later at the double-positive stage specifically inhibited $CD8^+$ single-positive maturation but did not affect $CD4^+$ single-positive cells. These results demonstrate that pharmacological γ-secretase inhibition recapitulates Notch1 loss in a vertebrate tissue and present a system in which rapid evaluation of γ-secretase-targeted pharmaceuticals for their ability to inhibit Notch activity can be performed in a relevant context.

Notch proteins are conserved cell surface receptors used repeatedly in metazoans to correctly select cell fates during development (1) and in the adult (2, 3). Notch receptors directly transduce a signal from the cell surface to the nucleus via regulated intramembrane proteolysis (RIP). Ligand binding promotes an ectodomain-shedding extracellular cleavage (4) followed by presenilin-mediated proteolysis within the transmembrane domain (5–8). These events release the Notch intracellular domain (NICD) (4, 6, 9); its translocation to the nucleus is essential for signal transduction *in vitro* (4, 9, 10) and *in vivo* (11).

Presenilins (PS1 and PS2), discovered as predisposing mutations in humans with familial Alzheimer's disease (AD) (12), are thought to contain the active site for γ-secretase (reviewed in refs. 13–15). The γ-secretase activity is one of few therapeutic targets for drugs that will ameliorate the amyloid plaque burden, whose accumulation is thought to cause AD (16, 17). The first of these drugs, currently in early clinical trials, was presented recently at the international Alzheimer's disease meeting by Bristol Myers Squibb.** The involvement of presenilin proteins in Notch signaling therefore is of extreme importance to the development of a pharmacological therapy for Alzheimer's disease. The discovery of a connection between γ-secretase and Notch, and the demonstration that γ-secretase inhibition of Notch and β-amyloid precursor protein (APP) has the same inhibition coefficient (6), led to the hypothesis that γ-secretase inhibition may result in Notch deficiency in the adult, most likely in hematopoiesis (6).

Genetic loss and gain of function experiments have provided compelling evidence for a role for Notch1 signaling in lymphopoiesis. In mice in which loss of *Notch1* was induced at birth by Cre-mediated recombination (19), or in mice reconstituted with $Notch1^{-/-}$ bone marrow (BM) (19, 20), T lymphocyte development is blocked. When Notch1 is completely missing, immature $CD3^-/CD4^-/CD8^-$ cells accumulate and no intermediate $CD4^+/CD8^+$ double-positive (DP) or mature $CD4^+$ single-positive (SP) or $CD8^+$ SP thymocytes are detected. Moreover, in mice reconstituted with bone marrow lacking Hes-1, one downstream target of Notch1 signaling, thymocytes are also arrested at the immature $CD4^-/CD8^-$ stage with impaired TCR-independent as well as TCR-dependent expansion (21). In addition to the block in T cell development, the thymus of *Notch1*-deficient animals contained elevated levels of early B cells. Conversely, in irradiated mice reconstituted with BM expressing a constitutively active form of Notch1, the bone marrow contained $CD4^+/CD8^+$ DP T cells and exhibited a simultaneous block in early B cell lymphopoiesis (22). Given the known consequences of *Notch1* loss in T cell development, the effects of γ-secretase inhibitors on lymphopoiesis can be evaluated and compared with the results obtained from genetic manipulation of *Notch1*.

Downstream of the decision to enter the $CD4^+CD8^+$ DP stage in thymocyte development, Notch signaling again is involved in maturation of $CD4^+CD8^+$ DP thymocytes into $CD4^+$ or $CD8^+$ SP cells. Recent studies reported that a constitutively active form of Notch1 driven by the *lck* promoter imparts resistance to glucocorticoid-induced apoptosis in DP thymocytes and promotes the maturation of DP cells to both $CD8^+$ SP and $CD4^+$ SP cells (23, 24). Conversely, using a novel *in vitro* differentiation system to study the maturation of DP cells (25), investigators report that lowering Notch1 activity after T cell receptor-directed lineage commitment into the $CD4^+$ or $CD8^+$ SP lineages repressed the maturation of $CD8^+$ SP thymocytes but not $CD4^+$ SP cells.

Collectively, these genetic and tissue culture-based experiments demonstrate an early requirement for Notch1 signaling in lymphopoiesis. Additional roles for Notch signaling in T cell development are also possible. Although uncertainties remain, thymocyte differentiation provides us with a system in which to test the hypothesis that γ-secretase inhibition will adversely impact aspects of hematopoiesis. Using fetal thymus organ culture (FTOC), we demonstrate here that γ-secretase inhibitors create the pharmacological equivalent of *Notch1* loss. These results not only offer means by which to evaluate the side effects of γ-secretase inhibitors before clinical trials but also validate γ-secretase inhibitors as powerful, reversible tools for the study of the role Notch signaling plays during individual steps in the development of vertebrate organs.

Abbreviations: FTOC, fetal thymus organ culture; DP, double-positive; SP, single-positive; DN, double-negative; NICD, Notch intracellular domain; AD, Alzheimer's disease; APP, β-amyloid precursor protein; dpc, days postcoitum; Cpd. 11, difluoro ketone γ-secretase inhibitor compound 11.

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Molinoff, B. P., Felsenstein, M. K., Smith, W. D. & Barten, M. D. (2000) *Neurobiol. Aging* **21, S136 (abstr.).

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Experimental Procedures

Transfections and Analysis of 293 Cells. Transient transfections of 293 cells with a Notch1 construct containing a deletion of the extracellular domain ($N\Delta E^{MV}$) were performed by the calcium phosphate precipitation method, as described previously (9). Generation of a dose-response curve (E.H.S. and R.K., unpublished data) was done by transfecting 10 μg of pCS2 + $N\Delta E^{MV}$ in 100-mm dishes. Twenty-four hours after transfection, multiple 100-mm dishes were trypsinized and the cells were pooled and plated onto six-well dishes for analysis. The difluoro ketone γ-secretase inhibitor compound 11 (Cpd. 11) (26) was diluted into medium used to feed Notch-expressing cells. ^{35}S (40 μCi/ml; Amersham Pharmacia) was added to the plates for 5 h in the presence or absence of inhibitor. Cells then were lysed and immunoprecipitated (9). Samples were separated by 6% SDS/PAGE and visualized by using a Molecular Dynamics Phosphorimager. IMAGEQUANT software was used to quantify both uncleaved $N\Delta E^{MV}$ and NICD. At least four data points were generated for each concentration of inhibitor. An inhibition coefficient (IC_{50}) value of less than 10 μM was calculated with PRIZM graphing software (GraphPad, San Diego), using the nonlinear regression analysis algorithm (data not shown, E.H.S. and R.K., unpublished data).

To determine the efficacy of inhibition of Notch1 proteolysis by Cpd. 11, transfected cells were plated in 30-mm culture dishes and allowed to recover overnight. Fresh medium containing 50 μM Cpd. 11 (6-fold higher than the IC^{50}) was added at time 0, 3.5, 7, and 10 h, after which medium was left unchanged. Samples for analysis by Western blotting were harvested every 2 h, starting before the addition of Cpd. 11, for a total of 18 h. One more sample was collected at 26 h.

FTOC. Fetal thymic lobes from 15.5-days postcoitum (dpc) C57BL/6 mice were dissected and cultured according to a previously described procedure (27). Briefly, dissected thymic lobes were placed at a liquid/air interface on transwell basket inserts (Costar) in 24-well plates containing 0.5 ml of medium (DMEM supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, glutamine, and penicillin/streptomycin). Samples were cultured in a humidified incubator at 37°C, 5% CO_2. Cpd. 11 (50 μM final concentration, delivered in DMSO at 1,000×) or the solvent DMSO alone was added at 12-h intervals over the relevant periods as indicated.

Flow Cytometric Analysis. After the culture period, thymocytes were harvested by pressing thymic lobes into a 70-μm nylon mesh filter (Falcon), washed in staining buffer (PBS with 2% FBS/0.09% NaN_3), and counted. Cells then were stained with biotin-CD3 Ab (PharMingen) on ice for 30 min, washed with staining buffer, and then stained with FITC-CD8 Ab, PE-CD4 Ab, and streptavidin-cychrome (PharMingen). Samples were analyzed with a Becton Dickinson FACScan flow cytometer by using CELLQUEST software.

Results

γ-Secretase Inhibitors Interfere with Early Thymocyte Development. We showed previously that γ-secretase inhibitors interfere with proteolytic formation of NICD from the Notch1 transmembrane receptor (6). To test the hypothesis that such inhibition may affect physiological aspects of Notch signaling, we studied the maturation of thymocytes in the presence of these inhibitors by using FTOC (27).

To establish a time course for treatment of thymic lobes with the difluoro ketone γ-secretase inhibitor Cpd. 11 (26) in these cultures, we first determined the efficacy of inhibition on steady-state Notch proteins in an established *in vitro* assay (9) by using 293 cells transiently transfected with a ligand-independent substrate of γ-secretase ($N\Delta E^{MV}$). In an attempt to mimic potential, clinical use of γ-secretase inhibitors, the treatment regiment should apply dosing severalfold the IC_{50} for Cpd. 11 to maintain the IC_{50} throughout the period of the experiment. Transfected cells were plated in 30-mm culture dishes and treated with fresh 50 μM Cpd. 11 (6-fold the IC_{50}; E.H.S. and R.K., unpublished data) at 0, 3.5, 7, and 10 h. Samples were harvested for protein every 2 h during the treatment period and after the final treatment, at 12, 14, 16, 18, and 26 h, to determine whether Notch proteolysis would recover. The steady-state amounts of the cleavage product NICD were estimated by Western blot analysis. The abundance of NICD reached a minimum by 8–10 h of treatment and did not increase above this minimum during the following 16-h chase period, when no fresh inhibitor is added (Fig. 1). Thus, the treatment regiment chosen constituted a dosing of 50 μM Cpd. 11 every 12 h.

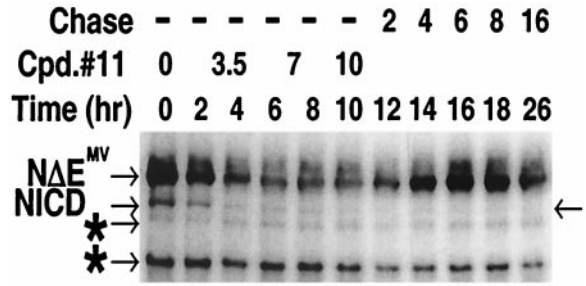

Fig. 1. Inhibition of Notch1 cleavage by the γ-secretase inhibitor, Cpd. 11. 293 cells were transfected with a ligand-independent Notch1 construct containing Notch1 with the extracellular portion deleted ($N\Delta E^{MV}$, precursor, arrow). Cells were treated with 50 μM fresh inhibitor at 0, 3.5, 7, and 10 h to reach maximal inhibition of the cleavage product NICD (arrow). Samples were taken for analysis at 2-h intervals during this treatment period and also at times 2, 4, 6, 8, and 16 h (chase period) after the final treatment. NICD levels remain low even after the 16-h chase period, suggesting Cpd. 11 activity remains for at least 16 h. Asterisk denotes degradation products of Notch1 with no or minimal signaling activity (9).

To examine the effect of lowering NICD levels with γ-secretase inhibitors on thymocyte maturation, we removed thymic lobes from 15.5-dpc murine fetuses and cultured them for 3 days without further treatment in the presence of solvent (DMSO) or in the presence of 50 μM Cpd. 11. Fresh Cpd. 11 (or control DMSO) was added immediately upon removal of thymic lobes into culture, and every 12 h subsequently, to effectively reduce γ-secretase activity during the 3-day culture period. Thymocytes isolated from freshly removed thymic lobes are primarily at an early $CD3^-/CD4^-/CD8^-$ triple-negative stage of maturation (28). After 3 days in culture, most of these thymocytes have progressed to the $CD4^+/CD8^+$ DP stage and a small percentage have progressed to the $CD8^+$ or $CD4^+$ SP stage (Fig. 2*A*, DMSO). In several preliminary experiments, we observed no difference in thymocyte development (total cellularity and relative percentages of each population) between nontreatment and DMSO-treatment controls (data not shown), so subsequent experiments compared only DMSO-treated controls with Cpd. 11-treated samples.

After 3 days in culture with Cpd. 11, there was a significant reduction in total thymocytes recovered relative to DMSO-treated cultures (Fig. 2*B*). This decrease in total cellularity resulted primarily from a relative decrease of DP cells; we detected a consistent, approximate 2-fold reduction in DP cell numbers in Cpd. 11-treated lobes relative to DMSO, whereas $CD4^-/CD8^-$ double-negative (DN) cell numbers were not changed significantly (Fig. 2*B*). These results suggest that γ-secretase inhibitors affect maturation of early thymocytes at a DN stage, in a manner consistent with loss of Notch1 signaling.

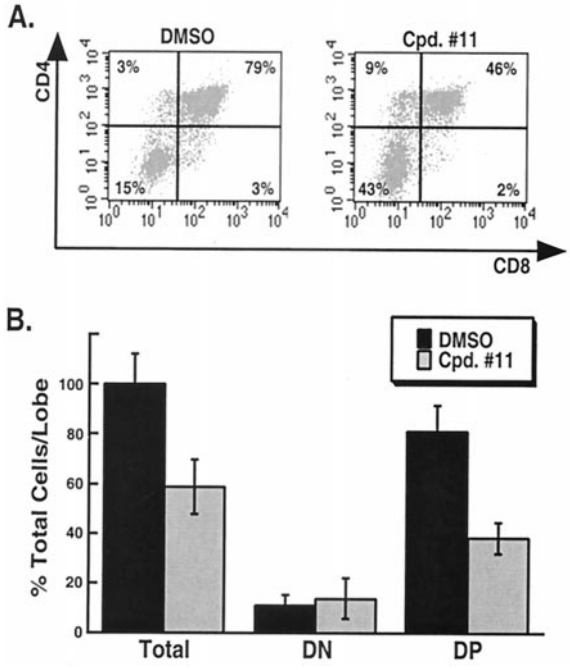

Fig. 2. Continuous treatment of FTOC with the γ-secretase inhibitor Cpd. 11 results in reduced total thymocyte cellularity, specifically affecting cellularity of $CD4^+CD8^+$ DP cells. Thymic lobes removed from 15.5-dpc C57BL/6 fetuses were cultured in the presence of Cpd. 11 (50 μM) or DMSO, added fresh every 12 h for 3 days. (*A*) FACS analysis of CD4 and CD8 expression from a representative sample, showing that relative percentages of the DP population were reduced after Cpd. 11 treatment in FTOC relative to DMSO treatment, whereas the relative percentages of DN cells were increased. (B) Total number of cells recovered per thymic lobe were reduced after 3-day treatment with Cpd. 11 in FTOC, relative to carrier, DMSO, alone ($P < 0.01$, Student's *t* test). Total cell numbers in DP populations also were reduced in Cpd. 11-treated samples relative to controls ($P < 0.01$, Student's *t* test). Although relative percentages of DN cells by FACS were increased, there was no significant difference in the total DN cells recovered per thymic lobe ($P > 0.3$, Student's *t* test). Total cells recovered per lobe varied from experiment to experiment from an average of 5.4×10^4 to 17×10^4 in DMSO samples, reflecting natural variation depending on exact age of thymocyte extraction. Thus, for each separate experiment, values for individual samples first were standardized relative to the average cellularity in control DMSO-treated samples for that experiment and then pooled for statistical analysis. Values for each class (DP, DN, total) are shown as a percentage of average total cells per lobe in DMSO-treated samples. Bars = SD of at least 10 total samples per treatment, from four independent experiments.

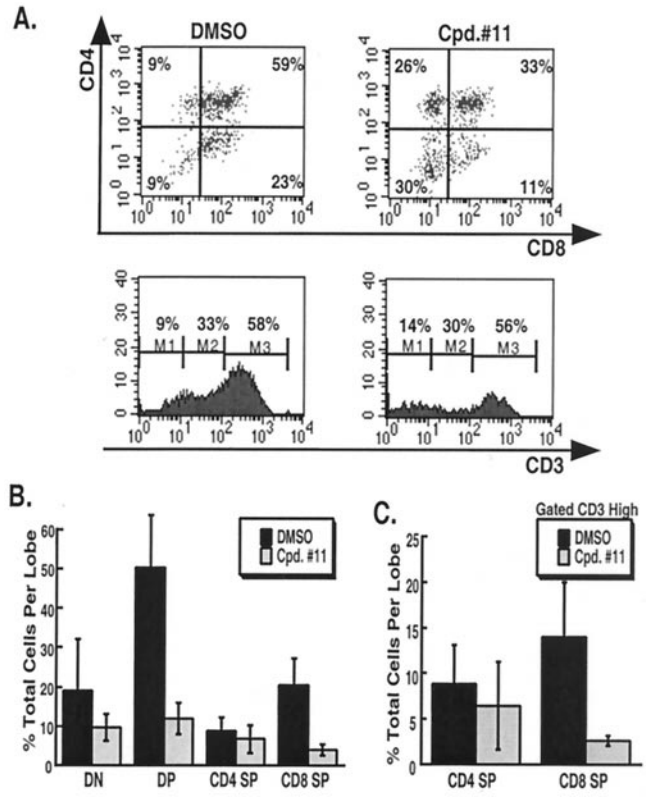

Fig. 3. Late treatment with the γ-secretase inhibitor Cpd. 11 in FTOC after progression of most thymocytes to the DP stage results in an inhibition of CD8 SP production without affecting CD4 SP progression. Thymic lobes removed from 15.5-dpc C57BL/6 fetuses were cultured for 3 days with no treatment followed by 3–4 days in the presence of Cpd. 11 (50 μM) or DMSO, added fresh every 12 h. (*A*) FACS analysis of CD4 and CD8 expression from a representative sample demonstrates a reduction in the percentage of CD8 SP thymocytes from FTOC after Cpd. 11 treatment relative to carrier (DMSO) treatment. Relative CD4 percentages, however, were increased for Cpd. 11 treatment. (*B*) Examination of total cell numbers recovered shows that CD8 SP and DP populations were largely reduced in Cpd. 11 samples relative to controls ($P < 0.01$ in both cases, Student's *t* test), whereas total DN and CD4 cell numbers were not significantly different from controls ($P > 0.1$ and $P > 0.3$, respectively, Student's *t* test). (*C*) When the CD3 high population (mature thymocytes, M3 in *A*) was gated, the same affect on $CD8^+$ SP cell numbers was seen ($P < 0.01$, Student's *t* test). Values were derived as in Fig. 2, and bars = SD of six samples per treatment, from two independent experiments. Several additional experiments carried out under the same conditions resulted in a similar distribution of cell populations, although total cell numbers were not determined.

γ-Secretase Inhibitors Interfere with CD8 SP Development. In some FTOC experiments, cultured 3 days and longer in the presence of Cpd. 11, we noticed a reduction of the $CD8^+$ SP populations (not shown), suggesting that γ-secretase inhibitors also may affect a later stage of thymocyte maturation. To test further this possibility, we cultured thymic lobes for 3 days without treatment followed by addition of Cpd. 11 or DMSO for an additional 3–4 days. The initial culture in the absence of inhibitor allows most thymocytes to progress to the DP stage (see Fig. 2*A*, DMSO). Then, during the second culture step, DP cells can differentiate to $CD4^+$ or $CD8^+$ SP cells (Fig. 3*A*). Therefore, this regimen specifically lowers NICD levels during the differentiation of SP cells. We found that in samples treated with Cpd. 11, there was a significant reduction in the numbers of $CD8^+$ SP cells relative to DMSO-treated samples (Fig. 3*B*). However, there was no reduction in the $CD4^+$ SP population, suggesting that γ-secretase inhibition preferentially inhibited the development of the $CD8^+$ SP lineage (Fig. 3*B*). We obtained the same results when only mature thymocytes (high CD3 expression, Fig. 3*A*) were analyzed (Fig. 3*C*), confirming that the effect was not a result of the elimination of immature $CD8^+/CD4^-$ cells known to be present transiently as DP cells form (38). We also found a significant reduction in total DP cells, suggesting that this population continued to require Notch signals for its differentiation and/or survival.

Discussion

Notch signaling is critical in the maturation events of multiple systems during development. Its role in the adult, although less characterized, is well established, particularly in regard to hematopoiesis (for review, see ref. 2). Various experiments *in vitro* have implicated Notch in aspects of myelopoiesis (30–34) and erythropoiesis (35, 36) and in development of multipotent hematopoietic progenitors (37–41). A role for Notch during lymphopoiesis and, specifically, thymocyte development has been established more strongly by studies using a variety of genetic and tissue culture manipulations (3, 19, 22, 42–44).

The connection between Notch and the presenilin proteins, mutations in which are related to predisposition to AD, potentially complicates the targeting of the activity of the presenilin proteins as a therapeutic strategy in dealing with AD progression. Presenilin-dependent γ-secretase activity cleaves truncated APP protein to form the amyloid-β plaque-producing Aβ42; however, the same activity is required for Notch1 cleavage and downstream cleavage-dependent signaling (13). Thus, global and persistent pharmacological inhibition of γ-secretase activity may result in side effects related to Notch1-dependent processes in the adult, particularly in hematopoiesis (6). To address these concerns, we have studied the affect of a class of γ-secretase inhibitors on thymocyte development by using an established FTOC system. This class of inhibitors was shown previously to inhibit both APP cleavage and Notch1 cleavage in tissue culture transfection assays (6). Here, we demonstrate that γ-secretase inhibition in thymic lobes interferes with thymocyte development in a manner consistent with genetic experiments involving gain and loss of Notch1 function (Figs. 2 and 3). Total thymocyte cellularity is decreased, similar to mice lacking *Notch1* or *Hes-1*, a downstream effector of Notch signaling (19, 21), specifically reducing the numbers of $CD4^+/CD8^+$ DP-stage thymocytes. The dose of Cpd. 11 (50 μM, about six times greater than the IC_{50} for this compound) and the treatment regiment were chosen to maintain an IC_{50} throughout the course of the experiment. The pharmacological effects, predictably, are less severe than those caused by complete loss of Notch signals, as some cells do progress to the DP stage. It may take more than 10 h after initial treatment before maximal inhibition of NICD production by inhibitor is achieved (Fig. 1), allowing some cells in FTOC to progress beyond an early checkpoint. Additionally, because production of NICD is not inhibited completely even by 50 μM Cpd. 11 treatment (Fig. 1), it is also possible that Notch signaling is sufficiently active in a fraction of cells, allowing their progression past this immature stage. Finally, given that penetration of thymic lobes by this inhibitor may be incomplete, some cells may develop exposed to relatively low concentrations of Cpd. 11. It is remarkable that despite all these caveats, thymocyte development was affected significantly.

When thymic lobes are allowed to progress to the DP stage (3 days in culture) and then treated with inhibitors for 4 subsequent days, we find specific reduction in the number of thymocytes differentiating into $CD8^+$ SP cells. Whereas $CD8^+$ SP cellularity is decreased, $CD4^+$ SP cellularity is unaffected relative to DMSO-treated controls (Fig. 3). Interestingly, total DP cellularity is also decreased in these two-stage, inhibitor-treated samples. This secondary phenomenon may be explained by the observation of Deftos *et al.* (23), who reported that an activated form of Notch1 imparted resistance to glucocorticoid-induced apoptosis in DP thymocytes through activation of bcl-2. Loss of Notch signaling in our FTOC experiments at the DP stage may render them vulnerable to cell death during maturation to the SP lineages, with the CD8 lineage preferentially affected over CD4. This is in agreement with recent findings demonstrating that, although Notch1 does not affect the CD4 vs. CD8 lineage decision, it is important for subsequent $CD8^+$ SP maturation from DP cells. $CD8^+$ SP maturation is blocked by reduction of Notch1 signaling by using antisense Notch1 or an anti-Notch1 antibody in a culture assay (25), with no effect on $CD4^+$ SP maturation. However, a contradictory observation was reported recently in mice in which Notch1 is conditionally deleted in late-stage DN thymocytes ($CD44^-/CD25^+$). In these animals, neither $CD4^+$ SP nor $CD8^+$ SP maturation is affected (45). Although Wolfer *et al.* (45) have specifically abolished Notch1, both Notch2 and Notch3 also are expressed in developing thymocytes (18) and may partially compensate for loss of Notch1. Our experimental paradigm targets all four mammalian Notch proteins (M. T. Saxena and R.K., unpublished results). Thus, although the discrepancy among various experiments regarding the role of Notch1 in later steps of thymocyte development remains to be resolved, our data suggest that Notch signaling, via one or more of the mammalian homologs, is critical for normal $CD8^+$ SP maturation. Further research will be necessary to determine which Notch protein(s) specifically acts to support this $CD8^+$ SP cell development.

In summary, by using an *in vitro* FTOC system, we have demonstrated that the effects of γ-secretase inhibition on thymocyte development mimic the effects of loss or inhibition of the Notch1 signal pathway in previous experiments involving thymocytes. Similar conclusions were reached independently by P. Doerfler and R. M. Perlmutter (unpublished results) by using different γ-secretase inhibitors, demonstrating that the effects are not caused by the choice of drug but rather its ability to antagonize Notch signaling. Collectively, these results suggest that elimination of γ-secretase activity in the adult may not be without risk and that careful study of the potential side effects of these inhibitors in an *in vivo* model at pharmacologically relevant doses is warranted. Thymocyte maturation in the developing embryo is distinct from that of the adult. However, because Notch signaling is thought to be involved in multiple processes in adult self-renewing cells, potentially harmful side effects may occur by interfering with various Notch-related aspects of hematopoiesis and tissue renewal. Therapeutic doses that reduce APP and Notch proteolysis without eliminating it are likely to present the best strategy for success because a significant reduction in Aβ42 should have the desired outcome in delaying onset of AD. These results also validate the FTOC as a relevant and rapid system in which to test dosage effects of γ-secretase-targeted pharmaceuticals.

Finally, these experiments demonstrate the potential use of γ-secretase inhibitors as valuable tools in probing the general function of Notch signaling at specific time points during a developmental process in various tissues.

We thank Alec Cheng for critical discussion and technical assistance, Petra Doerfler and MERCK for sharing unpublished information, and members of the Kopan lab for helpful discussions. R.K. is supported by National Institutes of Health Grant GM55479; B.K.H. is supported by the Medical Scientist Training Program and by Alzheimer's Association Grant RG991516, which also funded these experiments.

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