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Amyloid precursor family proteins are expressed by thymic and lymph node stromal cells but are not required for lymphocyte development

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Abstract

Pharmacological inhibitors that block amyloid precursor protein (APP) cleavage and the formation of senile plaques are under development for the treatment of familial Alzheimer's disease. Unfortunately, many inhibitors that block γ-secretase-mediated cleavage of APP also have immunosuppressive side effects. In addition to APP, numerous other proteins undergo γ-secretase-mediated cleavage. In order to develop safer inhibitors, it is necessary to determine which of the γ-secretase substrates contribute to the immunosuppressive effects. Because APP family members are widely expressed and are reported to influence calcium flux, transcription and apoptosis, they could be important for normal lymphocyte maturation. We find that APP and amyloid precursor-like protein 2 are expressed by stromal cells of thymus and lymph nodes, but not by lymphocytes. Although signals provided by thymic stromal cells are critical for normal T cell differentiation, lymphocyte development proceeds unperturbed in mice deficient for these APP family members.

Introduction

The major protein component of the senile plaques associated with familial Alzheimer's disease is the Aβ peptide that is generated when amyloid precursor protein (APP) undergoes sequential β- and γ-secretase-mediated cleavages. There has been a great deal of interest in the use of pharmacological γ-secretase inhibitors for treatment of Alzheimer's disease, based on the hypothesis that preventing cleavage of APP could potentially cure the disease. Unfortunately, γ-secretase inhibitors can have immunosuppressive side effects. Inclusion of γ-secretase inhibitors in fetal thymus organ cultures profoundly blocked thymocyte development (1,2). Moreover, γ-secretase inhibitors impaired proliferation and cytokine production by mature T cells *in vitro* (3–7).

Numerous proteins undergo γ-secretase-mediated cleavage including APPs, Notch 1-4, Delta1, Jagged2, CD44, ErbB4, E-cadherin, lipoprotein receptor-related protein (LRP), Nectin-1α and chemokines CX3CL1 and CXCL16 (8–18). These proteins are variably expressed in lymphocytes and lymphoid-associated stromal cells. The γ-secretase inhibitors that were used in experiments for impairing T cell responses and development are transition state analogs that bind to the active site of the γ-secretase complex, so as to inhibit the cleavage of all γ-secretase substrates (19). Because both lymphocytes and stromal cells express proteins that are γ-secretase substrates, it remains to be determined whether the adverse effects of the inhibitors reflect a direct effect on lymphocytes themselves or an effect on the stromal cells that direct lymphocyte maturation and/or activation. Thus, it is important to determine which substrates and cell types are associated with γ-secretase-mediated inhibition of lymphocyte development and function.

Prominent among the γ-secretase substrates is the APP family, consisting of three highly homologous proteins, APP and amyloid precursor-like proteins 1 and 2 (APLP1, APLP2). APLP1 expression is restricted to the nervous system (20,21). In contrast, APP and APLP2 mRNA are found in many tissues, including thymus (20,22–26). Cleavage of APP, APLP1 or APLP2 by γ-secretase releases a small intracellular fragment (27,28) which can associate with numerous cytosolic proteins including nuclear adaptor protein Fe65 and histone acetyl transferase Tip60 (27,29–32), scaffolding protein Jnk-interacting protein 1 (JIP1, IB1) (33,34), Grb2

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(35), *disabled* 1 (DAB1) (36), heme oxygenase (37), X11 (29), G protein G_0 (38), ShcA (39) and Notch inhibitory proteins Numb and Numb-like (40). Although the true physiological role of APP proteins remains elusive, the intracellular domain of APP has been shown to modulate phosphoinositide-mediated calcium flux and MEK/ERK activation (35,41), trigger apoptosis directly or enhance sensitivity of cells to other apoptotic stimuli (42), inhibit Notch signaling (40) and stimulate transcription (27,31,43,44). Notably, intracellular fragments of APP and Notch can co-localize in nuclear structures postulated to be sites of active transcription (45), and it is well established that Notch functions are important for T cell commitment, differentiation and maturation (46). Given the potential for cross-talk between APP and Notch in nuclear signaling and the finding that APP and APLP2 mRNA are expressed in lymphoid tissues, it is surprising that APP family proteins have not yet been studied in the immune system.

Here we have investigated the expression and function of APP family proteins in developing T cells. We find that APP and APLP2 proteins are expressed in thymus and lymph nodes (LNs), but exclusively by the stromal cells. These results preclude a functional role for these proteins in thymocytes themselves. Nevertheless, because thymocyte development and selection is absolutely dependent on signals provided by thymic stromal cells, it was possible that APP/APLP2 expression by stromal cells was required to support normal thymocyte development. However, studies of T cell development in mice deficient for APP, APLP2 or both revealed that T cell development proceeded unperturbed. Moreover, these mice had normal populations of peripheral T and B cells. While the physiological significance of the stromal cell expression remains to be determined, our results indicate that the defects in T lymphocyte maturation associated with γ-secretase inhibition do not involve APP family proteins.

Methods

Mice and genotyping

APP- and APLP2-deficient (germline null mutant) mice have been previously described (47,48) and were generously provided by Merck Research Laboratories (Rahway, NJ, USA) and Robert Cappai (University of Melbourne, Australia), respectivelyC57BL/6 (B6) mice were obtained from National Cancer Institute-Frederick. Mice were bred and maintained under specific pathogen-free conditions in National Institute of Allergy and Infectious Diseases (NIAID) Research Animal Facilities on protocols approved by the NIAID Animal Care and Use Committee. Mice were genotyped by PCR using the following primers: APLP2 forward 5′-ctgctgcaggtgg ctctgca-3′, APLP2 reverse 5′-cag ctctatacaag caaacaag-3′; APP forward 5′-ctgctgcaggtgg ctctgca-3′, APP reverse 5′-cag ctctatacaagcaaa caag-3′, and PGKNeo reverse 5′-cattgct-cagcggt gctgt-3′. The expected fragments are APLP2 wild type (WT), 400 bp; $APLP2^{-/-}$, 350 bp; APP WT, 250 bp, and $APP^{-/-}$, 470 bp.

Lymphocyte isolation

Lymphocytes were isolated from thymus and LNs. Single-cell suspensions were generated using 100 μm nylon mesh (PGC Scientifics, Garner, NC, USA). To enrich for mature $HSA^{lo/neg}$ thymocytes, single-cell suspensions of total thymocytes were incubated at 37° for 30 min with an anti-HSA (J11d) culture supernatant, a 1:10 dilution of Cedar Lane Low-Tox-M Rabbit Complement (Accurate Chemical & Scientific Corp., Westbury, NY, USA) and DNAse (Calbiochem, San Diego, CA, USA). After complement-mediated lysis, the remaining cells were washed twice with media and passed over a Ficoll gradient to remove dead cells and debris.

Antibodies

Antibodies used for immunofluorescence analysis were purchased from BD PharMingen (San Diego, CA, USA): anti-CD3ε–CyC or –APC (145-2C11); anti-CD4–CyC (RM4-5); anti-CD8–CyC or –APC (53-6.7), anti-CD19–PE (1D3); anti-CD25–FITC (7D4); anti-CD44–APC, or –biotin (IM7); anti-CD45–FITC (30-F11); anti-CD45R–CyC (RA3-6B2); anti-TCRγδ–PE (GL3); anti-TCRβ–FITC or –PE (H57.597); anti-IgD–FITC (11-26c.2a). A cocktail used to determine lineage negative (Lin−) thymocytes contained antibodies to CD4, CD8, CD19, TCRαβ and TCRγδ, all labeled with PE. Anti-CD4–PE (GK1.5) was purchased from Becton Dickinson Collaborative Technologies (Bedford, MA, USA). Anti-IgM–biotin was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Annexin V–FITC, propidium iodide and anti-CD8α–FITC or –PE (CT-CD8) were purchased from Caltag Laboratories (South San Francisco, CA, USA). Biotin-conjugated antibodies were visualized with streptavidin–Cy5 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). For microscopy, a purified anti-CD4 (BD PharMingen) was labeled with Alexa488 dye using a labeling kit (Molecular Probes/Invitrogen, Carlsbad, CA, USA).

Flow cytometry

A single-cell suspension of lymphocytes in HBSS, containing 0.2% BSA and 0.1% NaN_3, was incubated with properly diluted antibody at 4°C for 20 min. After staining, cells were washed twice with the same buffer and relative fluorescence was measured by flow cytometry using a FACS Calibur and Cell Quest Software (Becton Dickinson, San Jose, CA, USA).

In vitro survival assay

Thymocytes were plated at 1–1.5 × $10^6 ml^{-1}$ in RPMI supplemented with 10% FCS and cultured at 37°C. The number of live cells remaining was calculated daily. Live cells were identified based upon their ability to exclude Trypan Blue.

Protein lysates and western blotting

Single-cell suspensions or whole lymphoid tissues were lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM Na_3VO_4 and protease inhibitors (Roche complete tablets; Roche Applied Science, Indianapolis, IN, USA). Samples were incubated on ice for >30 min, inverting every 10 min to mix, and spun for 5 min at 12 000 r.p.m. at 4°C. Supernatants were stored at −80°C. For single-cell suspensions, thymus or LNs were crushed and filtered through 100 μm nylon mesh to remove connective tissue, resulting in a single-cell suspension containing >95% $CD45^+$ hematopoietic cells. For lysates of whole organs, intact thymus, LN or brain were homogenized in

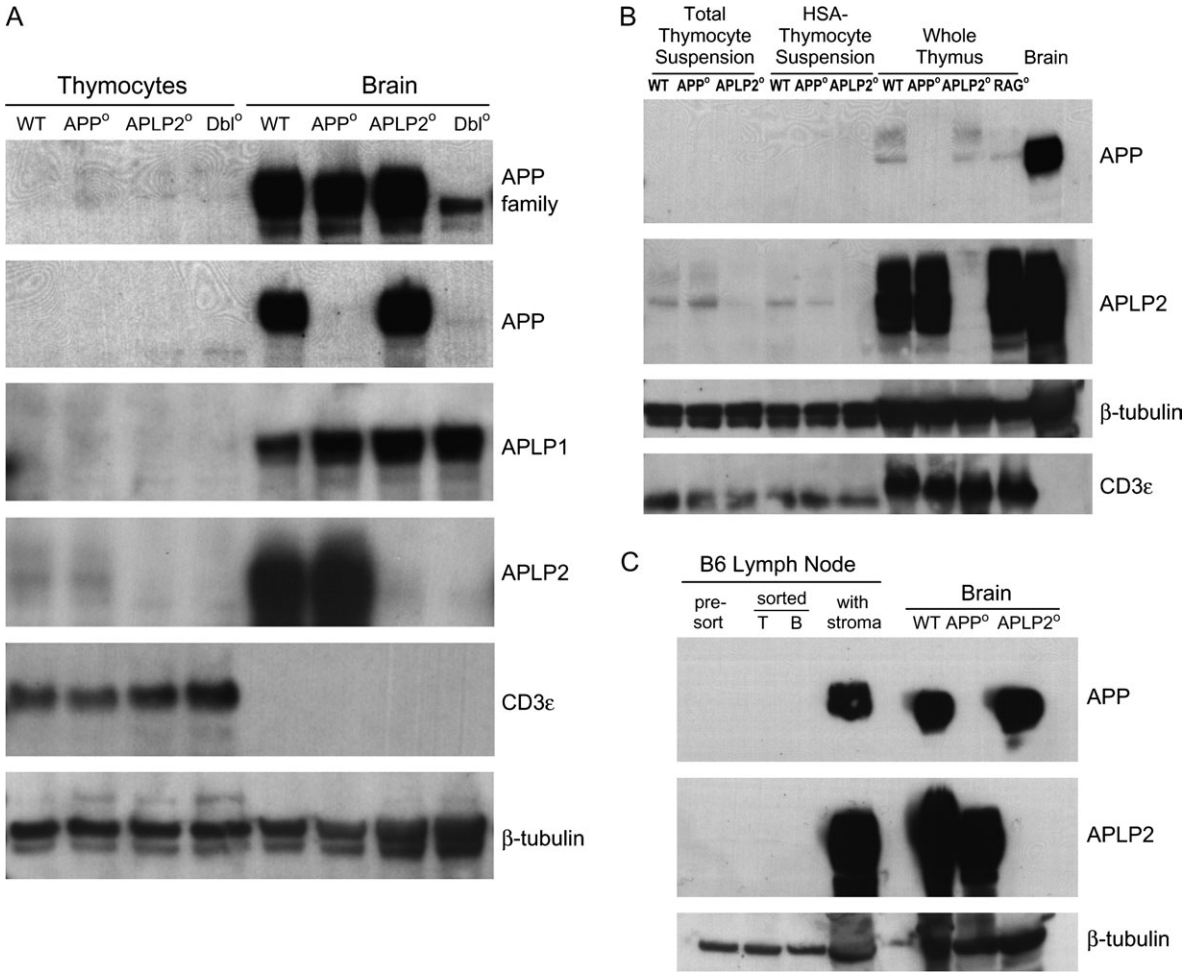

Fig. 1. APP and APLP2 are expressed by non-lymphoid cells in thymus and LN. To assess protein expression by western blot analysis, WT mice and mice deficient for APP (APP^{o}), APLP2 (APLP2^{o}) or APP/APLP2 double-deficient (Dbl^{o}) mice were used to obtain (A) cell suspensions of thymocytes and intact brain, (B) cell suspensions of total or HSA$^{neg/lo}$ thymocytes, intact thymus (which include stromal cells) or brain or (C) cell suspensions of B6 LNs (pre-sort) electronically sorted for T (TCR$\alpha\beta^{+}$) or B (CD19^{+}) cells, intact B6 LNs (which include stromal cells) or intact brain. Blots were hybridized with antisera against APP/APLP, APP, APLP1 or APLP2, as designated, or with anti-CD3ϵ and anti-β-tubulin for loading controls.

lysis buffer using a mortar and pestle, generating lysates composed of both connective tissue and hematopoietic cells.

Lysates were subjected to PAGE on Novex 8–12% NuPAGE gels with MES buffer (Invitrogen), and transferred to Hybond-P PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% non-fat dried milk in PBS/0.01% Tween 20, blots were hybridized overnight at 4°C with polyclonal antisera in PBS/0.01% Tween 20 with 2.5% non-fat dried milk. Anti-APLP1 (CT11) and anti-APLP2 (D2-II) were purchased from Calbiochem. Anti-CD3ε (M-20) and anti-β-tubulin (H-235) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-APP/APLP (B10.4) and anti-APP (B62.1) were developed as previously described for B10.4 (49). Secondary antibodies were HRP-conjugated anti-rabbit IgG (Roche) or anti-goat Ig (Jackson ImmunoResearch Laboratories, Inc.). Blots were developed with ECL western blotting analysis system (Amersham Pharmacia Biotech) or SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA).

Histology

Thymuses were fixed with a 4% PFA solution in PBS, then washed with PBS and treated with 20% sucrose solution in PBS. After washing, thymuses were immersed in O.C.T. compound (Tissue Tek) for 1 h and snap-frozen using a mixture of 2-methylbutane (Sigma–Aldrich, St Louis, MO, USA) and dry ice. Ten micrometer sections were obtained using a cryostat. The sections were fixed with 4% PFA for 15 min, washed with PBS, incubated with 50 mM NH_4Cl for 10 min, washed with PBS and blocked with a solution containing 5% normal goat serum, 3% BSA and 0.2% Triton X-100. Sections were incubated overnight with anti-APLP2 (D2-II) in PBS containing 5% normal goat serum and 3% BSA and then washed with PBS. Sections were stained 4 h with anti-CD4–Alexa-488, anti-CD8α–APC, and anti-rabbit IgG–Alexa-568 (Cat. no. A-11036, Molecular Probes) that was used to visualize anti-mouse

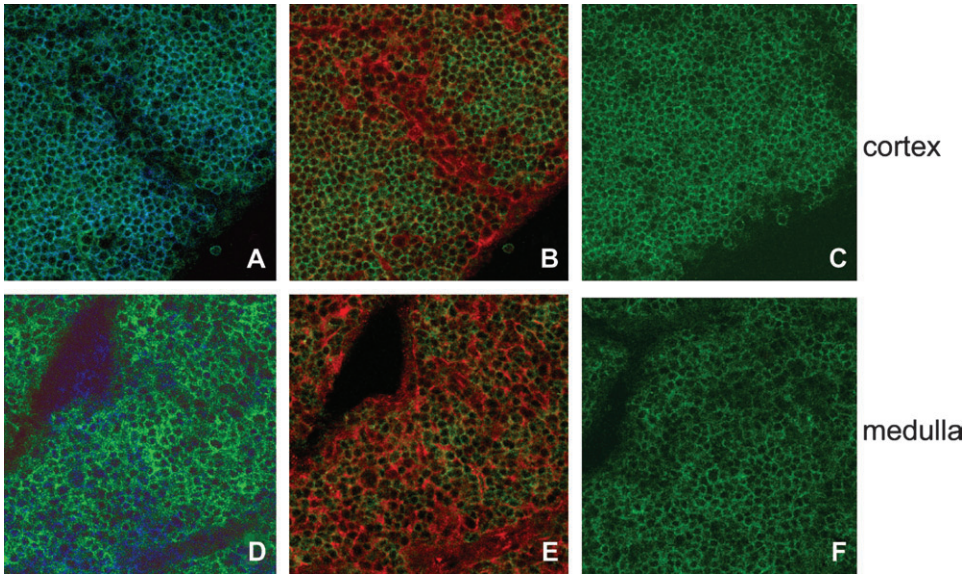

Fig. 2. APLP2 expression is localized to thymic stroma. Thymuses of WT B6 mice were fixed, sectioned and stained for co-expression of CD4 (green), CD8 (blue) and APLP2 (red). (A) The thymus cortex was identified by the presence of densely packed thymocytes co-expressing both CD4 and CD8. (B) Same image as in (A), showing immunofluorescence from anti-CD4 and anti-APLP2. (D) The thymus medulla was identified by the presence of less densely packed thymocytes expressing either CD4 or CD8. (E) Same image as in (D), showing immunofluorescence from anti-CD4 and anti-APLP2. (C and F) Serial sections of A/B or D/E, control showing immunofluorescence of anti-CD4 and secondary Alexa-586-labeled anti-rabbit-IgG without the primary anti-APLP2 antibody. Images were collected at ×63 magnification on a Leica LSR confocal microscope.

APLP2, and washed with PBS. Sections were mounted with ProLong Antifade (Molecular Probes), and immunofluorescence was analysed using a Leica LSR confocal microscope.

Statistical analyses

All two-tailed Student's *t*-tests were conducted using InStat Instant Biostatistics (GraphPad Software; San Diego, CA, USA). Error bars represent SEM.

Results

APP and APLP2 proteins are expressed in thymus and LNs

APP and APLP2 mRNA are expressed in thymus (20,22–26). To determine which thymic cell type(s) express APP family proteins, APP, APLP1 and APLP2 proteins were analysed by western blotting. Lysates made from thymus cell suspensions or intact brain of adult $APP^{+/-}$, $APP^{-/-}$, $APLP2^{-/-}$ or $APP^{-/-}APLP2^{-/-}$ mice were blotted with polyclonal rabbit sera raised against APP, APLP2 or APLP1. As expected, all three homologues were highly expressed in brain (Fig. 1). Consistent with previous reports that APLP1 expression is restricted to the nervous system, APLP1 was never observed in thymus lysates. Surprisingly, APP was not detected in thymocyte lysates under any conditions tested. In most analyses, APLP2 was also undetectable. However, in cases where a super sensitive extended duration substrate and long exposure times were used (see Methods), a faint APLP2 band was visualized in lysates made from large numbers of thymocytes. This result was consistent with either extremely low expression by many thymocytes or high expression by a minor fraction of cells, for example, by mature $CD4^+CD8^-$ or $CD4^-$ $CD8^+$ single-positive (SP) thymocytes which account for ~10% of total thymocytes.

Since thymocytes gradually downregulate expression of HSA (CD24) as they mature (50), we used this marker to isolate mature thymocytes for assay of APLP2 expression. Thymocytes of $APP^{+/-}$, $APP^{-/-}$ or $APLP2^{-/-}$ mice were depleted of immature HSA^{hi} cells using anti-HSA antibody (J11d) and rabbit complement. Prior to enrichment, thymocyte populations contained ~10% CD4 or CD8 SP thymocytes. After complement-mediated lysis, thymocytes were >90% mature SP (data not shown). As shown in Fig. 1(B), APLP2 expression was not enriched in lysates of SP thymocytes, making it unlikely that the faint band was due to a late onset of APLP2 expression in the most mature thymocytes.

Very few thymic stromal cells are recovered in thymocyte cell suspensions, whereas virtually all thymocytes and stromal cells are recovered if the intact thymus organ is used for preparing protein lysates. Thus, it was conceivable that the low level of APLP2 detected in lysates of thymocyte suspensions derived only from stromal cells. Indeed, lysates blotted for APP or APLP2 revealed a strong band for APLP2 using whole thymus of B6, $APP^{-/-}$ or recombination-activating $gene^{-/-}$($RAG^{-/-}$) mice and only a faint band for APP using whole thymus of B6, $APLP2^{-/-}$ or $RAG^{-/-}$ mice (Fig. 1B). These results suggested that thymic stromal cells express high levels of APLP2 and low levels of APP proteins, whereas thymocytes express neither family member.

To confirm the expression of APLP2 by thymus stromal cells, histological sections of WT B6 thymuses were stained with the same anti-APLP2-specific antisera used for western blotting in Fig. 1 and imaged using confocal microscopy (Fig. 2). Thymus cortex was identified by the presence of

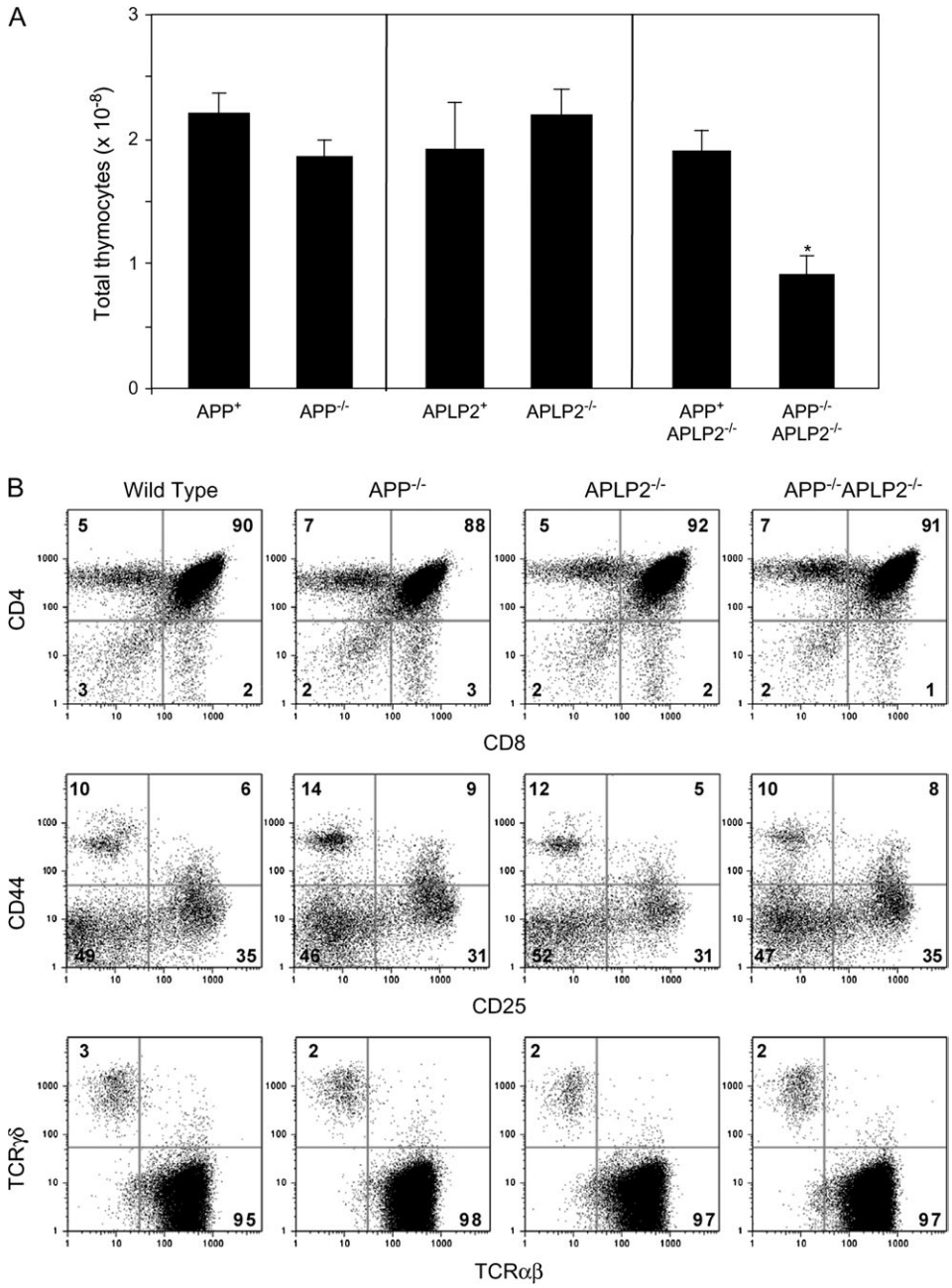

Fig. 3. APP and/or APLP2 are not required for normal thymocyte development from adult precursors. (A) Total thymus cellularity was calculated for 4- to 12-week old WT (APP$^+$ or APLP2$^+$) and APP$^{-/-}$ and/or APLP2$^{-/-}$ mice, as indicated. Asterisk denotes significance by *t*-test, $P = 0.02$. (B) Cells were gated for CD45$^+$ and analysed for expression of CD4 and CD8α (top), gated to exclude cells expressing CD4, CD8α, CD19, TCRαβ and TCRγδ (Lin−) and analysed for expression of CD44 and CD25 (middle) or gated for CD3$^+$ and analysed for expression of TCRαβ and TCRγδ (bottom). The data are representative of at least five mice analysed for each genotype.

densely packed thymocytes co-expressing both CD4 and CD8 (Fig. 2A). The thymus medulla was identified by the presence of less densely packed thymocytes expressing either CD4 or CD8 (Fig. 2D). By these analyses, APLP2 was expressed throughout the thymus by both the cortical and medullary stromal cells (Fig. 2B,E). Consistent with the western blotting data, no co-localization of CD4 and APLP2 expression was observed, confirming that APLP2 expression was restricted to non-T lineage cells.

To determine the expression pattern of APP and APLP2 in secondary lymphoid tissue, lysates made from B6 LNs were blotted for these proteins (Fig. 1C). The expression pattern of APP and APLP2 in LNs was similar to that of thymus. Both proteins were undetectable in lysates prepared from LN cell suspensions, or highly purified populations of T or B cells. However, these proteins were present in lysates made from intact, whole LNs, indicating that APP and APLP2 were derived from stromal cells. Therefore, the findings are

Table 1. Analysis of absolute cell number and relative proportion of thymocyte subsets in adult mice deficient in amyloid precursor family proteins[a]

Subset	WT	$APP^{-/-}$	$APLP2^{-/-}$	$APP^{-/-}APLP2^{-/-}$
Number of thymocytes ($\times 10^{-7}$)				
Total	22.97 ± 1.10	20.24 ± 1.44	22.12 ± 2.09	9.14 ± 1.58*
DN	0.51 ± 0.02	0.45 ± 0.03	0.47 ± 0.04	0.17 ± 0.04*
DP	20.21 ± 1.00	18.24 ± 1.27	20.29 ± 2.10	7.98 ± 1.32*
CD4 SP	1.64 ± 0.09	1.51 ± 0.10	1.60 ± 0.17	0.78 ± 0.20*
CD8 SP	0.60 ± 0.04	0.50 ± 0.03	0.54 ± 0.06	0.21 ± 0.05*
Number of thymocytes ($\times 10^{-6}$)				
γδ	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.05	0.11 ± 0.00*
Total Lin^-	5.47 ± 0.33	5.44 ± 0.95	4.12 ± 0.73	2.37 ± 0.67*
DN1	0.48 ± 0.11	0.36 ± 0.15	0.33 ± 0.09	0.13 ± 0.04*
DN2	0.45 ± 0.07	0.38 ± 0.08	0.24 ± 0.04	0.15 ± 0.06*
DN3	2.38 ± 0.41	2.16 ± 0.75	1.61 ± 0.55	0.98 ± 0.34*
DN4	2.15 ± 0.10	2.45 ± 0.30	1.91 ± 0.22	0.11 ± 0.31*
Percent of $CD45^+$ thymocytes				
γδ	1.49 ± 0.07	1.36 ± 0.07	1.51 ± 0.07	1.91 ± 0.94
DN	2.25 ± 0.07	2.21 ± 0.12	2.13 ± 0.12	1.75 ± 0.17
DP	87.96 ± 0.26	87.99 ± 0.28	88.51 ± 0.84	88.44 ± 1.89
CD4 SP	7.09 ± 0.13	7.33 ± 0.14	7.00 ± 0.38	7.61 ± 1.74
CD8 SP	2.61 ± 0.09	2.48 ± 0.10	2.36 ± 0.15	2.16 ± 0.27
Percent of Lin^- thymocytes				
DN1	8.97 ± 2.05	6.21 ± 1.85	7.68 ± 1.38	5.71 ± 1.18
DN2	8.13 ± 1.06	8.04 ± 2.63	6.17 ± 0.70	6.79 ± 3.02
DN3	42.47 ± 5.55	36.39 ± 5.55	35.07 ± 6.26	39.05 ± 3.85
DN4	40.03 ± 6.42	47.38 ± 6.42	50.09 ± 5.83	48.48 ± 6.55

[a]Values represent arithmetic means ± SEM; $*P \leqslant 0.03$; for WT $n = 32$, $APP^{-/-}$ $n = 26$, $APLP2^{-/-}$ $n = 18$, and $APP^{-/-}APLP2^{-/-}$ $n = 5$, with the exception of Lin^- (lineage negative, as described in Methods) $n = 5$ for each genotype.

consistent with previous reports of APP and APLP2 mRNA expression in lymphoid organs, but results here reveal that this expression can be attributed to non-lymphoid stromal cells.

Neither APP nor APLP2 are required for lymphocyte development from adult precursors

While we were unable to detect APP family protein expression in thymocytes, the possibility remained that these proteins performed some direct or indirect function in T cell development. Of relevance, thymic stromal cells are essential for promoting T cell differentiation; however, the signals they provide to developing thymocytes have not been fully defined. Therefore, the significance of high APLP2 expression by thymic stromal cells (Figs 1 and 2) was of particular interest. To pursue this issue, we investigated thymocyte development in mice deficient for APP family proteins. Mice with germline mutations in either APP or APLP2 have been described (47,48,51–53) and are grossly normal. In contrast, doubly deficient $APP^{-/-}APLP2^{-/-}$ mice have >80% neonatal mortality rate, and those mice that survive to adulthood are sterile and have multiple neurological and behavioral abnormalities (48,52). While the central nervous system of APP- and/or APLP2-deficient mice have been extensively studied (47,48,51–53), lymphocytes of these mice have not been characterized.

The more severe phenotype of double-deficient mice for APP and APLP2 demonstrates that these proteins are at least somewhat functionally redundant. To characterize thymocyte development in the absence of APP and APLP2, $APP^{+/-}APLP2^{-/-}$ mice were intercrossed to generate $APP^{-/-}APLP2^{-/-}$ animals. $APP^{-/-}APLP2^{-/-}$ pups were born with less than the predicted 25% Mendelian frequency (20:54:15); moreover, very few survived to weaning at 3 weeks of age. Of 260 mice from 50 litters genotyped, only 5 $APP^{-/-}APLP2^{-/-}$ mice survived to adulthood (94:161:5). Compared with control littermates, the mice that survived were smaller in overall body size and weight, failed to breed, appeared agitated and exhibited neurological problems such as forelimb weakness, spinning and difficulty righting.

Thymus cellularity of 4- to 12-week old mice, deficient for either APP or APLP2, was not significantly different from that of their WT littermates, but absolute numbers were decreased in the $APP^{-/-}APLP2^{-/-}$ mice (Fig. 3A and Table 1). However, when mice are stressed by poor health, it is common for thymus cellularity to be reduced due to an increased production of endogenous steroids. Although the double-deficient mice had a global reduction in thymocyte numbers, there was no indication of a developmental block by subset or linage analysis This interpretation was supported by the fact that the relative proportion of all subsets/lineages were comparable in APP and/or APLP2 mice (Table 1). Mean percentages of thymocytes at each distinct stage of development, double negative (DN, CD4−CD8−), double positive (DP, CD4+CD8+) and CD4 and CD8 SP, were all normal (Fig. 3B, top panels), and in the absence of APP and/or APLP2, there was no evidence of an accumulation of DN thymocytes. The relative contribution of each DN subset, DN1 (CD44+CD25−), DN2 (CD44+CD25+), DN3 (CD44−CD25+) and DN4 (CD44−CD25−) was unaffected (Fig. 3B, middle panels), and in $APP^{-/-}APLP2^{-/-}$ mice, the absolute number of all four subsets was similarly reduced (Table 1). WT and mutant mice

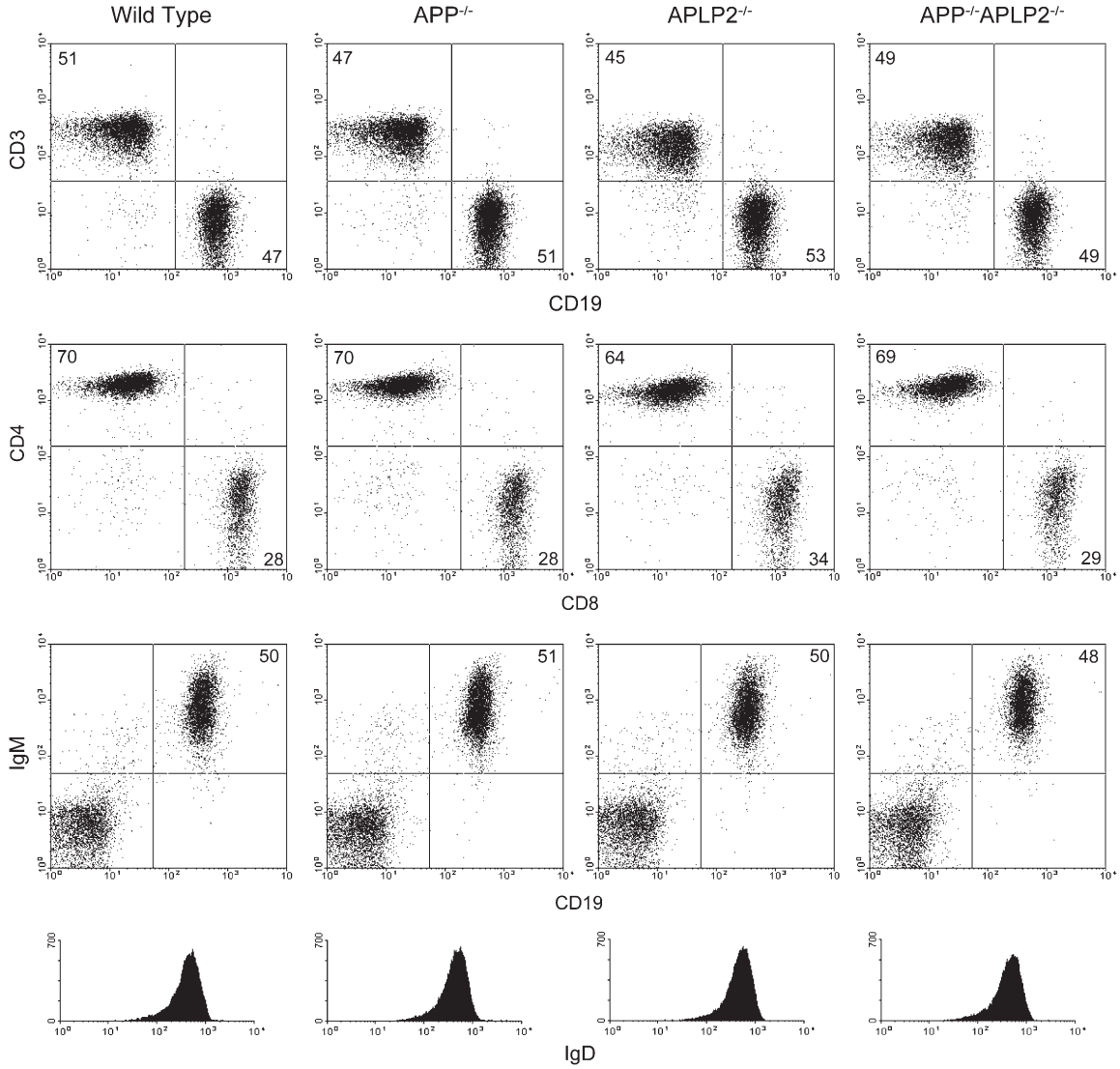

Fig. 4. Mature T and B cells appear normally in LNs in the absence of APP and/or APLP2. LN cells were gated for CD45$^+$ and analysed for expression of CD3 and CD19 (first row), gated for TCR$\alpha\beta^+$ cells and analysed for expression of CD4 and CD8α (second row) or analysed for expression of CD19 and IgM (third row). Cells co-expressing CD19 and IgM were analysed for expression of IgD (fourth row). The data are representative of at least three mice for each genotype.

generated TCR^{hi} mature thymocytes with similar frequency and the percentages of $\alpha\beta$ or $\gamma\delta$ thymocytes within the TCR^{hi} population were equivalent (Fig. 3B, bottom panels). Thus, T cell development proceeded normally with no evidence of a block at any stage, indicating that neither APP nor APLP2 are required for thymocyte maturation.

In the periphery, LNs from APP$^{-/-}$, APLP2$^{-/-}$ and APP$^{-/-}$ APLP2$^{-/-}$ mice all contained mature T and B lymphocytes at the expected frequency (Fig. 4). The CD3$^+$ T cell populations were normal, composed primarily of $\alpha\beta$ T cells with <2% of cells expressing TCR$\gamma\delta$ (data not shown). Within the $\alpha\beta$ T lineage, CD8 and CD4 T cells were present at normal frequencies, and LN B cells had the expected naive IgM$^+$IgD$^+$ phenotype. Taken together, these results indicate no role for APP family members in the development of lymphocytes originating from adult precursors.

Neither APP nor APLP2 are required for thymocyte development from fetal precursors

Several groups have utilized *in vitro* fetal thymus organ culture systems to assess the effects of γ-secretase inhibitors on thymocyte development (1,2). It is noteworthy in this regard that thymocytes developing in embryonic day 14–15.5, fetal thymus are derived from fetal liver precursors, whereas thymocytes of adults are derived from bone marrow. Thus, while APP and/or APLP2 were not needed for adult thymocyte development, it was possible that these proteins could be required for fetal development.

To assess T cell development from fetal liver precursors, thymocytes isolated from newborn APP- APLP2-deficient pups were analysed. APP$^{-/-}$APLP2$^{-/-}$ pups were born with less than the predicted Mendelian frequency, and APP$^{-/-}$

APLP2$^{-/-}$ neonates often had less milk in their stomach consistent with neurological deficits impairing their ability to suckle. In contrast to adult mice, only subtle differences in thymus cellularity were observed in the newborns ($P > 0.05$) (Fig. 5A). Phenotypic analyses of newborns revealed that all CD4/CD8 thymocyte subsets, as well as mature TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ thymocytes, were normal (Fig. 5B), similar to adults. Therefore, APP and APLP2 are dispensable for T cell maturation from fetal or adult precursors.

APP or APLP2 deficiency does not confer a survival advantage to thymocytes

APP is a positive regulator of programmed cell death. Forced expression of the intracellular domain of APP lowers the threshold for Fas-associated death in Jurkat T cells and induces apoptosis in HeLa and MCF7 cells (42). To determine sensitivity to programmed cell death, thymocytes of APP- APLP2-deficient mice were analysed directly *ex vivo* or at various times after culture in media in the absence of stromal cells or exogenous cytokines. The frequency of thymocytes undergoing apoptosis directly *ex vivo* or throughout the 4-day culture period was similar as determined by Annexin V and propidium iodide staining (Fig. 6A, and data not shown). Based on the ability to exclude Trypan Blue, absolute numbers of live cells remaining after culture were also assayed. At no time point was the mean number of live thymocytes recovered significantly different ($P > 0.26$) (Fig. 6B). Thus, in spite of a putative pro-apoptotic function for these proteins, the absence of APP and/or APLP2 did not confer a survival advantage to thymocytes.

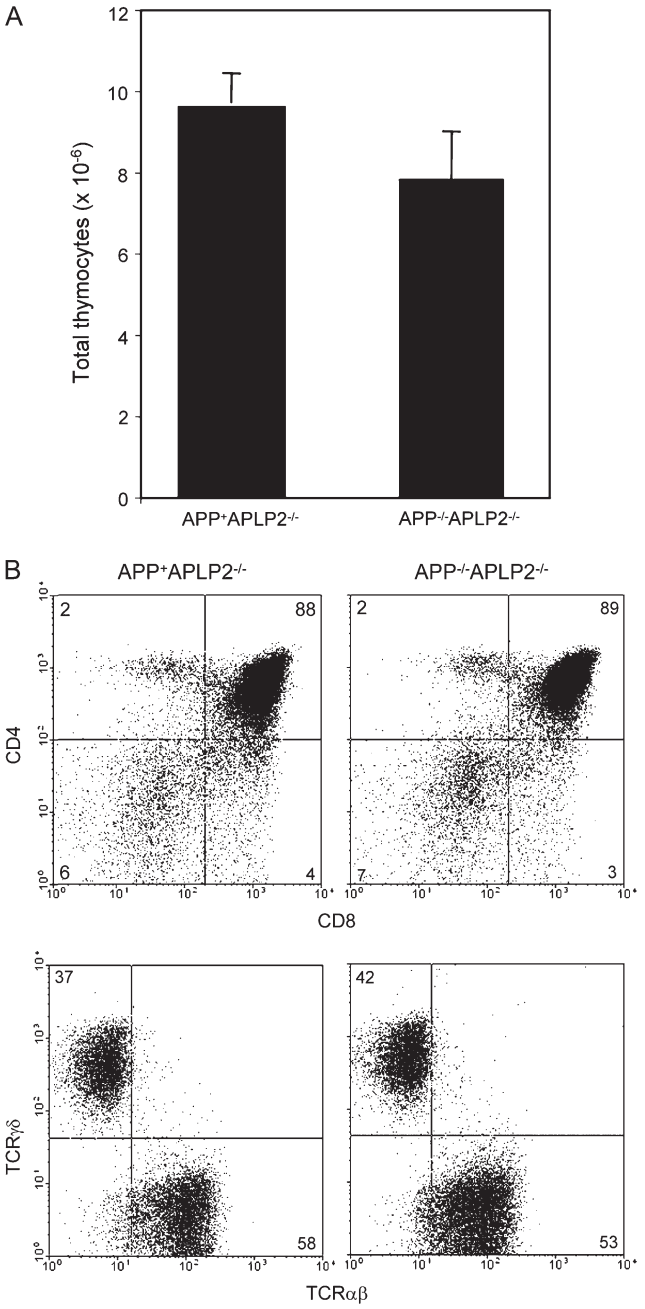

Fig. 5. APP and/or APLP2 are not required for normal thymocyte development in neonates. (A) Total thymus cellularity was calculated for newborn mice (*P*-values revealed no significant differences). (B) Thymocytes were gated for CD45$^+$ and analysed for expression of CD4 and CD8 (top) or for CD3$^+$ and analysed for expression of TCR$\alpha\beta$ and TCR$\gamma\delta$ (bottom). Because the APLP2$^{-/-}$ were equivalent to WT mice by every criteria tested, these mice were used as littermate controls since they were generated in the same cross.

Discussion

Studies using fetal thymus organ cultures demonstrate that γ-secretase inhibitors interrupt thymocyte maturation at several points (1,2). First, the transition of DN to DP thymocytes is impaired, and in late T cell development, the maturation of CD8 SP thymocytes is impeded. The end result is reduced thymus cellularity and an accumulation of DN thymocytes. These studies indicate that the activity of one or more of the γ-secretase substrates is important for T cell development. Moreover, they suggest that γ-secretase inhibitors, developed for treatment of Alzheimer's disease, could have immunosuppressive effects. APP family proteins were potentially the substrates affected because of the functions ascribed to these proteins as well as their reported expression in thymus. Thus, identifying γ-secretase substrates that operate in thymocyte development is important for elucidating the signals that drive differentiation and control cell fate. More significantly, identifying the target activities of γ-secretase inhibitors could aid in the development of more specific drugs for treating Alzheimer's disease with fewer immunosuppressive side effects.

From our results, it can be inferred that the inhibitors used in fetal thymus organ culture must have interfered with the processing of γ-secretase substrates other than APP/APLP2. We find that newborn mice deficient for APP and/or APLP2 had normal thymus cellularity and CD4/CD8 subsets. Moreover, adult mutant mice had a normal frequency of mature T and B cells in LNs. Efficient thymocyte development in APP- and/or APLP2-deficient mice, coupled with the absence of detectable APP, APLP1 or APLP2 protein expression by thymocytes or peripheral lymphoid cells, demonstrate that APP family members do not play a critical role in murine lymphocyte development. An

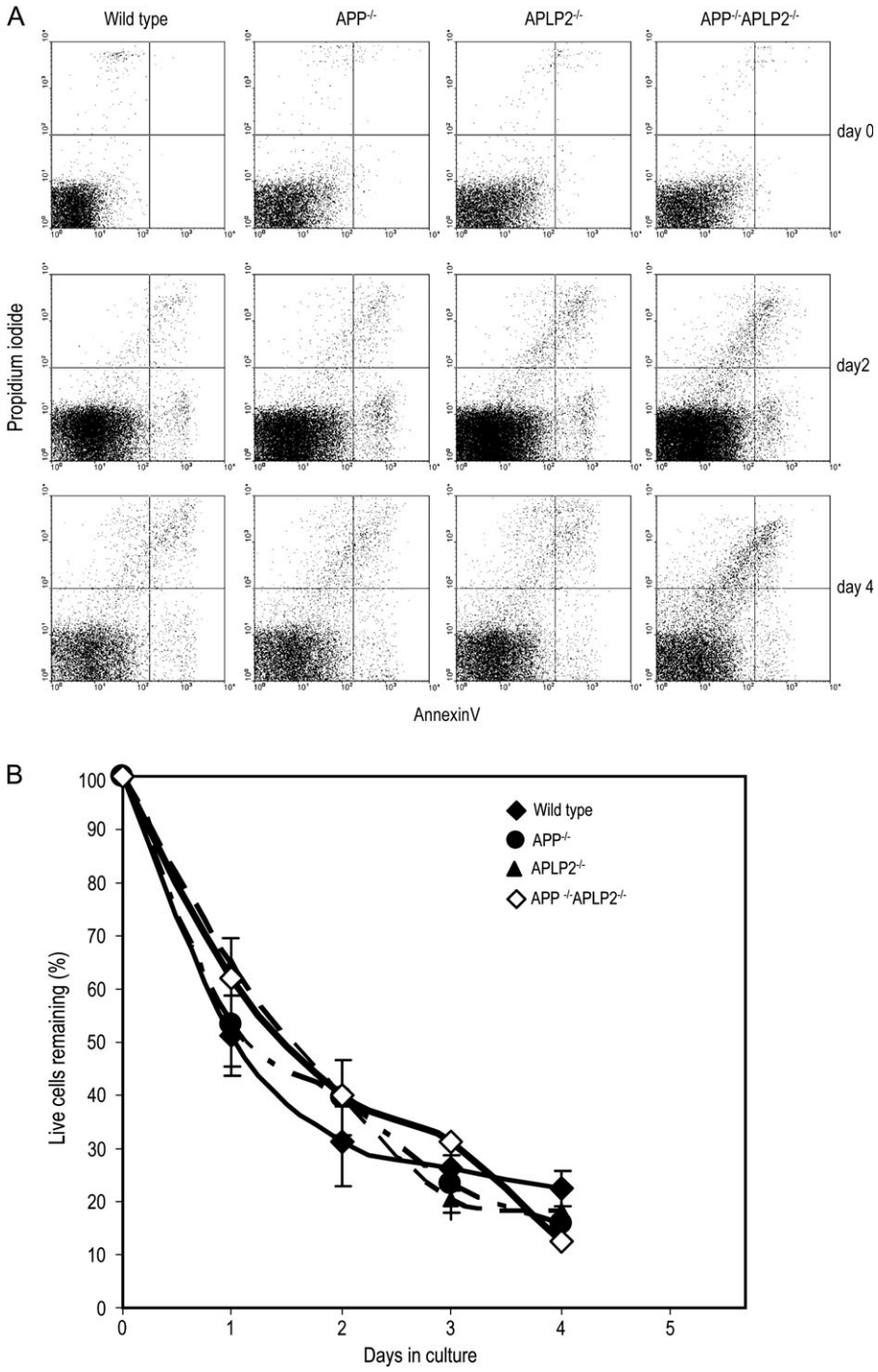

Fig. 6. Absence of APP and/or APLP2 confers no survival advantage on thymocytes. (A) Thymocytes were stained for Annexin V and propidium iodide *ex vivo*, or at various times after culture. (B) The number of live thymocytes harvested daily from cell culture was determined by Trypan Blue exclusion. Data represent the mean percentage of live cells remaining ±SEM. $P > 0.2$ for all time points.

important implication of these results is that pharmacological inhibitors that specifically inhibit cleavage of APP should not adversely affect lymphocyte development.

The first γ-secretase inhibitor described in 1998 was a transition state analog that worked by binding to and blocking the active site of the γ-secretase complex (19). Since then, numerous other γ-secretase inhibitors have been developed that work by a variety of mechanisms. The data presented here suggest that inhibitors that specifically interfere with γ-secretase-mediated APP cleavage are unlikely to have immunosuppressive side effects. Similarly, inhibitors that interfere with the first (BACE-mediated) cleavage of APP (54,55),

rather than the second γ-secretase-mediated cleavage, are unlikely to have immunosuppressive side effects on lymphocyte development or function. Consistent with this notion, BACE-deficient animals have no reported phenotype (56,57).

Our initial experiments using lysates of thymocyte cell suspensions revealed that mouse thymocytes do not express significant levels of APP or APLP2 protein. These findings were surprising in light of previous reports of mRNA expression in thymus (20,22–26). However, the earlier studies examined RNA prepared from intact thymus which recovers most of the stromal cells as well as the thymocytes. This discrepancy was reconciled when we prepared lysates from intact thymus or stained sections of frozen thymus. In assays that efficiently recover thymic stromal cells, it was clear that APLP2 protein was highly expressed and APP protein, at much lower levels. The latter results are consistent with findings that APP and APLP2 transcripts are both expressed in mouse thymus, with APLP2 > APP mRNA (23). Notably, in rats APP is reported to be expressed by astrocytes and microglia (22). The expression of APP in these myeloid cell types is consistent with our finding of APP and APLP2 in non-lymphoid cells of thymus and LN.

Using newly developed antisera specific for each member of the APP family, we were able to show for the first time that APP, APLP1 and APLP2 proteins are not expressed in mouse lymphocytes. Consistent with these data, APP mRNA is not detected in rat spleen (58). Most reports agree that human peripheral blood lymphocytes do not express APP constitutively, but APP can be induced in response to mitogenic stimulation (22,59). Some human T-lymphoma cell lines express APP, while other T cell lines do not. However, several issues must be considered when interpreting these data. Studies conducted prior to the cloning of APLP2 used antisera raised against the C-terminus of APP. Many of those antisera were later found to cross-react on APLP2 (23), making it unclear which protein(s) were detected. Moreover, in light of our present findings that APP and APLP2 are expressed by non-lymphoid cells in thymus and LN, the contribution of non-lymphoid cell in those preparations complicates any conclusions regarding lymphocyte expression. If, however, the dichotomy in APP/APLP2 expression patterns by murine and human lymphocytes is confirmed, then there may be fundamental differences in APP biology between mice and humans that must be taken in to account when developing experimental models for studying Alzheimer's disease in mice.

The significance of APP family protein expression in thymic stromal cells is unclear. Although thymic stromal cells provide essential signals for thymocyte maturation, T cells of APP-/APLP2-deficient mice develop normally. Identifying a role for APP and APLP2 in stromal cells could help in determining the physiological function of APP family members. Of relevance, a punctate staining pattern of APP on the cell surface of neurons in rat brain led to the hypothesis that APP mediates cell–cell or cell–matrix interactions (60). Therefore, if ligands of APP and APLP are discovered, it would be of interest to determine whether they are expressed by thymocytes.

APP and APLP2 mRNA have been found in all tissues examined to date. However, because of a potential role in Alzheimer's disease, most of the work investigating APP associations has been conducted using neuronal cells. As a result, many of the proteins found to associate with the intracellular domain of APP are specifically expressed by neurons. For example, G_0 is a brain-specific G protein (61), and expression of ShcC is restricted to the central nervous system (62). The intracellular domain of APP also associates with the nuclear adaptor protein Fe65 and Tip60 to form a complex that stimulates transcription (29–31). In this report, we have shown that APLP2 is highly expressed by stromal cells in both primary and secondary lymphoid tissues. It remains to be determined what proteins interact with APP/APLP2 in non-neuronal cell types and the function of these associations especially since the specific outcome of APP cleavage has been shown to be cell type dependent (42).

We have demonstrated that APP family proteins are not expressed in T lineage cells. This finding effectively excludes these proteins from the list of γ-secretase substrates that are required for normal T cell development. There are, however, several other γ-secretase substrates to be considered. Expression of substrates, ErbB4, Nectin-1α and LRP, by lymphocytes has not been reported, but immature T cells do express E-cadherin and CD44. Although E-cadherin plays a critical role in the development of a functional thymic environment, it is not required for thymocyte maturation within an established thymus microenvironment (63). Moreover, T and B cells develop and function normally in CD44-deficient mice (64). Recent data suggest that the most likely explanation for γ-secretase-mediated inhibition of thymocyte development is due to interference with Notch signaling. It is well established that Notch is expressed by thymocytes (60,65–68). In addition, Notch signaling is required for commitment of lymphoid precursors to the T cell lineage and for later stages of T cell development, including TCRβ selection of thymocytes and functional differentiation of peripheral CD4 helper cells (7,69–73).

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Abbreviations

APP	amyloid precursor protein
APLP1	amyloid precursor-like protein 1
APLP2	amyloid precursor-like protein 2
DN	double negative ($CD4^-CD8^-$)
DP	double positive ($CD4^+CD8^+$)
LN	lymph node
LRP	lipoprotein receptor-related protein
NIAID	National Institute of Allergy and Infectious Disease
RAG	recombination-activating gene
SP	single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$)
WT	wild type

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