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Article

Immunology

Alzheimer disease (AD) is characterized by the progressive deposition of the 42-residue amyloid  $\beta$  protein ( $A\beta$ ) in brain regions serving memory and cognition. In animal models of AD, immunization with  $A\beta$  results in the clearance of  $A\beta$  deposits from the brain. However, a trial of vaccination with synthetic human  $A\beta$ 1–42 in AD resulted in the development of meningoencephalitis in some patients. We measured cellular immune responses to  $A\beta$  in middle-aged and elderly healthy subjects and in patients with AD. A significantly higher proportion of healthy elderly subjects and patients with AD had strong  $A\beta$ -reactive T cell responses than occurred in middle-aged adults. The immunodominant  $A\beta$  epitopes in humans resided in amino acids 16–33. Epitope mapping enabled the identification of MHC/T cell receptor (TCR) contact residues. The occurrence of intrinsic T cell reactivity to the self-antigen  $A\beta$  in humans has implications for the design of  $A\beta$  vaccines, may itself be linked to AD susceptibility and course, and appears to be associated with the aging process.

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# Increased T cell reactivity to amyloid $\beta$ protein in older humans and patients with Alzheimer disease

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Alzheimer disease (AD) is characterized by the progressive deposition of the 42-residue amyloid  $\beta$  protein ( $A\beta$ ) in brain regions serving memory and cognition. In animal models of AD, immunization with  $A\beta$  results in the clearance of  $A\beta$  deposits from the brain. However, a trial of vaccination with synthetic human  $A\beta$ 1–42 in AD resulted in the development of meningoencephalitis in some patients. We measured cellular immune responses to  $A\beta$  in middle-aged and elderly healthy subjects and in patients with AD. A significantly higher proportion of healthy elderly subjects and patients with AD had strong  $A\beta$ -reactive T cell responses than occurred in middle-aged adults. The immunodominant  $A\beta$  epitopes in humans resided in amino acids 16–33. Epitope mapping enabled the identification of MHC/T cell receptor (TCR) contact residues. The occurrence of intrinsic T cell reactivity to the self-antigen  $A\beta$  in humans has implications for the design of  $A\beta$  vaccines, may itself be linked to AD susceptibility and course, and appears to be associated with the aging process.

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## Introduction

Alzheimer disease (AD) is the most common form of age-related cognitive failure in humans. It is characterized by the progressive accumulation of the amyloid  $\beta$  protein ( $A\beta$ ) in limbic and association cortices, where some of it precipitates to form a range of amorphous and compacted (fibrillar) extracellular plaques (1, 2). These plaques, particularly the more compacted ones, are associated with dystrophic neurites (altered axons and dendrites) (3), activated microglia, and reactive astrocytes. Cleavage of the amyloid precursor protein (APP) by the  $\beta$  and  $\gamma$  secretases releases both the  $A\beta$ 1–40 and  $A\beta$ 1–42 peptides, the latter being far more prone to aggregation and induction of neurotoxicity (4).

Inflammation may play an important role in mediating the neuronal and glial alterations that occur in AD. Evidence to date has suggested that this inflammation arises mainly from within the CNS (5–7). However, a recent study showed increased occurrence of T cells in the brains of patients with AD as compared with subjects with non-AD degenerative dementias and aged-matched controls (8). The inflammatory

changes — including microgliosis, astrogliosis, complement activation, cytokine elevation, and acute phase protein changes — are thought to represent, at least in part, a response to the early accumulation of  $A\beta$ 1–42 in the brain (9–13).

Although AD is thus associated with local innate immune responses, the induction of systemic adaptive immune responses to  $A\beta$  in mouse models of AD has been found to be beneficial for both the neuropathological and behavioral changes that these mice develop (14–20). However, a human clinical trial in which an  $A\beta$ 1–42 synthetic peptide was administered parenterally with the adjuvant QS21 to patients with AD was discontinued when approximately 5% of 300 treated patients developed what appeared to be a self-limited aseptic meningoencephalitis (21). The cause of this reaction is unknown, but one of the major hypotheses is that an immune reaction to  $A\beta$  was responsible (21–24).

Self-reactive T cells of low-to-moderate binding affinity are not necessarily deleted during negative selection in the thymus (25, 26), and some autoreactive T cells are positively selected and maintain the normal immune repertoire (27). Although such autoreactive T cells can mediate autoimmune diseases, they may also play a beneficial physiological role in immune regulation and maintenance of normal tissues (28–33). Furthermore, although the CNS has been described as immunologically privileged, it is now known that activated T cells can routinely penetrate the CNS (34–36).

Although  $A\beta$ -reactive B cells were previously observed in patients with AD (37), the presence of T cell reactivity to  $A\beta$  has not been previously described in patients with AD or shown to be related to the

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**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Nonstandard abbreviations used:** Alzheimer disease (AD); amyloid  $\beta$  protein ( $A\beta$ ); T cell receptor (TCR); amyloid precursor protein (APP); glatiramer acetate (GA); Mini Mental State Exam (MMSE); stimulation index (SI).

aging process. Because A $\beta$  antigen is progressively deposited in the CNS with age and in AD, we postulated that T cell reactivity to A $\beta$  could either decrease or increase with aging and in patients with AD, depending on whether the peptide was tolerogenic or immunogenic. Furthermore, the nature and magnitude of T cell reactivity to A $\beta$  in humans could have either beneficial or injurious effects for the host and may have important implications for A $\beta$  vaccination strategies in AD. We thus initiated a detailed investigation of T cell responses to A $\beta$  both in healthy young and elderly subjects and in patients with AD.

## Methods

**Antigens.** A $\beta$ 1–40 and A $\beta$ 1–42 synthetic peptides were from Biosource International (Camarillo, California, USA). We examined these peptides for fibril formation in 10 mM sodium phosphate (pH 7.4), using quasielastic light-scattering spectroscopy (38). Fibril formation was measured in three equivalent samples of 10  $\mu$ g/ml A $\beta$  for 5 consecutive days. Slight increase in particle size was detected in only one sample after a 1-day incubation, without a significant change up to day 5. Nested A $\beta$  peptides (A $\beta$ 1–15, A $\beta$ 4–18, A $\beta$ 7–21, A $\beta$ 10–24, A $\beta$ 13–27, A $\beta$ 16–30, A $\beta$ 19–33, A $\beta$ 22–36, A $\beta$ 25–39, and A $\beta$ 28–42) and alanine/glycine-substituted peptides were synthesized at Biosource International (Hopkinton, Massachusetts, USA). A $\beta$  peptides used for T cell assays were dissolved in DMSO (2 mg/ml). Glatiramer acetate (GA) was obtained at the Brigham and Women's Hospital pharmacy.

**Human subjects.** Subjects with AD (ages 65–90) were recruited from the Memory Disorders Unit at Brigham and Women's Hospital under an International Review Board–approved human studies protocol. The ages and educational background of the patients with AD were equivalent to those of the healthy older subjects, who were often the spouses of the patients. The diagnosis of AD was based on National Institute of Neurological Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINDS-ADRDA) criteria (39) and included use of the Mini Mental State Exam (MMSE) (40). Patients with mild-to-moderate AD having MMSE scores between 10 and 24 were selected for this study. Patients with severe AD (MMSE of less than 10) or whose history included a severe head injury, alcoholism, major psychiatric illness, epilepsy, or learning disability were excluded from the study. Overall, 27 adults, 35 older healthy individuals, and 42 patients with AD were evaluated in the study ( $n = 104$  subjects).

**Split-well culture system for A $\beta$  reactivity.** PBMCs were isolated from freshly drawn heparinized whole blood by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, according to the manufacturer's protocol. Cells were cultured in 30 wells of 96-well round-bottom plates at  $2 \times 10^5$  cells per well in RPMI medium (containing 2.5% nonautologous serum, 4 mM L-glutamine, 10 mM HEPES,

1 mM sodium pyruvate, and 10 mM nonessential amino acids) in the presence of pure human A $\beta$  peptide. At day 5, 10 U/ml of recombinant human IL-2 was added by replacing half of the medium with IL-2-containing medium. On day 7, medium was replaced with IL-2-free medium. On day 10, half the cells from each well were restimulated with irradiated autologous PBMCs in the presence and absence of 10  $\mu$ g/ml A $\beta$  for 48 hours and then pulsed with [ $^3$ H]thymidine for 12 hours. Cells were then harvested and radioactivity was measured (in cpm). The stimulation index (SI) (cpm in the presence of A $\beta$  divided by cpm in the absence of A $\beta$ ) was determined. Positive wells are those with an SI of at least 2.5 and a  $\Delta$ cpm of at least 2,000.

**Primary stimulation with  $\alpha$ CD3/CD28 or GA.** For  $\alpha$ CD3/CD28 stimulation, frozen PBMCs ( $2 \times 10^5$  cells per well) were cultured in five wells (U-bottom 96-well plate) in the presence of 1  $\mu$ g/ml soluble  $\alpha$ CD3 plus  $\alpha$ CD28 and in an additional five wells with cell culture medium alone and pulsed with [ $^3$ H]thymidine on day 2 after stimulation, and incorporation was measured 12 hours later. For GA stimulation, fresh PBMCs were cultured in five wells with 50  $\mu$ g/ml GA or cell culture medium alone and pulsed with [ $^3$ H]thymidine on day 6, and incorporation was measured 12 hours later.

**T cell lines, fine specificity, cytokine production, proliferation, and flow cytometry.** Positive wells obtained in the split-well assay were restimulated 14 days after their primary stimulation in the presence of autologous PBMCs ( $2 \times 10^5$  cells per well) and A $\beta$ 1–42. T cell lines were then supplemented with 10 U/ml IL-2 after 48 hours and every 2 days thereafter until they were completely resting. T cell proliferation was measured 72 hours after stimulation after 12 hours of incubation with [ $^3$ H]thymidine. Supernatants were collected 48 hours after T cell stimulation, and antigen-induced cytokine production was measured by sandwich ELISA. Recommended pairs of antibodies (coating and detecting) for IL-10 (R&D Systems Inc., Minneapolis, Minnesota, USA), INF- $\gamma$  (Endogen, Woburn, Massachusetts, USA), IL-13 (PharMingen, San Diego, California, USA), and TGF- $\beta$  (Promega Corp., Madison, Wisconsin, USA) were used according to the manufacturer's instructions. Surface and intracellular staining was performed on antibody-reactive T cells after stimulation. All antibodies were purchased from BD Biosciences (San Diego, California, USA), and staining was performed according to the manufacturer's instructions.

## Results

**Endogenous T cell responses to A $\beta$  in humans.** T cell responses to A $\beta$  were measured using a split-well assay that has been successful in demonstrating reactivity to self-antigens (41). Table 1 shows an example of the results of a split-well assay performed with PBMCs isolated from a healthy adult using 40  $\mu$ g/ml A $\beta$ 1–42. In this individual, 13.3% (4 of 30) of the wells

**Table 1**T-cell reactivity to A $\beta$  measured by a split-well assay system

A $\beta$ 1-42	-	+	SI	-	+	SI	-	+	SI	-	+	SI	-	+	SI
cpm	43,789	37,865	0.9	45,099	48,685	1.1	35,245	21,634	0.6	<b>8,745</b>	<b>69,821</b>	<b>8.0</b>	<b>45,723</b>	<b>142,880</b>	<b>3.1</b>
	173,254	141,713	0.8	<b>11,392</b>	<b>85,226</b>	<b>7.4</b>	16,037	22,330	1.4	36,315	35,369	0.9	63,579	50,010	0.8
	19,889	20,689	1.1	64,853	33,523	0.5	13,687	11,926	0.9	<b>12,871</b>	<b>56,147</b>	<b>4.4</b>	50,468	46,617	0.9
	5,874	5,566	0.9	26,719	57,697	2.1	41,186	31,531	0.8	25,355	19,537	0.8	18,268	16,304	0.9
	18,923	30,122	1.6	23,614	35,242	1.5	15,753	19,795	1.3	78,577	35,267	0.4	164,315	150,771	0.9
	18,816	17,705	0.9	15,029	24,260	1.6	7,300	8,183	1.1	67,300	78,350	1.2	1,542	427	0.3

PBMCs were isolated from an adult healthy individual and cultured in 30 wells of a 96-well plate with 40 mg/ml A $\beta$ 1-42. After 10 days, cells from each well were split and transferred to a new 96-well plate and cultured with irradiated autologous PBMCs in the presence or absence of A $\beta$ 1-42. After 48 hours the cells were pulsed with [ $^3$ H]thymidine for 12 hours, then harvested, after which cpm was measured. SI was calculated as the cpm in the presence of A $\beta$  divided by the cpm in the absence of A $\beta$ . SIs are in bold if  $\geq 2.5$  and if the  $\Delta$ cpm is  $\geq 2000$ .

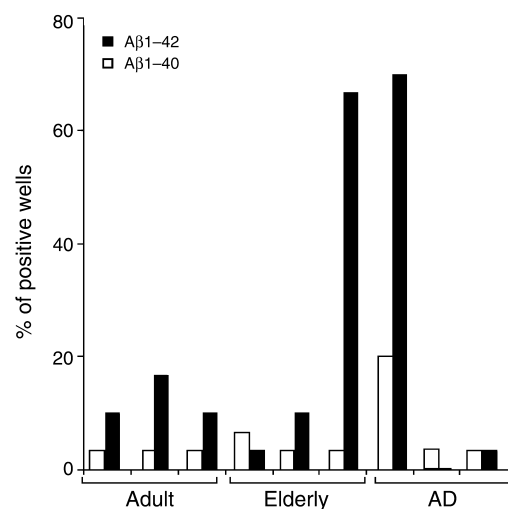
demonstrated A $\beta$ -mediated proliferation, with SIs ranging from 3 to 8 and a  $\Delta$ cpm higher than 30,000. Three wells had an SI of  $\leq 0.5$ . Split-well assays of PBMCs isolated from this subject were also performed using lower concentrations of A $\beta$ 1-42 (0.1, 1, or 10  $\mu$ g/ml), and these yielded 20%, 14%, and 20% positive wells, respectively. At these lower antigen concentrations, similar numbers of positive wells and SIs were obtained, with no wells demonstrating SIs  $\leq 0.5$  (data not shown); the latter is possibly secondary to activation-induced T cell death. Because A $\beta$ -reactive T cell lines usually proliferated maximally with 10  $\mu$ g/ml A $\beta$ 1-42, split-well assays were performed using 10  $\mu$ g/ml A $\beta$ 1-42 in our subjects.

*A $\beta$ 1-42 is more immunogenic than A $\beta$ 1-40.* Before testing large numbers of subjects using the split-well assay, we determined the relative antigenicity of the two major forms of A $\beta$ , 1-40 and 1-42. Split-well assays were performed in nine subjects (three young adults, 25-40 years old; three older healthy subjects, 50-85 years old; and three patients with AD, 58-90 years old) using either A $\beta$ 1-40 or A $\beta$ 1-42. Of these, six subjects (three adults, one older subject, and two patients with AD) showed a markedly higher percentage of positive wells when A $\beta$ 1-42 was used as the antigen, whereas only three subjects, who had low percentages of positive wells, showed similar or slightly higher reactivity evoked by A $\beta$ 1-40 (Figure 1). On the basis of these results, we chose to measure T cell responses to A $\beta$ 1-42 exclusively.

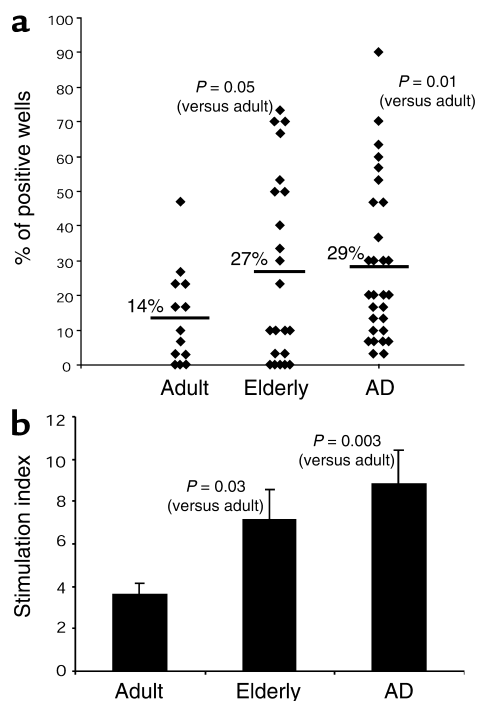
*Increased T cell reactivity to A $\beta$  in elderly subjects and patients with AD.* As described above (Figure 1), we found evidence of T cell reactivity to A $\beta$  in patients with AD and healthy adult and elderly subjects. To address the relative responses to A $\beta$ 1-42 among these three groups, we performed split-well assays on PBMCs derived from young adults (25-40 years old,  $n = 13$ ), older healthy subjects (50-85 years old,  $n = 22$ ), and patients with AD (58-90 years old,  $n = 29$ ), using A $\beta$ 1-42 (10  $\mu$ g/ml) as the stimulating antigen. The percentage of positive T cell reactivity to A $\beta$ 1-42 (as defined by an SI of at least 2.5 and a  $\Delta$ cpm of at least 2,000) was significantly higher in healthy elderly subjects (mean, 27%) than in normal adult subjects (mean, 14%) ( $P = 0.05$ ) (Figure 2a). Regression analy-

sis demonstrated a trend linking increased T cell reactivity with age ( $r = 0.29$ ,  $P = 0.09$ ). In addition, a significantly higher average of percent positive wells (29%) was also observed in the group of patients with AD (different from adults at  $P = 0.01$ ), whereas the elderly group did not differ from the AD group (Figure 2a). Furthermore, several of the elderly healthy subjects and the patients with AD had a markedly elevated percentage of positive wells (over 50%) (Figure 2a). Although the difference between the mean reactivities of the patients with AD and the elderly subjects was not significant, 23% (5 of 22) of the elderly subjects had no T cell reactivity to A $\beta$ , whereas this was not observed in patients with AD (0 of 29) ( $P = 0.03$ ).

Although the percent positive wells provide a measure of reactivity to A $\beta$ , they do not assess the

**Figure 1**

A $\beta$ 1-42 is more immunogenic than A $\beta$ 1-40. PBMCs were isolated from nine subjects (three adults, three older healthy subjects, and three patients with clinically mild or moderate AD) and cultured in U-bottom 96-well plates in the presence of human A $\beta$ 1-40 or A $\beta$ 1-42 (30 wells per peptide) as described in Methods. Cells were then restimulated at day 10 in the presence or absence of the peptide used initially. T cell proliferation was measured by [ $^3$ H]thymidine incorporation 72 hours after the secondary stimulation. The percentage of positive wells was obtained according to the number of wells (out of 30) with SIs (cpm in the presence of antigen divided by cpm in the absence of antigen) of at least 2.5 and a  $\Delta$ cpm of at least 2,000.



**Figure 2**

Aβ T cell reactivity is increased in elderly subjects and patients with AD. PBMCs were isolated from adult and elderly healthy subjects and patients with AD as described in Methods. Aβ-induced proliferation was measured after secondary stimulation in the split-well assay. (a) Percentage of positive wells was calculated for each individual as in Figure 1. (b) Average SIs ± SEM were calculated for each group on the basis of the average SIs for each individual (sum of positive SIs [each at least 2.5 with a Δcpm of > 2,000] divided by the number of positive wells). Two-tailed *P* values were calculated using the alternate Welch *t* test, assuming Gaussian populations with unequal SDs.

magnitude of the response. Accordingly, to determine whether the strength of the response to Aβ as measured by mean SIs was also higher in the elderly subjects and patients with AD, average SIs were calculated from the positive wells of each of the subjects tested. We found that average SIs were significantly higher in the healthy elderly and AD groups than in the adult group ( $P = 0.03$  and  $0.003$ , respectively) (Figure 2b).

The increased reactivity to Aβ was significant, given that T cell immunity tends to decrease with aging (42, 43), since older humans are more susceptible to infectious diseases and the effects of vaccination are reduced in the elderly (44). To determine whether the increased Aβ responses seen in our elderly and AD groups were also observed for other T cell responses, we examined T cell responses to αCD3/CD28 antibodies and to a universal antigen that stimulates T cells through the T cell receptor (TCR), GA (45). GA is a synthetic random polypeptide that stimulates a broad spectrum of HLA class II-restricted CD4<sup>+</sup> T cell populations in human PBMCs (46), and T cell reactivity to GA in humans does not require prior *in vivo* priming. Thus, αCD3/CD28 stimulation and primary T cell responses to GA were measured as an indication of global T cell activation and proliferation. As shown in Table 2, proliferative responses to αCD3/CD28 or GA were not increased in the elderly or in patients with AD. Indeed, there was a trend for patients with AD to have less T cell reactivity to both αCD3/CD28 and GA, as compared with the adult healthy group ( $P = 0.09$  and  $0.08$ ). Thus, the T cell reactivity we observed for Aβ among healthy elderly subjects and patients with AD was not related to a generalized increase in T cell responses of the subjects we tested.

#### Epitope mapping and cytokine profile of Aβ-reactive T cells.

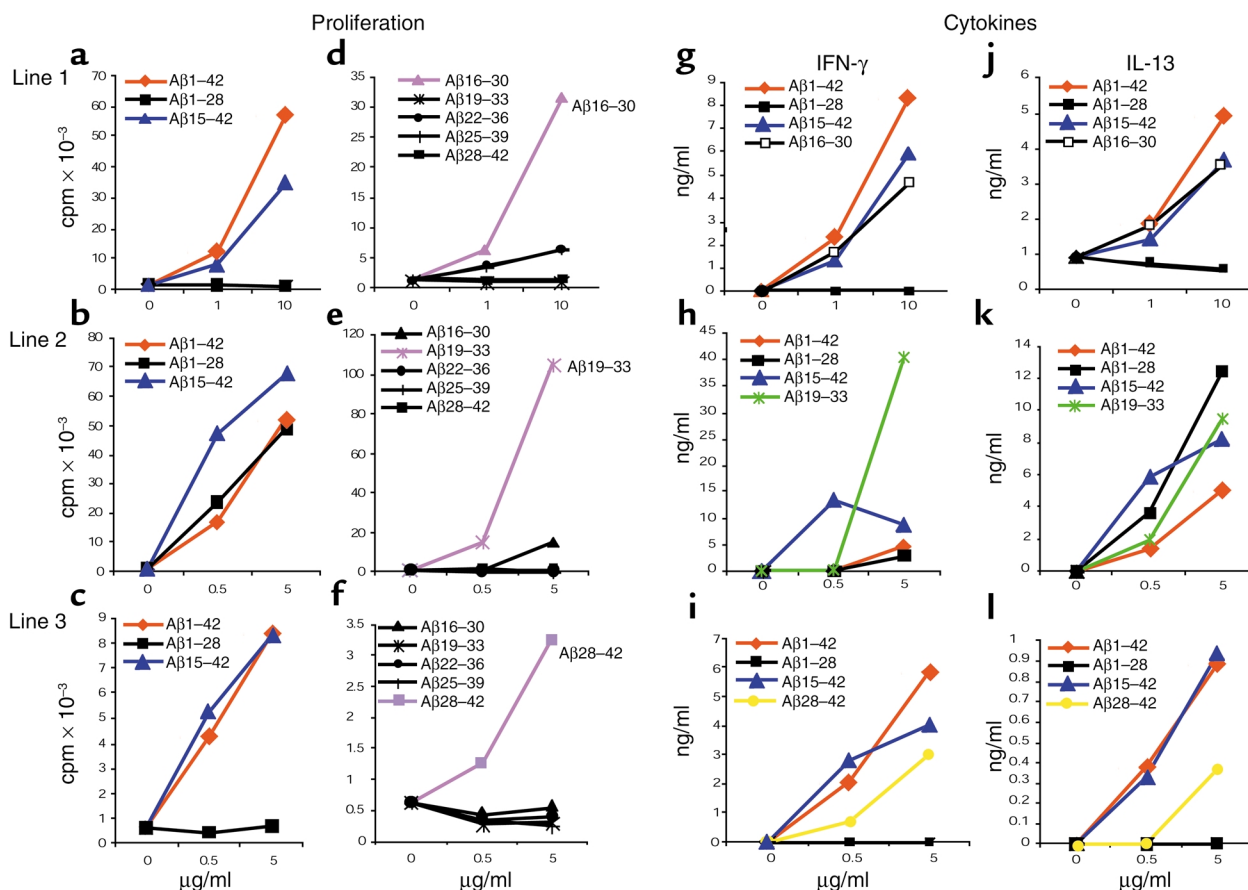
To determine if the T cell response to Aβ1–42 consisted of a response to a single T cell epitope or to multiple epitopes, we generated a panel of 24 short-term Aβ-reactive T cell lines as described in Methods. The specificity and reactivity of each T cell line was initially determined by a dose-dependent proliferation assay using Aβ1–42 and two overlapping Aβ fragments, Aβ1–28 and Aβ15–42. Proliferation of three representative Aβ1–42 T cell lines from three subjects is shown in Figure 3. T cell epitopes were located in the C terminus (Aβ15–42) in lines 1–3 (Figure 3, a–c) and also in the N terminus (Aβ1–28) in line 2 (Figure 3b). We then further investigated the epitope specificity of these three T cell lines using five overlapping peptides within Aβ15–42. As shown in Figure 3 (d–f), T cell epitopes were located in Aβ16–30, Aβ19–33, and Aβ28–42, respectively. Table 3 summarizes Aβ epitope analyses of T cell lines obtained from healthy subjects (6 adults and 12 older individuals) and 6 patients with AD. Aβ-reactive T cell lines were identified that were specific to Aβ16–30, Aβ19–33, and Aβ28–42, with the highest frequency of positive T cell lines induced by Aβ16–30. It should be noted that those T cell lines reactive to Aβ28–42 were unreactive to Aβ1–40 (data not shown), suggesting the importance of the two C-terminal residues. HLA restriction was tested in eight

**Table 2**

Proliferation of PBMCs stimulated with anti-CD3/CD28 or GA

	Adult	Elderly	AD subjects
αCD3/CD28 (mean cpm ± SEM)	23,593 ± 5,810	15,855 ± 3,315	11,377 ± 3,270
	(n = 10)	(n = 15)	(n = 13)
<i>P</i> value (vs. adults)		0.27	0.09
GA (mean cpm ± SEM)	35,342 ± 7,893	27,311 ± 4,781	19,403 ± 3,078
	(n = 13)	(n = 30)	(n = 52)
<i>P</i> value (vs. adults)		0.4	0.08

For αCD3/CD28 stimulation, PBMCs were cultured with 1 μg/ml soluble αCD3 plus αCD28 or cell culture medium alone, pulsed with [<sup>3</sup>H]thymidine on day 2 after stimulation, and incorporation was measured 12 hours later. For GA stimulation, cells were cultured with 50 μg/ml GA or cell culture medium alone and pulsed with [<sup>3</sup>H]thymidine on day 6. Mean cpm is the average cpm for each group; the average cpm for cells with culture medium alone was 825. PBMCs from all subjects stimulated with αCD3/CD28 showed proliferation. For GA, 90% of the subjects (from all groups tested) showed proliferation (SI of at least 5). Proliferative responses were calculated from these subjects. Two-tailed *P* values were calculated for the mean cpm using the alternate Welch *t* test, assuming Gaussian populations with unequal SDs.



**Figure 3**

Epitope specificity and cytokine profile of A $\beta$ -reactive T cell lines. Split-well assays were performed as described in Methods. To generate A $\beta$ -reactive T cell lines, positive wells were restimulated in the presence of irradiated PBMCs and A $\beta$  and then maintained with 10 U/ml IL-2. To determine A $\beta$  and epitope specificity, T cell lines were stimulated with A $\beta$ 1-42 and two overlapping peptides, A $\beta$ 1-28 or A $\beta$ 15-42, followed by their stimulation with nested peptides of the A $\beta$ 15-42 region. To measure A $\beta$ -induced cytokine production, supernatants were collected 48 hours after stimulation and examined by ELISA. T cell proliferation of three representative lines is shown: (a–c) induced by A $\beta$ 1-42 (a and c) A $\beta$ 15-42, and (b) A $\beta$ 1-28. T cell proliferation was also induced by (d) A $\beta$ 16-30 (e) A $\beta$ 19-33, and (f) A $\beta$ 28-42. A $\beta$ -induced secretion of IFN- $\gamma$  and IL-13 is shown in g through l.

representative positive subjects bearing different HLA class II alleles. T cell proliferation of these eight A $\beta$ -reactive T cell lines was completely blocked in the presence of the monoclonal anti-HLA-DR antibody LB3.1 but not by antibodies to DP or DQ (data not shown).

To investigate the functional properties of A $\beta$ -reactive T cells, we measured cytokine secretion 48 hours after stimulation with A $\beta$ 1-42 or with its various overlapping fragments in the same three representative cell lines used for epitope mapping (Figure 3). T cell lines 1, 2, and 3 secreted high amounts of IFN- $\gamma$  and lower amounts of IL-13 when stimulated with A $\beta$ 1-42 or C-terminal A $\beta$  fragments between 15 and 42 (Figure 3, g–l). In the one line that had shown reactivity to the N-terminal A $\beta$ 1-28 peptide (Figure 3b), A $\beta$ 1-28 also induced the secretion of high levels of IL-13 but not IFN- $\gamma$  (Figure 3, h and k). Similar patterns of cytokine secretion were observed in 16 T cell lines obtained from different subjects (data not shown). Significant levels of IL-4, IL-10, and TGF- $\beta$  could not be detected upon stimulation with any of the A $\beta$  epitopes tested (data not shown).

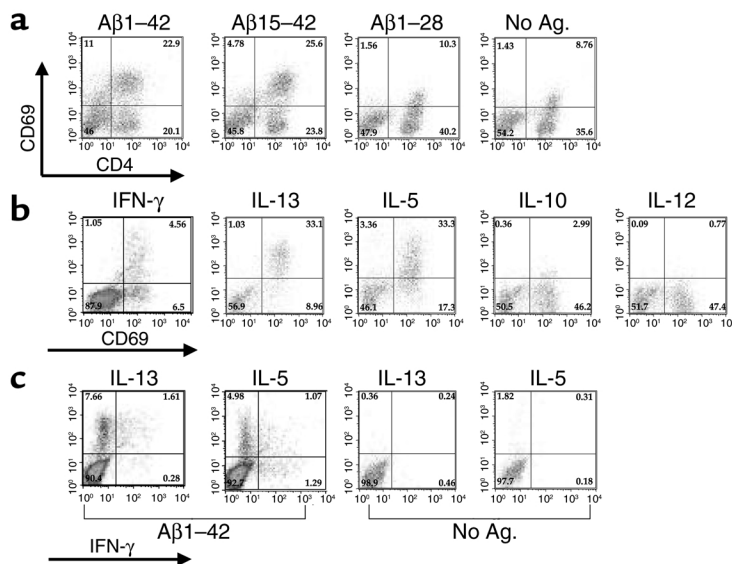
To further determine the phenotype of A $\beta$ -reactive T cells, a representative T cell line also was tested by FACS for Th1 and Th2 cytokine expression. Resting T cells were stimulated with irradiated autologous PBMCs in

**Table 3**

Distribution of A $\beta$  T cell epitopes in human PBMCs

	Number (percent)
A $\beta$ 1-28	2 (8%)
A $\beta$ 15-42	22 (92%)
Fine specificity	
A $\beta$ 7-21	1 (4%)
A $\beta$ 16-30	10 (42%)
A $\beta$ 16-33	6 (25%)
A $\beta$ 28-42	5 (20%)

A $\beta$ 1-42-reactive T cell lines were obtained from 24 different subjects (6 adults, 12 healthy older subjects, and 6 patients with AD) from the split-well assay and maintained in vitro as described in Methods. After two or three rounds of stimulation with A $\beta$ 1-42 and autologous PBMCs, T cell mapping was obtained by stimulating the cells with A $\beta$ 1-28 and A $\beta$ 15-42 followed by fine mapping of 22 T cell lines from 20 different subjects, using 15-amino-acid overlapping peptides of either A $\beta$ 1-28 or A $\beta$ 15-42.



**Figure 4**

Activated Aβ-reactive T cells exhibit Th1, Th2, and Th0 phenotypes after stimulation with autologous PBMCs and Aβ peptides. Aβ-reactive T cells were established from a secondary stimulation assay as described in Methods. Resting cells were cultured with autologous PBMCs alone or stimulated in the presence of Aβ1-42, Aβ15-42, or Aβ1-28 and harvested after 30 hours. Cells were stained with Cy-chrome-labeled anti-CD4 and FITC-labeled anti-CD69 (**a**) followed by intracellular staining with phycoerythrin-labeled anti-IFN-γ, -IL-13, -IL-5, -IL-10, and -IL-12 (**b**). Cells also were stained with Cy-chrome-labeled anti-CD4 followed by intracellular staining with FITC-labeled anti-IFN-γ and phycoerythrin-labeled anti-IL-13 or anti-IL-5 (**c**). Positive cells were not observed when intracellular staining was performed with isotype controls (data not shown). For (**b**) and (**c**), analysis was gated on CD4<sup>+</sup> cells. No Ag., no antigen.

the presence of Aβ1-42, Aβ15-42, or Aβ1-28 and analyzed by FACS 30 hours later. In Figure 4a, we measured activation of CD4<sup>+</sup> T cells for Aβ1-42, Aβ15-42, and Aβ1-28 and found increased expression of the CD69 activation marker on CD4<sup>+</sup> cells stimulated with Aβ1-42 and Aβ15-42 but not with Aβ1-28. We then tested the cytokine profile of the activated T cells as identified by CD69 expression (Figure 4b) and found that cells expressed IL-5 (33.3%) and IL-13 (33.1%), with a smaller number of cells expressing IFN-γ (4.56%) and IL-10 (2.99%) and very few cells expressing IL-12 (0.77%). We further asked whether the cytokine profiles represented primarily Th2 lineages by testing for the expression of INF-γ and IL-13/IL-5 in the same cells. We found that the majority of IL-13- and IL-5-producing cells did not make IFN-γ, though we could identify a small number of double-positive (IL-13/IL-5 and IFN-γ) cell populations (Figure 4c).

Because the T cell epitopes were most frequently localized to Aβ16-30, we further mapped this epitope for each of its constituent amino acids by using alanine-substituted peptides in the T cell proliferation assay (the naturally occurring alanines at positions 21 and 30 were changed to glycines). Substitution of phenylalanine-20, glutamic acid-22, and aspartic acid-23 with alanines blocked or significantly decreased T cell proliferation at all three concentrations used, whereas substitutions of phenylalanine-19, valine-24, and glycine-29, respectively, had inhibitory effects only at 0.1 or 1 μg/ml (Table 4). Substitution of lysine-16, alanine-21, serine-26, or asparagine-27 increased T cell proliferation more than twofold (Table 4).

## Discussion

Here, we show, we believe for the first time that circulating Aβ-reactive T cells are present in patients with AD and increase with aging. In addition, we define DR-restricted T cell epitopes reactive with Aβ in humans.

There has been only one previous study in humans that measured Aβ-reactive T cells (47). These investigators reported that Aβ-induced T cell proliferation was similar in young and elderly controls and that such T cells could not be detected in patients with AD. The authors postulated that Aβ-reactive T cells were anergized in the patients with AD. Our study clearly shows T cell responses in AD, and this finding may be related to the peptide we studied and the assay system we used. We investigated the more immunogenic peptide Aβ1-42 instead of Aβ1-40 as the stimulating antigen, and our assay system is more sensitive for detection of self-reactive T cells than is the primary

**Table 4**

Proliferation of Aβ16-30-specific T cells with alanine/glycine-substituted peptides

Peptide (μg/ml)	0.1	1	10	
Aβ16-30	1,648	6,780	17,339	
Substituted				Effect on T cell proliferation
Phe 20 Ala	1,288	2,239	4,216	
Glu 22 Ala	1,145	840	517	↓↓↓
Asp 23 Ala	1,145	1,685	612	
Phe 19 Ala	986	4,354	17,943	
Val 24 Ala	944	6,160	19,869	↓
Gly 29 Ala	622	3,014	14,513	
Leu 17 Ala	2,242	11,805	24,480	
Val 18 Ala	7,216	17,427	26,814	
Gly 25 Ala	2,426	13,763	24,979	↑
Lys 28 Ala	5,050	15,475	29,156	
Ala 30 Gly	2,123	12,027	15,395	
Lys 16 Ala	5,357	22,304	36,456	
Ala 21 Gly	12,901	29,634	36,448	↑↑
Ser 26 Ala	18,808	36,287	38,335	
Asn 27 Ala	23,636	36,588	34,983	

T cells specific to Aβ16-30 were stimulated in the presence of alanine/glycine-substituted peptides and autologous PBMCs. T cell proliferation was measured by [<sup>3</sup>H]thymidine incorporation 72 hours after stimulation. Numbers represent the average cpm of each antigen concentration tested (average cpm in the absence of antigen was 911).

stimulation system used by Trieb et al. (47). These factors may also explain why we found increased reactivity in older subjects, whereas Trieb et al. did not. Our findings were unexpected given that aging is associated with increased susceptibility to infectious agents and defective T cell priming (42, 43), a trend we also observed in our subjects when cells were stimulated with  $\alpha$ CD3/CD28 or GA.

The A $\beta$ -reactive T cell response with respect to antigen specificity, HLA class II restriction, and cytokine pattern has not been previously described in humans. CD4 T cell epitopes were identified primarily in the A $\beta$ 15–42 peptide, which is segregated from the dominant B cell epitopes identified in A $\beta$ 1–15. Of the several peptides within this region that induced T cell proliferation, the A $\beta$ 16–30 peptide was the most common epitope. Replacement of amino acids within this epitope with alanine or glycine either completely blocked T cell proliferation and cytokine production or had an inhibitory effect, suggesting that these residues maintain TCR-MHC interactions. Interestingly, glutamic acid-22 and aspartic acid-23, the replacement of which by alanine blocked T cell proliferation, are the sites of missense mutations in humans that lead to early onset of AD and/or hereditary cerebral hemorrhage with amyloidosis. We also identified T cell epitopes in A $\beta$ 28–42. These T cells were specific to A $\beta$ 1–42 and could not be stimulated by A $\beta$ 1–40, which partially explains the observed higher immunogenicity of A $\beta$ 1–42 over A $\beta$ 1–40. T cells were infrequently stimulated by the N-terminal region 1–28 residues. We found that monoclonal anti-DR inhibited virtually all T cell lines tested. The analysis of A $\beta$  T cell epitopes and their restriction to HLA-DR class II further demonstrates that A $\beta$  is processed and presented by APCs in the context of MHC and that A $\beta$ -specific T cell proliferation is mediated through MHC-TCR interactions. Thus, A $\beta$  induces adaptive immune responses in the periphery in addition to innate immune responses in the CNS.

It is unknown why A $\beta$ -reactive T cells are maintained throughout life and why they increase in the elderly and in patients with AD in the face of decreasing T cell priming that occurs with aging. A $\beta$ 1–42 is a self-antigen, and self-reactive T cells have been implicated in variety of activities, such as immune regulation, self-maintenance and repair, and autoimmune diseases (29, 30, 32, 33, 48–50). In the present study, A $\beta$ -reactive T cells were detected in almost all subjects tested, suggesting that these cells either escape central and peripheral tolerance or are positively selected to maintain the normal T cell repertoire. The cytokine repertoire of these T cells was of Th1, Th2, and Th0 phenotypes. It is possible that the activation and expansion of A $\beta$ -reactive T cells in the elderly and patients with AD found in our study indicates that A $\beta$  is captured by local APCs in the brain in the context of A $\beta$  deposition, and that these APCs migrate to secondary lymph nodes and induce T cell activation.

Although A $\beta$  deposition occurs in elderly humans that do not have overt signs of AD, there appears to be increased T cell reactivity to A $\beta$  in patients with AD, since in contrast to elderly subjects, all patients with AD tested had some A $\beta$  reactivity. Such reactivity could reflect an endogenous reaction to A $\beta$  deposition in the brain in the context of the local innate immune response that occurs in AD.

In a recent A $\beta$  vaccination trial, some patients with AD developed brain inflammation, hypothesized to be due to induced T cell responses (24). On the basis of our findings of endogenous reactivity to A $\beta$  in patients with AD, the use of a full-length A $\beta$  peptide and a Th1 adjuvant (QS21) might be expected, in retrospect, to lead to T cell-mediated CNS inflammatory effects, particularly in patients with AD having a pre-existing high frequency of A $\beta$ -reactive T cells. Of note, increased T cell reactivity to A $\beta$  was not observed preclinically in APP transgenic mice, perhaps because of their very high levels of peripheral A $\beta$  and the consequent induction of T cell tolerance (51). However, it has recently been shown that immunization of naive C57BL/6 mice with A $\beta$ 1–42 emulsified in CFA and injected with pertussis toxin resulted in a mild form of experimental autoimmune encephalomyelitis (52). In addition, the encephalitis may also be due to antigen spreading and expansion of T cells specific to CNS myelin antigens such as myelin basic protein and proteolipid protein. Furthermore CNS peptides associated with neurodegeneration were immunogenic in Lewis rats, and among these peptides was  $\beta$ -synuclein, which was found to be expressed in myelin, induced acute paralytic encephalomyelitis (53).

T cells may have regulatory properties as well as causing harmful autoimmune responses. We found that A $\beta$  administered intranasally to APP transgenic mice induced anti-A $\beta$  antibodies and partial clearance of A $\beta$  plaques, accompanied by infiltration into the CNS of small numbers of mononuclear cells expressing the anti-inflammatory cytokines IL-4, IL-10, and TGF- $\beta$  (17). It was recently shown that overexpression of TGF- $\beta$  in the CNS of APP transgenic mice resulted in a significant reduction of A $\beta$  plaque burden by promoting microglial clearance of the peptide (54). Since almost all human A $\beta$ -reactive T cell lines we studied also showed a Th2 phenotype, it is possible that mucosal immunization could boost this lineage and thus enhance clearance of A $\beta$  by both stimulating A $\beta$  antibody production (24, 55) and modulating microglial activation at sites of A $\beta$  plaques, with a minimal risk of harmful T cell responses in the CNS.

In summary, in addition to the clear relevance of our findings of intrinsic A $\beta$  T cell reactivity for vaccination strategies in AD, the increased responses to A $\beta$  we observed with age may be an important link to physiological immune responses to A $\beta$  that may have either beneficial or harmful effects for the host. Given that T cell immunity tends to decrease with age, the increased reactivity to A $\beta$  we observed may represent an unusual



immunologic response that could provide insights into the aging process and its relationship to A $\beta$ .

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