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# NEUROLOGY

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# Inflammatory markers and the risk of Alzheimer disease

## The Framingham Study

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**ABSTRACT Objective:** To examine whether serum cytokines and spontaneous production of peripheral blood mononuclear cell (PBMC) cytokines are associated with the risk of incident Alzheimer disease (AD). **Methods:** We followed 691 cognitively intact community-dwelling participants (mean age 79 years, 62% women) and related PBMC cytokine production (tertiles of spontaneous production of interleukin 1 [IL-1], IL-1 receptor antagonist, and tumor necrosis factor  $\alpha$  [TNF- $\alpha$ ]) and serum C-reactive protein and interleukin 6 (IL-6) to the risk of incident AD. **Results:** Adjusting for clinical covariates, individuals in the top two tertiles (T2 and T3) of PBMC production of IL-1 or the top tertile (T3) of PBMC production of TNF- $\alpha$  were at increased risk of developing AD (multivariable-adjusted hazard ratio [HR] for IL-1 T2 = 2.84, 95% CI 1.09 to 7.43;  $p = 0.03$  and T3 = 2.61, 95% CI 0.96 to 7.07;  $p = 0.06$ ; for TNF- $\alpha$ , adjusted HR for T2 = 1.30, 95% CI 0.53 to 3.17;  $p = 0.57$  and T3 = 2.59, 95% CI 1.09 to 6.12;  $p = 0.031$ ) compared with those in the lowest tertile (T1). **Interpretation:** Higher spontaneous production of interleukin 1 or tumor necrosis factor  $\alpha$  by peripheral blood mononuclear cells may be a marker of future risk of Alzheimer disease (AD) in older individuals. These data strengthen the evidence for a pathophysiologic role of inflammation in the development of clinical AD.

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Experimental studies suggest that inflammation plays a fundamental role in the pathogenesis of Alzheimer disease (AD).<sup>1</sup> Postmortem studies of the brain in AD demonstrate the presence of acute-phase reactants (including C-reactive protein [CRP], proinflammatory cytokines, and activated complement cascade proteins) in the senile plaques and neurofibrillary tangles.<sup>2,3</sup>

However, clinical observations on the potential role of inflammation in AD have yielded inconsistent results. Whereas several community-based studies have linked anti-inflammatory interventions to a lowered risk of developing AD,<sup>4</sup> a randomized, placebo-controlled clinical trial failed to demonstrate a beneficial effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the progression of AD.<sup>5</sup> Other observational studies that have evaluated the relations of markers of systemic inflammation to AD risk have been inconclusive; circulating cytokines have been reported to be elevated,<sup>6,7</sup> decreased,<sup>8</sup> or unaltered<sup>9,10</sup> in AD patients compared with cognitively intact controls.

The objectives of the present investigation were to compare the relations of serum cytokines (CRP and interleukin-6 [IL-6]) vs peripheral blood mononuclear cell (PBMC) production of cytokines (the proinflammatory cytokines interleukin 1 [IL-1] and tumor necrosis factor  $\alpha$  [TNF- $\alpha$ ], and the anti-inflammatory cytokine IL-1 receptor antagonist [IL-1RA]) to the risk of developing AD in the community-based Framingham Study cohort.

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**Disclosure:** The authors report no conflicts of interest.

**METHODS Study population.** The Framingham Study is a longitudinal community-based observational study of 5,209 participants (2,336 men and 2,873 women) who have been evaluated biennially since 1948 for cardiovascular risk factors and the development of cardiovascular disease. Of these, 2,842 participants constitute a dementia-free inception cohort that is under continuous surveillance for the development of neurologic disorders, including stroke and AD. Participants who were alive, were free of dementia, and attended the 22nd examination cycle (1990 to 1994) were eligible for the present investigation (n = 1,016). Of these, 691 individuals (430 women) with available measurements of CRP and PBMC production of cytokines (IL-1, TNF- $\alpha$ , IL-1RA) estimated at this 22nd examination, while they were cognitively intact, constituted our study sample. A subset of these participants (n = 295) also had available measurements of serum CRP at a remote examination (cycle 16), and these individuals were included in analyses relating “remote antecedent” inflammation (as reflected by CRP) to the risk of AD. The study was reviewed by the Boston University Institutional Review Board, and all participants (or their health care proxy) signed informed consent.

**Dementia evaluation.** The methods used for dementia screening and follow-up by the Framingham dementia study have previously been described.<sup>11</sup> Briefly, surviving cohort members who attended biennial examination cycles 14 and 15 were given a standardized neuropsychological test battery to establish a dementia-free cohort. Beginning at examination cycle 17 (1982), the Mini-Mental State Examination (MMSE)<sup>12</sup> was administered biennially to the cohort. An MMSE score below the education-specific cutoff score, a decline of 3 or more points on subsequent administrations, or a decline of more than 5 points as compared with any previous examination prompted more in-depth testing.<sup>13</sup> For each case of possible dementia, neurologic and neuropsychological examinations were performed. A panel consisting of at least one neurologist and a neuropsychologist reviewed cases designated as moderate or severe dementia (Clinical Dementia Rating [CDR]<sup>14</sup> greater than or equal to 1) by the examining neurologists. Mild dementia cases (CDR = 0.5) were reexamined within 1 or 2 years and reassessed, at least biennially for the onset of moderate to severe dementia. We used data from the neurologist’s examination, neuropsychological test performance, Framingham study records, hospital records, information from primary care physicians, CT and MRI records, and autopsy confirmation when available. All subjects identified to have dementia satisfied the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* criteria<sup>15</sup> and had dementia of severity greater than or equal to 1 on the CDR scale, and had symptoms of dementia for a period of at least 6 months. All subjects identified as having Alzheimer dementia meet the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association<sup>16</sup> criteria for definite, probable, or possible AD.

**Measurement of cytokines and CRP.** The detailed methods followed for specimen collection and analyses of cytokines and CRP have been described elsewhere.<sup>17,18</sup> Briefly, attendees of the 22nd examination cycle underwent phlebotomy in the supine position and in a nonfasting state during the early afternoon. Twenty milliliters of blood (17 mL in heparinized tubes, 3 mL serum) was obtained from each attendee, and the collected specimen was immediately transported from Framingham to the US Department of Agriculture Human Nutrition Research on Aging in Boston. PBMCs were isolated by Ficoll–

Hypaque centrifugation, washed, and cultured for 24 hours in 96-well flat-bottom plates with ultrafiltered, pyrogen-free RPMI 1640 medium (Sigma) that was supplemented with 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin, with 1% autologous heat-inactivated pooled serum and 1% L-glutamine. After incubation, plates were then frozen at  $-80^{\circ}\text{C}$  until assay. The average time from phlebotomy to isolation of PBMC was 75 minutes (range 35 to 120 minutes). Total PBMC production of IL-1, TNF- $\alpha$ , and IL-1RA were measured in unstimulated cells (spontaneous production). Serum IL-6 was measured with an ELISA (R&D Systems). CRP was measured with an immunoprecipitation assay (IncStar). The interassay variability was  $<10\%$  and the intraassay variability was  $<5\%$  for both inflammatory markers.<sup>19</sup>

Serum concentrations of CRP at examination cycles 16 and 17 (1980 to 1982) were measured with an ultrasensitive enzyme immunoassay using monospecific polyclonal and monoclonal antibodies produced by immunization with highly purified CRP. The immunoglobulin G fraction of polyclonal goat anti-human CRP antiserum was immobilized on the inner surface of microwell plates, and 100  $\mu$ L (at 1:100 dilution) of each serum sample was introduced into the test wells. The titer plates were incubated for 30 minutes at room temperature and washed four times with rinsing solution. Aliquots (100  $\mu$ L) of secondary rabbit anti-human CRP antibody conjugated to horseradish peroxidase were added to each well and incubated for 30 minutes at room temperature. The plates were then rinsed, and horseradish peroxidase activity was determined. After 30 minutes of incubation at room temperature, the enzymatic reaction was stopped, and the optical density of the product was quantified. A standard curve was generated by using known concentrations of human serum CRP as an internal control in each experiment. The concentration of CRP in the test samples was determined from the standard curve. The correlation coefficient of split specimen was 0.86.

**Statistical analyses.** We evaluated the relations of serum CRP and IL-6 levels and spontaneous production of IL-1, IL-1RA, and TNF- $\alpha$  by PBMC (all measured at the 22nd examination) to the risk of incident AD on follow-up (separate analyses for each marker) using Cox proportional hazards regression. We confirmed that the assumption of proportionality of hazards was met. Serum CRP levels were categorized into low (0), intermediate (1 to 2 mg/dL), or high ( $\geq 3$  mg/dL),<sup>20,21</sup> whereas serum IL-6 and PBMC production of IL-1, TNF- $\alpha$ , and IL-1RA were divided into tertiles (T1, lowest; T2, middle; and T3, highest). In these analyses, the lowest category (low CRP or first tertile for other cytokines) served as referent with which the individual upper categories/tertiles were compared. We constructed models adjusted for age and sex; and adjusted for age, sex, ApoE  $\epsilon 4$  allele status (present or absent), history of stroke (present or absent), educational achievement (dichotomized at high school completion), homocysteine levels (continuous variable, log-transformed), current smoking (present or absent), body mass index (BMI; continuous variable), and  $\beta$ -hydroxy- $\beta$ -methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor use (yes/no). Because ApoE status is related strongly to both CRP levels<sup>22</sup> and risk of AD, we performed additional analyses stratifying our sample according to the presence or absence of the ApoE  $\epsilon 4$  allele.

In exploratory analyses, we evaluated whether combinations of the three PBMC cytokines (high levels of IL-1 and TNF- $\alpha$  and low levels of IL-1RA) were related to AD risk. The cutpoints that defined high IL-1 and TNF- $\alpha$  and low IL-1RA

**Table 1** Baseline characteristics at examination cycle 22

Characteristic	Women, n = 430	Men, n = 261
Age, years	79 ± 5	78 ± 4
Follow-up, years	7 ± 3	7 ± 3
ApoE ε4, %	19%	20%
Body mass index, kg/m <sup>2</sup>	27 ± 5	27 ± 4
High school degree, %	71%	69%
Current cigarette smoking, %	10%	5%
Prevalent stroke, %	5%	4%
HMG-CoA reductase inhibitor use, %	5%	6%
NSAID use, %	19%	13%
Plasma homocysteine, μmol/L	12 ± 5	12 ± 5
Median (range)	11 (4-42)	11 (4-40)
CRP, mg/dL	1.5 ± 3.9	2.6 ± 8.0
Median (range)	0 (0-28)	0 (0-63)
PBMC IL-1, ng/mL	4.5 ± 4.2	4.6 ± 3.7
PBMC TNF-α, ng/mL	4.7 ± 3.7	5.3 ± 4.3
IL-1RA, ng/mL	11.8 ± 8.2	12.4 ± 7.2
Serum IL-6, pg/mL	7.2 ± 16.7	9.6 ± 20.6
Median (range)	3.5 (3.1-274.0)	4.3 (3.1-207.4)

Values are mean ± SD except as indicated.

HMG-CoA =  $\beta$ -hydroxy- $\beta$ -methylglutaryl-coenzyme A; NSAID = nonsteroidal anti-inflammatory drug; CRP = C-reactive protein; PBMC = peripheral blood mononuclear cell; IL-1 = interleukin 1; TNF- $\alpha$  = tumor necrosis factor  $\alpha$ ; IL-2RA = interleukin 2 receptor antagonist; IL-6 = interleukin 6.

were selected based on the results of primary analysis, i.e., the category above or below which risk of AD escalated. Additionally, we examined the relation of CRP measured at the 16th examination cycle (1979 to 1982) to the development of incident AD beginning more than 12 years later (from the 22nd examination cycle onwards). Analyses were conducted using SAS software (SAS Institute Inc., Cary, NC, 2006).

**RESULTS** The characteristics of participants in our sample at the baseline examination are presented in table 1. A comparison of characteristics of participants with and without PBMC data are presented in table 2. The two groups are similar, except the participants who were excluded due to lack of PBMC

**Table 2** Baseline characteristics among participants in the dementia cohort with follow-up for dementia who attended examination cycle 22, by PBMC data availability

Characteristic	Excluded (no PBMC data), n = 325	Included (PBMC data available), n = 691
Age, years	82 ± 6	79 ± 4
Male, %	30%	38%
Nursing home residence, %	13.4%	0.3%
ApoE ε4, %	19%	19%
Body mass index, kg/m <sup>2</sup>	26 ± 5	27 ± 5
High school degree, %	65%	70%
Current cigarette smoking, %	9%	8%
Prevalent stroke, %	10%	5%
HMG-CoA reductase inhibitor use, %	4%	5%
NSAID use, %	13%	17%
Plasma homocysteine, μmol/L	13 ± 6	12 ± 5

Values are mean ± SD except as indicated.

PBMC = peripheral blood mononuclear cell; HMG-CoA =  $\beta$ -hydroxy- $\beta$ -methylglutaryl-coenzyme A; NSAID = nonsteroidal anti-inflammatory drug.

**Table 3** Multivariable Cox proportional models examining the relations between PBMC cytokine production and the risk of AD

Cytokine measured	Tertile	No. of cases/subjects	HR, age and sex adjusted	p Value	No. of cases/subjects	HR, multivariable adjusted	p Value
PBMC IL-1	T1	8/229			6/181		
	T2	18/236	2.47 (1.07-5.70)	0.03	16/196	2.84 (1.09-7.43)	0.033
	T3	18/223	2.32 (1.00-5.40)	0.05	14/179	2.61 (0.96-7.07)	0.06
PBMC TNF- $\alpha$	T1	12/234			9/183		
	T2	13/236	0.92 (0.42-2.03)	0.84	11/195	1.30 (0.53-3.17)	0.57
	T3	18/216	1.67 (0.80-3.47)	0.17	15/176	2.59 (1.09-6.12)	0.03
PBMC IL-1RA	T1	16/216			15/180		
	T2	13/211	0.76 (0.37-1.59)	0.47	9/171	0.71 (0.31-1.65)	0.43
	T3	11/216	0.62 (0.29-1.35)	0.23	8/164	0.54 (0.22-1.33)	0.18

Multivariable model adjusted for age, sex, ApoE  $\epsilon$ 4 allele status, history of stroke, educational achievement, homocysteine levels, current smoking history, body mass index, and  $\beta$ -hydroxy- $\beta$ -methylglutaryl-coenzyme A reductase inhibitor use. PBMC = peripheral blood mononuclear cell; AD = Alzheimer disease; HR = hazard ratio; IL-1 = interleukin 1; TNF- $\alpha$  = tumor necrosis factor  $\alpha$ ; IL-1RA = interleukin 1 receptor antagonist.

data were more likely to reside in a nursing home (13.4 vs 0.3%). During a mean follow-up of 7 years (range 4 to 10 years), 44 participants (25 women) developed AD, and 262 individuals (147 women) died.

**PBMC IL-1, TNF- $\alpha$ , and IL-1RA production and the risk of AD.** On follow-up, AD developed in 44 of 688 participants (6.40%; 25 women) with available PBMC IL-1 levels, 43 of 686 individuals (6.27%; 24 women) with TNF- $\alpha$  levels, and in 40 of 643 people (6.22%; 23 women) with IL-1RA levels (table 3). After adjusting for covariates (age, sex, ApoE  $\epsilon$ 4 allele status, history of stroke, education, homocysteine levels, current smoking history, BMI, and HMG-CoA reductase inhibitor use), we found that compared with those with the lowest (T1) production of IL-1, subjects in the second highest tertile (T2) of IL-1 production levels were at increased risk of developing incident AD (adjusted hazard ratio [HR] = 2.84, 95% CI 1.09 to 7.43;  $p$  = 0.03), and subjects with the highest tertile of IL-1 production (T3) showed a trend toward an increased risk of developing incident AD that was of borderline significance (adjusted HR = 2.61, 95% CI 0.96 to 7.07;  $p$  = 0.06). Subjects with the highest (T3) production of TNF- $\alpha$  were at greater risk of developing AD vs those with the lowest production (adjusted HR for T2 = 1.30, 95% CI 0.53 to 3.17;  $p$  = 0.57 and T3 = 2.59, 95% CI 1.09 to 6.12;  $p$  = 0.03). In contrast, subjects with the highest (T3) production of IL-1RA did not have a decreased risk of developing AD compared to those with the lowest (T1) production (HR = 0.54, 95% CI 0.22 to 1.33;  $p$  = 0.18). Secondary analyses that additionally adjusted for NSAID use did not effect a statistically significant change in the results.

**Combination of PBMC cytokines and the risk of AD.** Based on primary analysis, we defined high-risk individuals as those with high levels of IL-1 (top two tertiles) or TNF- $\alpha$  (top tertile). In our sample, 27.6% of women and 29.9% of men had levels of these two markers of enhanced inflammation indicative of high risk (>T1 for IL-1, >T2 for TNF- $\alpha$ , or <T2 for IL-1RA), but this group accounted for 41.9% of all AD cases. Compared with individuals with favorable levels (low IL-1, TNF- $\alpha$ ), individuals with unfavorable levels had an increased risk of AD (adjusted HR = 1.61, 95% CI 1.13 to 2.29;  $p$  = 0.009).

**Serum CRP and IL-6 at baseline and the risk of AD.** As shown in table 4, 44 of 691 subjects with available CRP levels at examination cycles 22 and 33 of 540 individuals with available serum IL-6 developed AD. Neither CRP nor serum IL-6 levels were related to the risk of AD in any of the models. Among individuals with serum CRP levels available at the 16th examination cycle, 23 of 295 persons (7.80%; 13 women) developed AD on follow-up and in multivariable analyses; we found no relations between remote CRP levels and risk for incident AD. Additional adjustment for NSAID use did not alter these findings.

**DISCUSSION** Several lines of scientific evidence implicate inflammation in the pathogenesis of AD. Proinflammatory cytokines alter the expression and processing of  $\beta$ -amyloid precursor protein,<sup>23,24</sup> and fibrillar  $\beta$ -amyloid in turn promotes the production of proinflammatory cytokines by microglial and monocytic cell lines.<sup>25</sup> IL-1 also increases neuronal tau phosphorylation<sup>26</sup> and activates astrocytes.<sup>27</sup> On a parallel note, polymorphisms of some inflam-



**Table 4** Cox proportional hazards model examining the relationship between serum cytokine levels and the risk of AD

	Tertile	No. of cases/subjects	HR, age and sex adjusted	p Value	No. of cases/subjects	HR, multivariable adjusted	p Value
CRP 16	Low	3/24			3/22		
	Intermediate	12/140	0.36 (0.10–1.32)	0.123	9/109	0.29 (0.07–1.23)	0.093
	High	8/131	0.23 (0.06–0.91)	0.036	7/108	0.36 (0.07–1.78)	0.209
CRP 22	Low	30/476			24/388		
	Intermediate	3/85	0.64 (0.19–2.10)	0.460	3/70	0.75 (0.22–2.57)	0.651
	High	11/130	1.53 (0.76–3.07)	0.234	9/101	1.68 (0.75–3.71)	0.208
IL-6	T1	9/195			8/160		
	T2	11/165	1.48 (0.61–3.60)	0.694	9/127	1.22 (0.14–3.34)	0.703
	T3	13/180	1.23 (0.52–2.94)	0.385	10/147	1.27 (0.46–3.53)	0.644

Multivariable model adjusted for age, sex, ApoE  $\epsilon$ 4 allele status, history of stroke, educational achievement, homocysteine levels, current smoking history, body mass index, and  $\beta$ -hydroxy- $\beta$ -methylglutaryl-coenzyme A reductase inhibitor use. AD = Alzheimer disease; HR = hazard ratio; CRP = C-reactive protein; IL-6 = interleukin 6.

matory genes, including IL-1, IL-6, and TNF- $\alpha$ , have been associated with an increased risk of developing AD,<sup>28–31</sup> thereby indirectly incriminating inflammatory responses in the development of the disease.

Cross-sectional analyses have explored the association between circulating inflammatory cytokines and AD. Both IL-1<sup>32</sup> and TNF- $\alpha$ <sup>33</sup> blood levels have been shown to be elevated in patients with AD in some studies, whereas others found no association.<sup>9</sup> High plasma levels of CRP and IL-6 have been associated with poorer cognitive performance at baseline and with a greater risk of cognitive decline over a 2-year follow-up period<sup>34</sup> in some studies, although a recent report from the Women's Health Study found no evidence of a link between high sensitivity CRP and decrements in cognitive function.<sup>35</sup> One possible explanation for the conflicting findings of studies on the relation of serum cytokines and AD may be that circulating levels of cytokines reflect systemic inflammation but may not adequately mirror intracerebral inflammatory responses due to the presence of the blood brain barrier. Thus, some previous studies have reported higher CSF levels of TNF- $\alpha$  relative to serum levels in AD patients.<sup>36</sup> In this context, it is noteworthy that perivascular macrophages and microglia in the brain that participate in intraparenchymal inflammation are derived from circulating macrophages.<sup>37</sup> Moreover, a case-control study showed a significant increase of PBMC-released cytokines, including IL-1 and IL-6 in AD patients compared with healthy controls and a decrease in PBMC cytokine production in AD patients treated with acetylcholinesterase inhibitor. Therefore, measurement of the spontaneous production of cytokines (such as IL-1 and TNF- $\alpha$ ) by PBMC may better reflect their po-

tential to contribute to intracerebral inflammation compared with assessment of serum cytokine levels.

Further, these observations raise the possibility that circulating cytokines and PBMC production of cytokines may be elevated early in the course of AD and could predict the occurrence of AD in individuals free of the condition at baseline. The present investigation examines these hypotheses prospectively in an elderly community-based sample.

We observed that higher levels of PBMC production of the inflammatory cytokines IL-1 or TNF- $\alpha$  were associated with an increased risk of developing AD. This relationship was not linear for IL-1, because the most pronounced elevation of risk was observed in the second (T2) and not the highest (T3) tertile. Additionally, a composite of unfavorable levels of several PBMC cytokines was associated with an approximately twofold increase in risk of AD compared with those with favorable levels. In contrast, levels of circulating inflammatory cytokines (CRP, IL-6) were not associated with the risk of AD. Likewise, the hypothesis that a potential explanation for the variable results of studies relating serum cytokines to AD risk may be that measurement of inflammatory biomarkers in close proximity to (but before) presumed disease onset may be confounded by incipient disease was not confirmed in this study. We measured CRP levels at time periods close to and relatively remote from the "age at risk" of AD onset and found that neither recent nor remote CRP levels were related to the risk of AD.

There are several possible explanations for the observed lack of association between baseline serum levels of IL-6 and CRP and AD risk. First, as noted previously, circulating cytokine levels may not reflect tissue levels, especially so in the brain because of the blood-brain barrier. Second, sys-

temic factors that are unrelated to degenerative brain processes may influence circulating levels of inflammatory markers. In fact, several studies have shown that aging alone can cause an increase in peripheral cytokines, including IL-6<sup>38,39</sup> and TNF- $\alpha$ .<sup>40</sup> Third, we evaluated participants who attended a routine Heart Study examination and did not have data on cytokines on institutionalized people. It is conceivable that nonattendees were sicker and more likely to have elevated cytokine levels; their exclusion would bias us toward the null hypothesis of no association between cytokines and AD risk. Fourth, we used low-sensitivity CRP and may have misclassified individual's CRP levels. Fifth, our results may have been influenced by a survival bias; persons with elevated serum levels of CRP and IL-6 were at greater risk of dying during the follow-up period (adjusted HR for CRP T3 vs T1 was 1.57, 95% CI 1.17 to 2.11;  $p = 0.003$  and adjusted HR for IL-6 T3 vs T1 was 1.76, 95% CI 1.25 to 2.49;  $p = 0.001$ ), whereas elevated PBMC production of cytokines was not associated with a greater risk of mortality. Finally, we had insufficient power to determine a modest association between circulating cytokines and AD. An additional limitation of our investigation was the almost exclusively European origin and older age of our study sample. These reduce the generalizability of our results to other ethnicities and younger age groups. Thus, our results should be considered hypothesis generating and validated in other samples.

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