**CD4⁺ T Cells Prevent Spontaneous Experimental Autoimmune Encephalomyelitis in Anti–Myelin Basic Protein T Cell Receptor Transgenic Mice**

By Fabienne Van de Keere and Susumu Tonegawa

From the Howard Hughes Medical Institute, the Center for Cancer Research, and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

**Summary**

Autoimmune diseases result from a failure of tolerance. Although many self-reactive T cells are present in animals and humans, their activation appears to be prevented normally by regulatory T cells. In this study, we show that regulatory CD4⁺ T cells do protect mice against the spontaneous occurrence of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. Anti–myelin basic protein (MBP) TCR transgenic mice (T/R₁) do not spontaneously develop EAE although many self-reactive T cells are present in their thymi and peripheral lymphoid organs. However, the disease develops in all crosses of T/R⁺ mice with recombination-activating gene (RAG)-1 knockout mice in which transgenic TCR-expressing cells are the only lymphocytes present (T/R₂ mice). In this study, crosses of T/R⁺ mice with mice deficient for B cells, CD8⁺ T cells, NK1.1 CD4⁺ T (NKT) cells, or γ/δ T cells indicated that α/β CD4⁺ T cells were the only cell population capable of controlling the self-reactive T cells. To confirm the protective role of CD4⁺ T cells, we performed adoptive transfer experiments. CD4⁺ T cells purified from thymi or lymph nodes of normal mice prevented the occurrence of spontaneous EAE in T/R⁻ mice. To achieve full protection, the cells had to be transferred before the recipient mice manifested any symptoms of the disease. Transfer of CD4⁺ T cells after the appearance of symptoms of EAE had no protective effect. These results indicate that at least some CD4⁺ T cells have a regulatory function that prevent the activation of self-reactive T cells.

Key words: autoimmune disease • experimental autoimmune encephalomyelitis • CD4⁺ T cells • regulatory cells • adoptive transfer

Several mechanisms of tolerance normally prevent the immune system from responding to self-antigens. In the thymus, tolerance is induced by negative selection of developing T cells with high affinity to self-antigens (1). Low-affinity self-reactive T cells, or T cells expressing a receptor for antigens not presented intrathymically, escape to the peripheral tissues. Although some self-reactive T cells are prevented from causing disease by T cell anergy (2) or apoptosis (3), others appear to remain silent simply because the antigens they recognize are not present in immunogenic forms. Interestingly, a high incidence of autoimmune diseases has been observed in rodents rendered lymphopenic (for review see reference 4). In these models, the spontaneous activation of disease-causing T cells has been attributed to the lack of regulatory CD4⁺ T cells (5, 6).

CD4⁺ T cells can develop into functionally distinct populations that differ in their patterns of cytokine production. Th1 cells secrete proinflammatory cytokines such as IFN-γ and TNF-β and are responsible for certain organ-specific autoimmune diseases such as multiple sclerosis, diabetes, and rheumatoid arthritis. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. These cytokines activate B cells and enhance eosinophil proliferation and function. The cytokines produced by Th1 and Th2 cells antagonize each other's development and activity (7). Because of this cross-regulation between the Th1 and Th2 subsets, it was thought that Th1 cell-mediated autoimmune diseases could be prevented or cured by Th2 cells. However, using a mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE),¹ we could not show a protective function of anti–myelin basic protein (MBP) Th2 cells (8). Indeed anti-MBP Th2 cells not only failed to protect mice against

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¹Abbreviations used in this paper: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; KO, knockout; MBP, myelin basic protein; NOD, nonobese diabetic; RAG, recombination-activating gene.
pathogenic anti-MBP Th1 cells but did themselves induce EAE, at least in immunodeficient mice. Pakala et al. made similar observations using nonobese diabetic (NOD) mice (9).

In this work, we used the EAE model to characterize the regulatory lymphocytes that normally prevent disease induction by autoreactive T cells EAE is caused by CD4+ T cells that recognize peptides derived from proteins of the central nervous system (CNS) in association with MHC class II molecules. Paralysis results from T cell infiltration in the CNS and subsequent degradation of myelin. The disease can be induced in susceptible animals by immunization with CNS proteins such as MBP or by transfer of encephalitogenic T cell lines (10), or may occur spontaneously in mice expressing a transgenic TCR specific for MBP (Ac1-11) (11, 12). Interestingly, Lafaille et al. (12) observed a striking difference in the incidence of spontaneous EAE in anti-MBP TCR transgenic mice, referred to hereafter as T/R mice, and their crosses with recombinant-activating gene (RAG)-1 deficient mice, referred to hereafter as T/R KO mice. Although EAE occurred in only ~10% of the T/R+ mice, all T/R− mice became sick. This finding indicated that lymphocytes present in the T/R− mice but absent in the T/R+ mice were able to control the self-reactive anti-MBP T cells. Having already ruled out a role of anti-MBP Th2 cells in this protection (8), we now show, by crossing the T/R+ mice with mice deficient in subsets of lymphocytes and by adoptive transfer experiments, that CD4+ T cells carrying receptors other than the MBP-specific TCR prevent the spontaneous development of EAE in anti-MBP TCR transgenic mice.

Materials and Methods

Mice. Anti-MBP TCR transgenic mice have been described previously (12). Mice expressing a TCR specific for MBP Ac1-11 were generated in C57BL/6 mice. These mice were crossed to mouse strains with various gene defects, all on a C57BL/6 background. The Iaβ restriction element was introduced by crosses with B10.PL mice. H-2d mice were used in all experiments. The B10.PL (72N S)/Sn, β2-microglobulin (β2-m) knockout (KO), TCR-α KO, and IgM μ KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The RAG-1 KO and TCR-β KO mice were available in our laboratory. All mice were maintained in autoclaved cages with autoclaved bedding, food, and water. Animals were typed for the expression of the transgenic TCR by flow cytometry analysis of blood lymphocytes using anti-Vβ8.1, 8.2-FITC, and anti-CD4-PE antibodies (PharMingen, San Diego, CA). Flow cytometry analysis and PCR were used to determine the expression of the RAG-1, IgM μ, TCR-α, and β2-m genes. The expression of the TCR-β gene was determined by PCR only. Two sets of primers, one set spanning the disrupted site of the specific gene and another set spanning the neomycin resistance marker, were used to perform the PCR reactions. Flow cytometry analysis of blood lymphocytes using anti-CD3-FITC and anti-B220-PE antibodies (PharMingen) determined the expression of the RAG-1 and IgM μ genes. TCR-α and β2-m gene expression was established by using the anti-CD8-FITC and anti-CD3-PE antibodies (PharMingen).

CD4+ T Cell Purification. Peripheral CD4+ T cells were purified by magnetic microbeads as previously described (13). The cells were first labeled for 45 min at 4°C with biotinylated anti-γδ TCR antibody (GL3; Pharmingen), then washed and incubated for 20 min at 4°C with a mixture of streptavidin-coupled beads and beads directly coupled to anti-CD8 antibodies (Milenyi Biotec). Washed cells were passed through a column inserted into the SuperMacs magnet, and the cells that did not bind to the column (CD4+−) were collected. For the purification of the CD4+ T cells from lymph nodes, beads directly coupled to anti-B220 antibodies were added to the labeling mixture. To evaluate the efficiency of the cell separation, aliquots of purified cells were stained with anti-CD3-FITC and anti-CD4-PE antibodies (PharMingen) and analyzed by flow cytometry. Typically, we obtained a purity >95 or >70% for CD4+ T cells isolated from lymph nodes or thymi, respectively.

Flow Cytometry. CD4+ T cells either just before the transfer or recovered from mesenteric lymph nodes of EAE-free mice 9 wk after the transfer were analyzed by using fluorescein-conjugated antibodies against CD3 (145-2C11), CD4 (RM4-5), Vβ8.1,8.2 (MR5-2), CD45R B (23G2), CD44 (IM-7), and CD62L (ME-L-14), all from Pharmingen, and CD3 (CT-CD3), from Caltag (Burlingame, CA). About 0.5 × 10⁶ cells were incubated with antibodies for 45 min at 4°C, washed, and analyzed with a FACScan® and the CellQuest software (Becton Dickinson, Mountain View, CA). Dead cells were excluded based either on forward scatter and size or by propidium iodide staining.

Figure 1. Incidence of spontaneous EAE in anti-MBP TCR transgenic RAG-1−/− (T/R−) and RAG-1+/− (T/R+) mice. (a) Percentage of mice with EAE among T/R− (white bar; n = 45) and T/R+ (striped bar; n = 30) mice. All mice were monitored for EAE symptoms for at least 5 mo starting at 1 mo of age unless they developed EAE and died. (b) Time course of the development of spontaneous EAE in T/R− mice.

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propidium iodide staining or on their forward and side scatter profiles.

Cell Transfer. Purified CD4+ T cells were washed twice with PBS and injected intravenously into T/R− recipient mice in 200 μl of PBS.

EAE Scoring. Clinical symptoms of EAE were graded as follows (14): level 0, no symptoms; level 1, limp tail; level 2, partial paralysis of hind legs; level 3, complete paralysis of hind legs; level 4, paralysis of fore and hind legs; level 5, moribund.

Results

EAE Develops Spontaneously in T/R− Mice at a Narrow Age Window. We crossed the T/R+ mice in a C57BL/6-8-B10.PL background with RAG-1-deficient mice (15) in a C57BL/6 background. The incidence of spontaneous EAE was 100% in T/R− littermates but only 4.4% in T/R+ littermates (Fig. 1A). In T/R− mice the disease developed in a narrow time window (Fig. 1B), between days 35 (no disease) and 65 (100% disease incidence). This result indicated that T/R− mice contain regulatory cells that prevent the anti-MBP T cells from becoming pathogenic, and protect the T/R− mice from an early onset of the disease.

The Absence of CD41 T Cells in Anti-MBP TCR Transgenic Mice Abrogates the Protection against Spontaneous EAE. T/R− mice differ from T/R+ mice in that they have B cells, CD81 T cells, some CD4+ T cells expressing non-transgenic T cell receptor chains, NK1.1 CD41 T cells (NKT cells), and γ/δ T cells. To investigate the putative protective role of any of these lymphocyte subsets, we crossed the T/R+ mice to several mouse strains, each of which lacked one or more lymphocyte subsets due to a gene KO. All strains of mice used for these crosses were in a C57BL/6 background. In each cross we compared the disease incidence of anti-MBP TCR transgenic littermates homozygous and heterozygous for the disrupted gene. We did not observe a significant difference in the incidence of EAE in the anti-MBP TCR transgenic mice that lacked B cells, CD8+ T cells, or γ/δ T cells due to the targeted disruption of the IgM μ chain gene (16), the β2-m gene (17),

Table 1. Protection of T/R− Mice against EAE after Adoptive Transfer of CD4+ T Cells

| Experiment | Source of CD4+ T cells | No. of cells injected (× 10^6) | EAE incidence (%) | Fatality | Individual maximum score |
|------------|------------------------|--------------------------------|-------------------|--------|------------------------|
| 1          | Lymph node             | 0.5                            | 2/4 (50)          | 0      | 2, 0, 0, 2             |
| 2          | Lymph node             | 1                              | 1/2 (50)          | 0      | 1, 0                   |
| 3          | Lymph node             | 2.5                            | 3/3 (100)         | 0      | 2.5, 2.5, 3            |
| 4          | Lymph node             | 2.5                            | 0/2 (0)           | 0      |                        |
| 5          | Lymph node             | 5                              | 0/2 (0)           | 0      |                        |
| 6          | Thymus                 | 1                              | 1/2 (50)          | 0      | 1, 0                   |
| 7          | Thymus                 | 1                              | 0/2 (0)           | 0      |                        |
| 8          | Thymus                 | 1                              | 0/1 (0)           | 0      |                        |
| 9          | Thymus                 | 1                              | 0/2 (0)           | 0      |                        |
| 10         | Thymus                 | 2.5                            | 3/4 (75)          | 0      | 2, 3, 2, 0             |
| 11         | Thymus                 | 2.5                            | 1/4 (25)          | 0      | 0, 0, 1, 0             |
| 12         | Thymus                 | 5                              | 2/4 (50)          | 0      | 1, 0, 0.5, 0           |
| Control    | –                      | –                              | 14/14 (100)       | 57     | 5, 3.5, 5, 2.5, 5, 3.5, 2.5, 3, 5, 5, 5, 5, 3.5, 5 |

*28–43-d-old mice were injected and monitored every other day for 9 wk.
or the TCR-α chain gene (18), respectively (Fig. 2). In contrast, mice homozygous for a mutation in the TCR-α gene, resulting in the absence of CD4+ and CD8+ T cells (19), were not protected. In these mice, the incidence of spontaneous EAE was similar to that observed in the RAG-1-defective T/R− mice (Fig. 2). Taken together, these data showed that the non-TCR transgenic α/β CD4+ T cells that are present in T/R+ mice but not in T/R− mice prevent spontaneous EAE. It should be noted that the incidence of EAE in all mice described above that do have α/β CD4+ T cells was ~25%. This is clearly higher than the incidence of EAE in T/R+ mice, which is only 4.4%. We do not know the reason for this. It could possibly be due to minor differences in the genetic background that arose in these crosses. Indeed B6 and B10 mice, although genetically very close, do differ at multiple loci (20, 21).

A adoptive transfer of CD4+ T cells protects young T/R− mice against spontaneous EAE. To confirm the protective role of CD4+ T cells, we performed adoptive transfer experiments. CD4+ T cells were purified from lymph nodes and thymi of B10.PL mice. Different numbers of these cells were transferred into young T/R− mice that did not yet show any signs of disease. We monitored the reconstituted T/R− mice every other day for 9 wk for the spontaneous development of EAE. The transfer of as few as 106 CD4+ T cells protected the recipient T/R− mice. The protection was not always completely effective. Some of the recipients developed mild symptoms and either remained sick or recovered from the disease (Table 1, Fig. 3a). In contrast, the disease developed in all control T/R− mice and was lethal in >50% of them during the observation period of 9 wk (Table 1). CD4+ T cells isolated from both lymph nodes and thymi were protective (Table 1, Fig. 3a). No protection was observed when CD4+ T cells were transferred to T/R− mice that were already sick at the time of the injection (data not shown). To assess the time-window during which protection could be accomplished, we injected 5 × 106 CD4+ T cells into groups of T/R− mice that differed by age. Protection was observed as long as the recipient mice were 40 d old or younger (Fig. 3b). Recipient mice that were older than 45 d at the time of injection developed EAE as severely as untreated mice, although mice that were 41–45 d old developed a very mild form of the disease (mean EAE score of 1.5). Thus, to achieve full protection the CD4+ T cells must be transferred before the T/R− mice reach the age of 40 d.

We also adoptively transferred CD8+ T cells or B cells in T/R− mice. All recipients developed EAE symptoms similar to the uninjectected control T/R− mice (data not shown). Taken together, these findings showed that CD4+ T cells but no other lymphocyte subsets protect against spontaneous development of EAE.

A her transfer, naive CD4+ T cells acquire a memory phenotype. We analyzed the phenotype of the CD4+ T cells before transfer and studied their fate in the recipient mice. A typical CD3/CD4 cytfluorometry profile of the transferred cells is shown in Fig. 4A. At the time of injection, the lymph node cells expressed high levels of CD45RB and CD62L and an intermediate level of CD44 (Fig. 4B, a). This surface marker profile is characteristic of naïve T cells (22). The CD4+CD8− thymocytes expressed CD45RB and CD44 at intermediate levels, and ~50% of them expressed CD62L (Fig. 4B, b). 9 wk after transfer, the reconstituted mice were killed and their T cell populations were analyzed. The injected CD4+ T cells recovered from mesenteric lymph nodes constituted ~12% of the total T cell population and had acquired a phenotype of memory T cells (22); CD45RB+CD62LlowCD44hi (Fig. 5B, a). Very similar results were obtained with lymph node T cells from T/R− littermates. In these mice, ~10% of all CD4+ T cells were nontransgenic and most of them exhibited a memory phenotype (Fig. 5B, b).

NKT cells do not protect against spontaneous EAE. NKT cells are a small lymphocyte population that expresses a Vα8-biased TCR repertoire, and that produces large amounts of IL-4 after TCR ligation. It is thought that they...
may play a role in autoimmunity (23). A small number of NKT cells were present in the protective CD4⁺ T cell population that we transferred in T/R⁻ mice. They constituted ~0.1 and ~2% of the cells purified from the lymph nodes and thymocytes, respectively (data not shown). However, it is unlikely that these cells played a role in the protection against spontaneous EAE. First, despite the 20-fold difference of concentration of NKT cells between the transferred peripheral T cell and the thymocytes, both populations were equally competent in the protection. Second, although β2-m−deficient mice lacked NKT cells (24), we did not observe any difference of disease susceptibility between transgenic mice heterozygous and homozygous for the defect of the β2-m gene (Fig. 2).

Discussion

Using the EAE model, Lafaille et al. showed that most mice carrying a transgenic TCR specific for MBP did not spontaneously develop EAE despite the presence of many self-reactive T cells (12). Interestingly, the disease developed in all crosses of T/R⁻ mice with RAG-1 KO mice (T/R⁻ mice), indicating that regulatory lymphocytes must exist and control the spontaneous development of EAE. In this work we showed that the striking difference between the incidence of spontaneous EAE in T/R⁺ and in T/R⁻ mice was due to the presence of protective CD4⁺ T cells in the latter mice. Protection against EAE was lost in T/R⁻ mice crossed to homozygosity for a defect in the TCR-α gene. Crosses of T/R⁻ mice with mice deficient for other

Figure 4. Flow cytometry analysis of the donor CD4⁺ T cells. (A) CD4/CD3 profile of aliquots of the donor cells isolated from lymph nodes (a) or from thymi (b) of female B10.PL mice. The percentage of CD4⁺CD3⁺ T cells is indicated at the upper right corner of each panel. (B) Histograms of the expression of the indicated markers on the surface of the CD4⁺-gated donor cells from lymph nodes (a) or thymi (b).

Figure 5. Flow cytometry analysis of lymphocytes recovered from a T/R⁻ recipient of CD4⁺ T cells and from a T/R⁻ littermate. (A) CD4/Vβ8.2 profile of mesenteric lymph node lymphocytes from a T/R⁻ recipient mouse 9 wk after the transfer of CD4⁺ T cells from lymph nodes of B10.PL mouse (a), and from an age-matched T/R⁻ littermate that received no injection (b). The percentage of CD4⁺Vβ8.2⁻ (i.e., CD4⁺ nontransgenic cells) and CD4⁺Vβ8.2⁺ (i.e., mostly CD4⁺ transgenic cells) is indicated on each panel at the upper left and right quadrants, respectively. (B) Histograms (filled area) of the expression of the indicated markers on the surface of the CD4⁺Vβ8.2⁻-gated mesenteric lymph node lymphocytes from a T/R⁻ recipient mouse 9 wk after the transfer of CD4⁺ T cells from lymph node cells, or from a T/R⁻ littermate (b). In B (a), the histograms of the donor cells shown in Fig. 4 B, a are reproduced for comparison (open area). The experiments were carried out with 11 T/R⁻ recipient mice and with 5 T/R⁻ littermates. The data shown are representative of these experiments. Similar experiments were also carried out with mesenteric lymph node lymphocytes from T/R⁻ mice that received CD4⁺ T cells from B10.PL thymi. The results were similar to those shown here in part a of A and B.
genes that resulted in the lack of B cells, CD8+ T cells, NKT cells, or γδ T cells had no significant effect on the incidence of spontaneous EAE. This indicated that CD4+ T cells must be responsible for the protection observed in T/R+ mice. The ability of CD4+ T cells to protect against EAE was confirmed by the adoptive transfer of such cells, purified from normal mice, into T/R- mice. This demonstrated that the protective cells develop not only in the T/R+ mice but also in normal nontransgenic mice. To achieve full protection, the cells had to be transferred before anti-MBP TCR-expressing cells had caused any disease symptoms. Apparently, the protective T cells prevented the MBP-reactive T cells from expressing their disease-inducing potential. They could not suppress already ongoing responses of pathogenic T cells (our unpublished data), nor could they protect T/R+ mice against EAE induced by immunization with MBP (25). The inability of regulatory CD4+ T cells to suppress already activated disease-causing cells was also observed by their failure to protect recipient mice of in vitro-activated encephalitogenic T cell clones against EAE (8, 10).

The CD4+ cells we injected contained a small percentage of NK1.1 CD4+ T cells. However, our data did not support a role for these cells in the protection against EAE. In contrast to our results, Hammond et al. have shown that NOD mice could be protected from diabetes upon adoptive transfer of α/β-TCR+CD4-CD8+ thymocytes (NKT cells) through their secretion of IL-4 (26), and Wilson et al. have observed a reduced frequency of NKT cells and a loss of their capacity to secrete IL-4 in diabetic patients (27). Even though a reduced frequency of NKT cells has been observed in mice (28, 29) and humans (30) prone to autoimmune diseases, thus far no report has shown a decreased frequency of NKT cells in multiple sclerosis patients. It is unlikely that NKT cells play a protective role in every autoimmune disease.

The most extensively studied model of a spontaneous autoimmune disease is the diabetes of NOD mice. The NOD mouse model differs from our EAE mouse model in that diabetes occurs with high frequency in non-TCR transgenic mice. However, diabetes development is accelerated in NOD mice expressing a transgenic TCR derived from a diabeticogenic T cell clone (31). A recent study by Luhrer et al. (32) has shown that diabetes development is delayed and its incidence is reduced in these TCR transgenic mice if the development of more endogenous CD4+ T cells was promoted by crossing them to mice with an alternate MHC class II locus. The protective effect of the mixed MHC class II loci was reversed in mice that were homozygous for a defect in the TCR-α gene. The authors concluded that the spontaneous development of diabetes was delayed and prevented, in at least some mice, by regulatory CD4+ T cells (32). However, they did not address the role of CD8+ T cells in the protection observed. Indeed, the crosses they performed affected not only the CD4+ but also the CD8+ T cell population. Although the number of CD8+ T cells was increased in crosses with an alternate MHC class II locus, no CD8+ T cells were present in the crosses with the TCR-α defect. Thus, a protective role for CD8+ T cells cannot be ruled out in this model of autoimmune diabetes.

We observed that in T/R+ mice, which do not develop spontaneous EAE at a high frequency despite the presence of high numbers of anti-MBP TCR transgenic T cells, most non-TCR transgenic CD4+ T cells expressed memory cell markers. This is similar to the findings that, in mice carrying a transgenic TCR recognizing pigeon cytochrome c, the transgenic cells maintain a naive phenotype throughout their lives, whereas the endogenous CD4+ T cells have a memory phenotype (33). In our transfer experiments, protection against spontaneous EAE was achieved by the injection of thymic and peripheral CD4+ T cells expressing a naive phenotype. However, 9 wk after the transfer, most of these cells had acquired markers of memory cells CD45RBlowCD62LlowCD44high. This is probably the result of an extensive proliferation of the injected cells, which give rise to memory-phenotype cells (34). The spontaneous development of autoimmune diseases has also been observed in other animal models, and protection against these diseases was accomplished by transfer of memory CD4+ T cells. Diabetes developed in rats rendered lymphopenic by adult thymectomy and sublethal γ irradiation. The disease could be completely prevented by the injection of CD4+ T cells that expressed CD45RC at low levels, a marker for memory T cells (5). Protection by memory CD4+ T cells has also been observed in a model of inflammatory bowel disease. This disease was induced in SCID mice by the transfer of CD4+ CD45RBlowCD62LlowCD44high. This is similar to the findings that, in mice carrying a transgenic TCR recognizing pigeon cytochrome c, the transgenic cells maintain a naive phenotype throughout their lives, whereas the endogenous CD4+ T cells have a memory phenotype (33).

In a series of experiments with the same TCR transgenic mouse model used in our study but conducted independently, Olivares-Villagomez et al. also found that CD4+ T cells protect T/R- mice against spontaneous EAE (35).

In this study we have examined the role of each of the major classes of lymphocytes in the development of EAE. Tolerance could be reestablished and autoimmune disease prevented by reconstituting T/R- mice with CD4+ T cells. This model will be helpful to study the specificity of the regulatory cells and the mechanism by which they protect against autoimmune diseases.

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Address correspondence to Susumu Tonegawa, Center for Cancer Research, Massachusetts Institute of Technology, 40 Ames St., E17-353, Cambridge, MA 02139. Phone: 617-253-6459; Fax: 617-258-6893; E-mail: tonegawa@mit.edu

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