# The Long-Term Maintenance of Cytotoxic T Cell Memory Does Not Require Persistence of Antigen

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## Summary

I have used the transfer of primed lymphocytes into syngeneic irradiated recipients to investigate whether the persistence of antigen is required in the long-term maintenance of cytolytic T cell memory to influenza virus. Animals were immunized with influenza virus (A/WSN) and used 17 wk later as either donors for T cells or as lethally irradiated recipients. Naive age-matched mice served as controls. At intervals of 4, 8, 16, and 25 wk after T cell transfer, experimental and control groups were immunized with a heterologous virus (A/JAP) and splenocytes tested for lytic activity to influenza virus 3 and 6 d after immunization. Lytic activity 3 d after infection (a property exclusive to a memory cytotoxic T cell response) (Effros, R. B., J. Bennink, and P. C. Doherty. 1978. Cell. Immunol. 36:345.; and Hill, A. B., R. V. Blanden, C. R. Parrish, and A. Müllbacher. 1992. Immunol. Cell. Biol. 70:259.), was only observed by primed and naive irradiated recipients reconstituted with memory T cells. No day 3 responses were observed when naive T cells were transferred into irradiated primed or unprimed recipients. These observations demonstrate that cytolytic T cell memory to influenza virus is long lived in the absence of antigen.

One of the characteristics of an immune response is the acquisition of memory which manifests itself as an accelerated and elevated response to a second stimulation by the same or closely related antigen. The establishment and long-term maintenance of immunological memory underpins all protective vaccination strategies.

The B cell memory response is rather well characterized and is the consequence of antigen-driven B cell proliferation and affinity maturation (1). The question as to what mechanisms operate in the T cell compartment is still unresolved. T cell memory manifests itself in second set graft rejections (2) and enhanced cytotoxic T (Tc) cell responses in vitro (3–5) and in vivo (6, 7). Memory Tc cells have been shown to have less stringent requirements for in vitro activation than naive Tc cells (8–11). There are no known phenotypic cell surface markers that distinguish virgin from memory cells. Most markers thought to be associated with memory cells are by and large activation markers (12, 13). However, quantitative changes in the expression of cell adhesion molecules have been documented (14).

The cellular basis of Tc cell memory has been thought to reside in an increased precursor frequency as a result of the priming and longevity of such T cells (14). Recently it has been postulated that the maintenance of memory requires continuous restimulation of the memory Tc cell pool, due to either cross-reactions with third party antigen, idiotype networks (15), or persistence of antigen from the priming inoculum (16). A requirement for antigen in the maintenance of memory has been argued by Gray and Matzinger (16) in the Tc cell response to the minor histocompatibility antigen H-Y.

Here we readdress this important question and show that long-lasting Tc cell memory to influenza virus does not require the persistent presence of the priming antigen.

## Materials and Methods

*Animals.* Specific pathogen-free female BALB/c and CBA/H mice were supplied by the Animal Breeding Establishment of the John Curtin School of Medical Research.

*Viruses and Immunization.* Influenza virus strains A/WSN (H1N1) and A/JAP (H2N2) were grown and titrated as previously described (17). Female 11-wk-old BALB/c mice were immunized with $10^3$ hemagglutinin units (HAU) of A/WSN intraperitoneally.

*Cell Transfer.* Splenocytes from 32 naive animals or 32 animals immunized 17 wk previously were passed through nylon wool columns and treated with monoclonal $Ia^d$ antibody (HB3) (American Type Culture Collection, Rockville, MD) plus complement (Cedarlane Laboratories, Ltd., Hornby, ON, Canada). $2.5 \times 10^7$ splenocytes plus $10^7$ unfractionated bone marrow (BM) cells from naive animals were transferred into 24-h previously irradiated (850 rad from $Co^{60}$ source) recipients of either memory or naive type. (See Fig. 1 for flow diagram of the experimental protocol.)

*Generation of Effector Tc Cells.* One or two individual mice from the six experimental groups were immunized with $10^3$ HAU A/JAP intraperitoneally 3 or 6 d before assay, respectively. Splenocyte suspensions were prepared and assayed for lytic activity directly.

For secondary in vitro responses, aliquots from splenocytes described above ($10^7$) were cocultured for 5 d with 2 × $10^6$ syngeneic splenocytes that had been infected for 1 h with 100 HAU A/WSN in 5 ml of culture medium (Eagle's MEM [GIBCO BRL, Gaithersburg, MD] supplemented with 5% heat-inactivated fetal bovine serum [FBS; Pacific Bioindustries, Sydney, NSW, Australia], antibiotics [100 U/ml penicillin and 100 μg/ml streptomycin], and 5 × $10^{-5}$ M 2-ME) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Alloreactive Tc cells were generated by coculture of aliquots of 2 × $10^6$ splenocytes with $10^6$ irradiated (2,000 rad from a $Co^{60}$ source) CBA/H splenocytes in 2 ml culture medium for 5 d.

*Target Cells.* P815 mastocytoma (H-$2^d$) and L929 (H-$2^k$) target cells were grown and labeled with $^{51}$Cr. P815 targets were infected with influenza virus or modified with a synthetic peptide with a sequence derived from influenza virus nucleoprotein (NPP) as has been described in detail (18).

*Cytotoxicity Assay.* The standard $^{51}$Cr-release assay has been reported previously (18). Results are expressed as percent specific lysis calculated as 100 × [(experimental $^{51}$release−spontaneous release)/(maximal release−spontaneous release)]. Lytic units per spleen were calculated according to the formula: cytotoxicity units = $Y_0$.ln (1-Y), where $Y_0$ = number of target cells per assay well, Y=[(experimental $^{51}$Cr-release − spontaneous release)/(maximal release − spontaneous release)].

## Results and Discussion

The Tc cell response to influenza virus provides an ideal system to ascertain whether antigen is required to maintain memory. After initial immunization with one serotype of influenza virus, memory Tc cells can be recalled by a secondary immunization, up to at least 1 yr later, with a serologically distinct virus. The predominant response is directed against a subtype-conserved antigenic determinant encoded within the nucleoprotein gene of the virus. Such in vivo influenza-immune Tc cell responses have all the characteristics of immunological memory. The reach peak activity on day 3 and only slowly decline with still high lytic activity 10 d after secondary immunization. Primary responses, on the other hand, are absent on day 3, peak at day 6, and decline rapidly thereafter with negligible lysis by day 10 (6, 7). The T cell immune response to influenza virus does not depend on extensive viral replication or viral persistence. No influenza virus genetic material can be demonstrated 14 d after infection by PCR (19), and yet T cell memory is long-lived (5) as it is also in response to other viral infections (20). In addition, long-term Tc cell memory can be induced in mice using influenza virus inactivated by γ-irradiation (21). Here we used a long-term cell transfer system to ascertain the longevity of influenza virus–specific memory Tc cells in the absence of antigen. An outline of the experimental protocol is given in Fig. 1. Age-matched BALB/c mice, naive or primed with A/WSN influenza virus 17 wk previously were used as T cell donors and recipients. Splenocytes from a total of 32 A/WSN virus–primed mice and 32 naive animals were pooled, respectively, passed through a nylon wool column, and treated with monoclonal IA$^d$ Ab plus complement. FACS® analysis (Becton Dickinson & Co., Mountain View, CA) revealed that treated splenocytes were ≥91% from naive animals and ≥96%
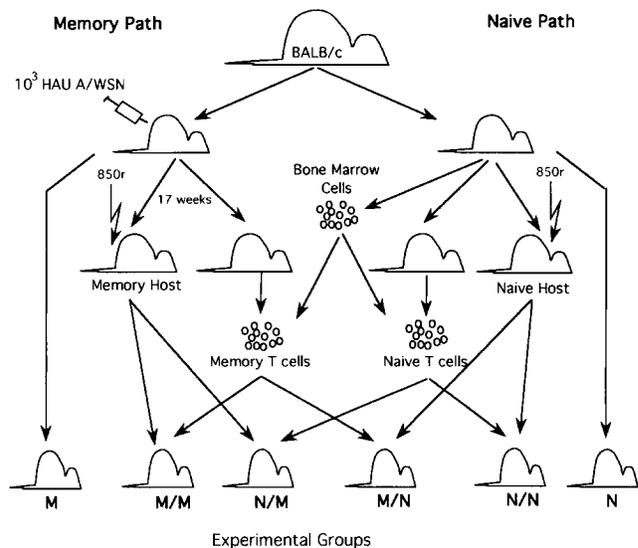


Figure 1. Schematic representation of experimental protocol.
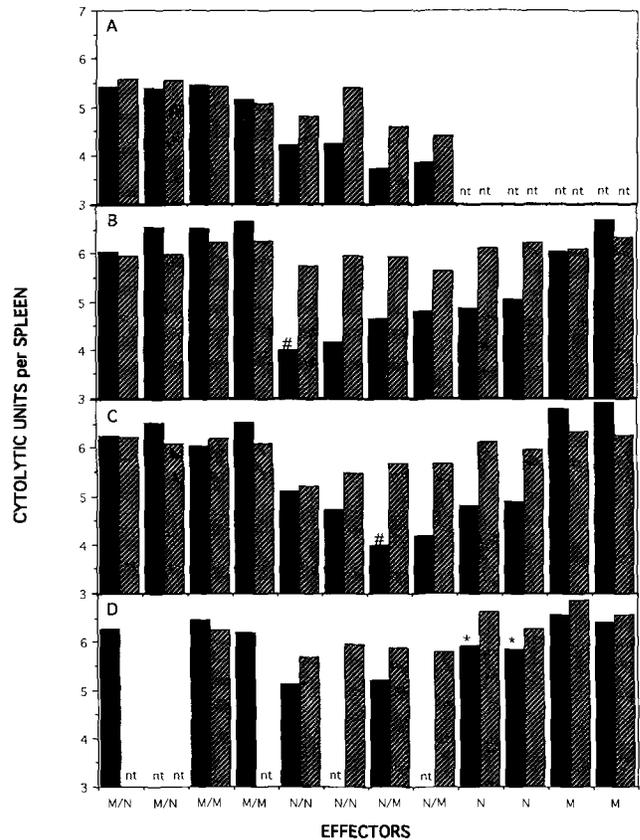
T cells from memory mice (data not shown). Memory or naive T cells (2.5 × $10^7$) plus BM cells ($10^7$) from naive syngeneic animals were then transferred into syngeneic naive or virus-primed recipients 24 h after lethal irradiation. Four experimental groups were maintained for up to 25 wk after reconstitution: memory T cells into naive recipients (M/N), memory T cells into memory recipients (M/M), naive T cells into naive mice (N/N), and naive into memory (N/M). In addition, two nonirradiated and nonreconstituted groups consisted of age-matched naive (N) and memory mice (M). One or two animals from each group were boosted in vivo with an intraperitoneal injection of the heterologous virus A/JAP 3 and 6 d before the assay of their spleen cells for viral-specific cytotoxicity. Assays were carried out at intervals of 4, 8, 16, and 25 wk after the transfer of T cells plus BM. Splenocytes from individual animals were tested on MHC-matched P815 target cells left uninfected, modified with NPP or infected with A/WSN. Fig. 2 shows the titration curves of effector cells 16 (*A* and *B*) and 25 wk (*C* and *D*) after reconstitution. Fig. 2, *A* and *C* shows effector titration curves 3 d after infection and Fig. 2, *B* and *D*, 6 d after infection. On day 3 after infection, lysis of NPP-modified target cells was observed only with splenocytes from memory animals (controls) and animals that received memory but not naive T cells, irrespective of whether the recipients were naive or had been primed. Thus, any residual long-term persisting antigen in irradiated memory recipients, if at all present, is not sufficient to prime naive T cells. However, we cannot formally exclude that irradiation with 850 rad does not irreversibly destroy an APC presenting antigen over a long time. If this was indeed the case, all M/M experimental animals would be equivalent to M/N animals, and all M/M mice gave significant day 3 responses. The lack of a requirement for antigen in the maintenance of memory is directly demonstrated by the fact that naive animals that received memory T cells gave a significantly greater lytic response on day 3, even 25 wk after transfer and a full 41 wk after primary immunization, than naive, age-
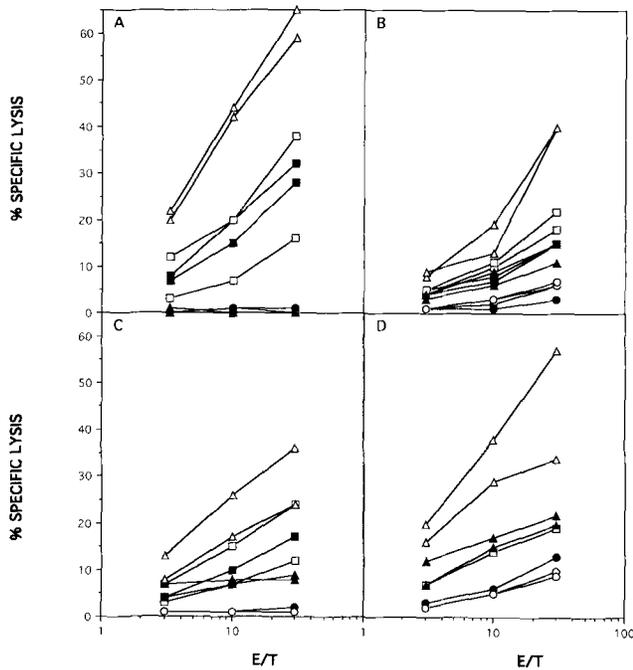
**Figure 2.** Lysis of P815-NPP modified target cells by splenocytes from experimental animals 16 (*A* and *B*) and 25 wk after reconstitution (*C* and *D*). Effector cells were assayed 3 (*A* and *B*) or 6 (*B* and *D*) days after infection. Experimental groups were as follows: (▲) naive (N), (△) memory (M), (□) memory T cells/memory recipients (M/M), (■) memory T cells/naive recipients (M/N), (○) naive T cells/memory recipients (N/M), (●) naive T cells/naive recipients (N/N). Assay duration was 6 h. All values are means of triplicates with SE never >3%.



**Figure 3.** Cytolytic units per spleen derived from the linear part of a fourfold E/T titration curve. Values for individual animals are given. Splenocytes were tested 3 (■) or 6 (▨) d after infection. (*A–D*) Splenocytes obtained for animals 4, 8, 16, and 25 wk after T cell transfer. (*nt*) Not tested. (#) No target cell lysis occurred. (*) Lysis of unmodified P815 was equal to P815-NPP.

matched controls. As expected, splenocytes from reconstituted animals gave lower lysis than their appropriate age-matched control naive or memory mice. This is a general observation with whole body irradiation transfer models and, in our study, could partially be due to the lack of, or reduced numbers, of B cells that have been shown able to act as helper cells for influenza virus–immune Tc cells (22, 23). The lytic responses 6 d after infection were consistent with those obtained in previous studies for primary and memory responses (6, 7, 17); however animals that were reconstituted with naive T cells gave very low lysis. Identical responses were found when splenocytes were tested on A/WSN virus–infected targets (data not shown), and no lysis was observed on unmodified and uninfected targets. A summary of the lytic activity of splenocytes from all the animals tested from 4 to 25 wk after transfer is given in Fig. 3. The results are expressed as lytic units per spleen to give an index of the total lytic potential. The data establish definitively that naive recipients that received memory T cells up to at least 25 wk earlier retain the memory phenotype.

No statistically significant elevation of lytic activity could be observed when memory T cells were transferred into memory recipients, thus arguing against the notion of a sequestered reservoir of antigen required for the continuous stimulation of antigen-specific T cells to maintain memory

(16). In addition, naive mice or animals that received naive T cells, showed no signs of priming by either environmental cross-reactive antigens or airborne influenza virus. This is evidenced by the absence of lytic activity 3 d after priming and by the similarity of lytic activity of splenocytes from young mice compared to old and long-term reconstituted mice. This is also corroborated by the absence of elevated lytic activity in animals where naive T cells were transferred into memory recipients vs naive recipients.

To test the overall immunological competence of the animals, set numbers of splenocytes ($10^7$) from individual mice reconstituted 16 and 25 wk previously and tested for in vivo Tc activity (Figs. 2 and 3) were stimulated in vitro for 5 d with syngeneic splenocytes infected with A/WSN and culture aliquots tested on A/WSN-infected target cells (Fig. 4, *A* and *B*). Splenocytes tested 25 wk after reconstitution were also tested for their ability to generate alloreactive Tc cells (Fig. 4 *C*). The data indicate that T cells from reconstituted animals were immunologically as competent as naive nonreconstituted animals. However, the responses varied widely between and within groups, suggesting that in vitro boosting after influenza virus restimulation in vivo may trigger complex immune regulatory events.
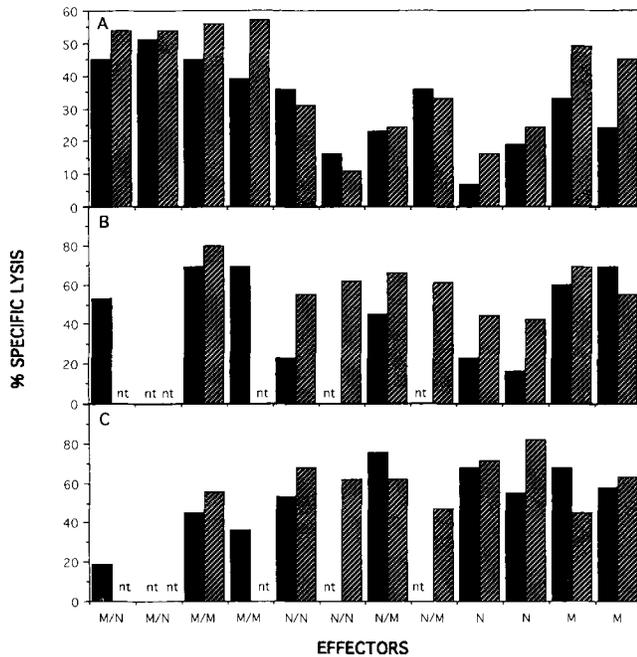
**Figure 4.** Percent specific lysis of *in vitro*-generated effector Tc cells. (*A* and *B*) Cytolytic activity on P815-NPP target cells of splenocytes from mice 16 (*A*) and 25 wk (*B*) after T cell transfer, boosted *in vitro* with syngeneic A/WSN–infected stimulator cells for 5 d. Values given are from a fourfold titration curve and values are derived from a logarithmic regression analysis solved at 1/45 aliquot of culture. (*C*) Cytolytic activity of L929 target cells of splenocytes from mice 25 wk after reconstitution with T cells and stimulated for 5 d with allogeneic CBA splenocytes. Values given were obtained as for *A* and *B*.

The possibility of generating in vitro artefacts was the prime reason to use an in vivo model not requiring in vitro restimulation. The data presented here are partly at variance with those published recently by Gray and Matzinger (16) which showed that in the absence of antigen 6 wk after primary immunization, Tc cell responses declined and were absent 16 wks after primary immunization. However, two out of five experimental animals tested 28 wk after immunization did show retention of Tc cell memory in the absence of antigen, as is observed with all animals in our study. It is difficult to explain their failure to observe memory Tc cell responses at earlier time intervals after reconstitution. One possible difference between our study and that of Gray and Matzinger is the number of T cells transferred. In our study, we used approximately a spleen equivalent ($2.5 \times 10^7$), fivefold higher than in their study, and we therefore expect our complement of memory T cells, and hence the memory response, in our mice reconstituted with memory cells, to reflect that in the intact animal.

There exists good evidence that B cell memory requires continuous restimulation by antigen, which is facilitated by follicular dendritic cells (24, 25) presenting the trapped B cell epitope in its original immunogenic form. T cells, however, recognize epitopes consisting of MHC molecules plus processed antigen. Continuous processing of a nonreplicating antigen places a time limit on its availability for the maintenance of T cell memory, if required, or other more esoteric mechanisms would need to be invoked (15, 16). On the other hand, our observations are consistent with the proposal that the longevity of T cells follows from qualitative and or quantitative changes after priming, and that such longevity is all that is required for the Tc cell memory response to be maintained.

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