10. $D^{b-/-}$ and $K^{b-/-}$ mice [S. Pascolo *et al.*, *J. Exp. Med.* **185**, 2043 (1997)] were back-crossed to each other and then to $\beta_2M^{-/-}$ mice to obtain $D^{b-/-} \times K^{b-/-} \times \beta_2M^{-/-}$ mice. All of the mice were on a C57BL/6 background.
11. Y. Vugmeyster *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12492 (1998).
12. This hyperproliferation of endogenous CD8 T cells of $D^{b-/-} \times K^{b-/-} \times \beta_2M^{-/-}$ mice is unlikely to be due to stimulation with MHC class I present on the transferred CD8 T cells because even in $-/-$ mice that have not received any cell transfer, the endogenous CD8 T cells exhibit a similar pattern of hyperproliferation (*8*).
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15. Although Tanchot *et al.* (*6*) reached a different conclusion, there are many similarities between their study and ours. In agreement with us, Tanchot *et al.* (*6*) found that naïve CD8 T cells were more dependent on MHC class I molecules than memory T cells. For example, they showed that HY-specific memory CD8 T cells (which recognize HY peptide presented by D^b) were able to expand in $D^{b-/-}$ mice, whereas naïve CD8 T cells disappeared. Similar to our finding, they also reported proliferation of HY-specific memory CD8 T cells in MHC class $I^{-/-}$ ($D^{b-/-} \times \beta_2M^{-/-}$) mice. However, in contrast to our results, they found that despite this proliferation, HY-specific memory CD8 T cells declined (>20-fold drop in 2 weeks) in MHC class $I^{-/-}$ mice. A potential complication of the Tanchot *et al.* (*6*) study was that they transferred HY-specific memory CD8 T cells from B6 mice into $D^{b-/-} \times \beta_2M^{-/-}$ mice that were on a 129 × B6 background. It is well established that mice with 129 background can pose problems in adoptive transfer experiments and in fact when we used the same mice that Rocha used in her study neither CD8 nor CD4 T cells from B6 mice survived after adoptive transfer (*20*). However, new preliminary experiments with MHC class $I^{-/-}$ mice backcrossed to B6 show the same results with HY transgenic memory T cells (B. Rocha, personal communication). Thus, it is possible that differences between our findings may reflect a unique property of the HY-specific transgenic CD8 T cells. Consistent with this is the observation that unlike most naïve CD8 T cells (either transgenic or polyclonal), naïve HY transgenic T cells do not divide even in +/+ lymphopenic mice. The study by Markiewicz *et al.* (*6*) is difficult to compare with ours because they examined the survival of in vitro–activated HY-specific effector and naïve CD8 T cells after transfer into $TAP^{-/-}$ mice. They found that the activated cell population was more dependent on MHC class I for their survival than naïve CD8 T cells. Thus, it is conceivable that survival of CD8 effectors (which are highly susceptible to apoptosis), and not memory CD8 T cells, was being analyzed in their study.
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21. Thy1.1$^+$ C57BL/6 mice were immunized with 2×10^5 PFU of LCMV Armstrong and rested for 3 to 8 months. Spleen cells from groups of these mice were pooled, stained for surface CD8, sorted in a FACSVantage (Becton Dickinson), and transferred intravenously into irradiated (550R) Thy1.2 recipients. Before transfer, LCMV-specific memory CD8 T cell numbers were determined by staining with MHC class I tetramers and by single-cell assays (ELISPOT and intracellular stain) measuring IFN-γ production (*2*). After adoptive transfer, the donor CD8 T cells were identified by the Thy1.1 marker, and LCMV-specific memory cells were quantitated with MHC class I tetramers and by cytokine assays (*2*). Memory cells transferred into MHC class I–deficient mice were analyzed for cytokine production by adding spleen cells from uninfected +/+ mice (1:1 ratio) to present the peptide. LCMV-specific memory CD8 T cells were distinguished from effector cells on the basis of minimal to no ex vivo cytolytic activity (>20-fold lower than effector cells on a per cell basis), low levels of CD69, low IL-2Rα (CD25), and low transferrin receptor (CD71) expression. They also differed from naïve cells on the basis of high expression of cell surface markers CD44 and Ly6C and their ability to induce rapid cytokine responses.
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Class II–Independent Generation of CD4 Memory T Cells from Effectors

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The factors required for the generation of memory CD4 T cells remain unclear, and whether there is a continuing requirement for antigen stimulation is critical to design of vaccine strategies. CD4 effectors generated in vitro from naïve CD4 T cells of mice efficiently gave rise to small resting memory cells after transfer to class II–deficient hosts, indicating no requirement for further antigen or class II recognition.

Signals through the T cell receptor (TCR), provided by high doses of peptide antigen bound to class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs), are critical for the activation of naïve CD4 T cells and for their transition to effector and memory cells (*1*). However, transfer of effectors, generated in vitro from naïve CD4 T cells, to adoptive hosts results in the development of long-term CD4 memory even though no further antigen is introduced (*2*), contradicting some studies stressing the importance of antigen persistence (*3*), but agreeing with others arguing against such a role (*4*). Different strengths of TCR interaction with peptide-MHC are needed for T cell response at different stages of T cell differentiation and can induce different outcomes. In the thymus, low-avidity TCR interaction with self peptides bound to self MHC induces positive selection (*5*). Naïve CD8 T cells in class I–deficient hosts (*6*) and naïve CD4 T cells in class II–deficient mice (*7, 8*) have shortened life-spans, suggesting that interactions between TCR and MHC prolong naïve T cell life-span. Naïve CD4 T cells require high avidity/density TCR interactions for induction of cytokine synthesis, whereas effector and memory cells respond efficiently at lower avidity/density (*9*). Thus, effector and memory cells might be expected to overcome this MHC dependence. However, in two recent studies, activated CD8 T cells did not generate long-term memory after transfer to class I–deficient hosts (*10*). Using a model where effectors are generated in vitro and then transferred to adoptive hosts, which do or do not express class II, we found that neither antigen recognition nor interaction with class II is necessary for the generation of memory or for its persistence.

T helper cell 1 (T_H1) or T_H2 cytokine-polarized effectors were generated in vitro from naïve CD4 T cells of AND TCR transgenic (Ig) mice (*11*) by stimulation with PCCF (a fragment of pigeon cytochrome) and mitomycin-treated I-E^k transfected fibroblast, DCEK-ICAM, or T cell–depleted APC from B10.BR mice (*2, 9*). The added APCs are no longer present in the cultures after 24 to 48 hours (*12*). In aged AND mice, Tg^+ memory cells do not develop, suggesting a lack of environmental antigens capable of stimulating cross-reacting responses (*13*). The effectors generated are >99% $CD4^+$, Tg^+ cells and contain no detectable APCs or APCs capable of mediating their restimulation (*14*).

To evaluate the possibility that endogenous TCR chains could contribute to memory generation or persistence, we crossed the AND mice to RAG-2–deficient mice [RAG knockout (KO)] (*15*). Effector cells were generated from naïve CD4 T cells, and aliquots were transferred to T cell–deficient hosts (*16*) created by adult thymectomy, lethal irradiation, and bone marrow reconstitution (ATXBM) (*17*). Both donor and recipient were on a B6 background (*17*), making allogeneic reactions unlikely. Because the hosts are devoid of T cells, there is ample opportunity for transferred cells to receive other, potentially important, non–TCR mediated signals. Equivalent numbers of Tg^+ CD4 T cells were seen in hosts 3, 8, and 13 weeks after transfer. The recovered cells were small, $CD44^{hi}$ with a memory phenotype (*17*). Equal numbers of Tg^+ recovered memory cells were restimulated ex vivo, and cytokine titers in the supernatants were determined (*18*). The cytokine pro-

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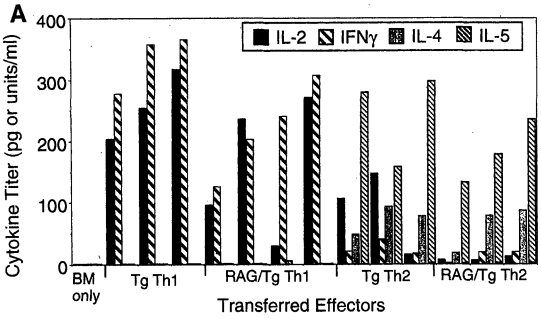
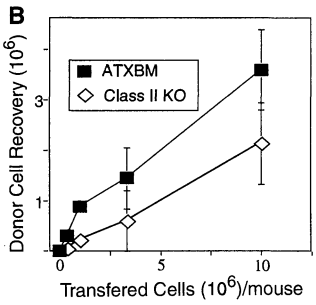




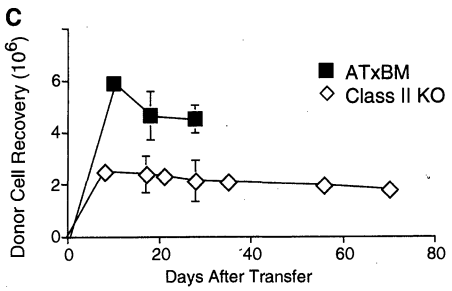
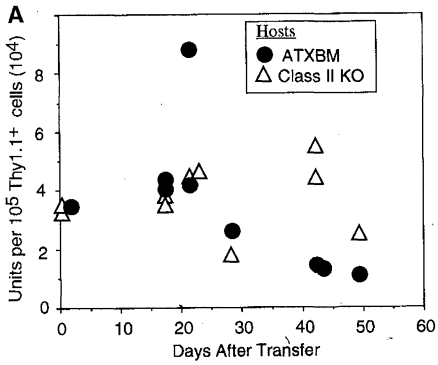


Fig. 1. Recovery of memory CD4 T cells after adoptive transfer of effectors. **(A)** Development of memory with effectors from RAG KO mice. Naïve CD4 T cells were purified from the spleens of AND TCR transgenic or AND TCR RAG-2 KO mice, and T_H1 effectors and T_H2 effectors were generated (*4, 14*). Effectors were transferred to B6 ATXBM mice (*15, 16*). **(B)** Memory is proportional to number of transferred cells. T_H2 effectors were generated from AND.PL.(Thy1.1) mice. Graded numbers of T_H2 effectors were transferred into ATXBM and class II KO mice. Four weeks after transfer, total lymph node and spleen cells of each mouse were recovered and the number donor cell per organ determined (*18*). **(C)** Recovery of CD4 memory cells in hosts without class II. T_H2 effectors were transferred into ATXBM and class II KO hosts. At various times, hosts were killed and donor cell recovery was determined. Each time point represents the mean and standard error of results from two to three mice, except for class II KO mice at week 5 and 10, for which there was only one mouse.



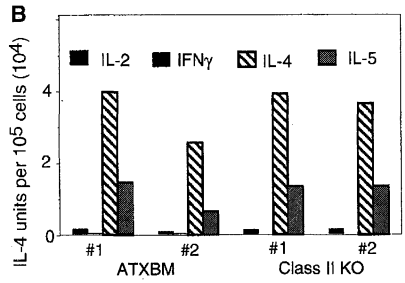


Fig. 2. Cytokine production of recovered memory cells. **(A)** Maintenance of cytokine production capacity. At different time points after transfer of $Thy1.1^+$ T_H2 effectors, Thy1.1 donor T cells were recovered from ATXBM or class II KO recipients. CD4 T cells were purified and recultured at 1.5×10^5 per milliliter with DCEK-ICAM APCs at 0.5×10^5 per milliliter and 5 μM PCCF. Supernatants were collected at 40 hours and assayed for IL-4 production (*18*). **(B)** Cytokine production by memory cells. Six weeks after adoptive transfer, Thy1.1 T cells were recovered and restimulated as in Fig. 2A. Supernatants were collected at 40 hours and assayed for IL-2, IL-4, IL-5, and IFN-γ production.

files produced 8 weeks after transfer (Fig. 1A) indicate no differences between the two groups. Thus, the lack of endogenous TCR expression among naïve CD4 T cells had no impact on the generation or persistence of functional CD4 memory cells.

To evaluate the necessity for TCR-peptide/class II interactions for memory generation from effectors, we transferred graded numbers of T_H2-polarized AND.Thy1.1 effectors to ATXBM and class II KO mice (*8*), and evaluated the number of $CD4^+$, Tg^+ memory cells recovered 4 weeks later (*16*). There was a linear relation between the number of transferred cells and the size of the memory population recovered in both hosts (Fig. 1B). Thus, the recovery of memory is a good measure of the efficiency of memory generation, and both hosts were equivalent in their abilities to support this process. The transferred cells did not expand to repopulate the host to the extent suggested for transferred whole-spleen populations (*19*). Naïve CD4 T cells that were transferred to class II KO hosts had a shortened life-span (*20*), as expected from earlier studies (*7, 8*), indicating that contaminating APCs [which are 20 to 30% in naïve populations but undetectable in effectors (*14*)] are unable to provide necessary signals to promote MHC-dependent survival.

T_H2 effectors were transferred to groups of T cell–deficient ATXBM and class II KO mice. The number of donor CD4 memory cells recovered at various times after transfer is indicated (Fig. 1C). In both hosts, the numbers of donor cells recovered initially were high, but fell to somewhat stable levels, which then persisted throughout the 10 weeks of testing. Class II KO mice had smaller lymph node and spleens compared to ATXBM mice, and they contained CD8 T cells, either of which may contribute to the lower absolute numbers recovered in the class II KO hosts. Most CD4 T cells recovered at all times after 2 weeks were small (>95%) cells expressing intermediate to high levels of CD44; of these, most (>80%) were Tg^+. Similar results were seen when Th1-polarized effectors were transferred (*21*).

Recovered cells were purified and restimulated ex vivo (*18*). The titers of interleukin-2 (IL-2), interferon-γ (IFN-γ), IL-4, and IL-5 in collected supernatants were determined (*2*). In each case, high production of appropriately polarized cytokines was seen. IL-4 production is shown in Fig. 2A. Over 7 weeks, recovered memory cells produced high levels of IL-4, comparable to those produced by initial effector cells (day 0). At the later times, cytokine production by memory cells recovered from class II KO mice was slightly higher than from ATXBM hosts (Fig. 2A). The cytokine profiles at 6 weeks (Fig. 2B) are representative of the more than 25 mice analyzed.

Although class II KO mice do not express class II or contain class II–restricted CD4 T cells (*11*), they do express CD-1 and class I, so the TCR in the AND mice could interact with these alternate MHC molecules. To examine this possibility, wild-type and class II KO mice were lethally irradiated and injected with bone marrow from AND.Thy1.1 mice. The extent of positive selection was evaluated in each host at week 6 by examining the appearance of single positive $CD4^+$ donor T cells in the thymus and periphery (Fig. 3A). In control hosts, a large population of single positive cells developed (43% of the lymphocyte gate), indicating efficient positive selection, and in the periphery, 6% of the cells were $Thy1.1^+$ (donor) $CD4^+$ cells. In contrast, there were virtually no single positive CD4 T cells in the thymus of class II KO hosts and no detectable $Thy1.1^+$, $CD4^+$ cells (less than in the staining control) in the periphery. Thus, even the weak signals required for positive selection are not available in the class II KO recipients.

In adoptive transfer experiments, minor histocompatibility differences may exist, which could lead to allogeneic reactions. In TCR Tg mice, genes in regions around the Tg may be selectively retained. Moreover, KO mice may themselves recognize on donor cells products of the gene they lack. This should not be a problem in our transfers to class II KO mice, because the hosts are T deficient and the transferred T cells do not express class II. If there are any unexpected responses of donor T cells, we would expect them to undergo some division. The CD4 T cells we recovered were small and resting, but it has been suggested that in normal animals, memory cells divide occasionally as detected by the incorporation of bromodeoxyuridine (BrdU)

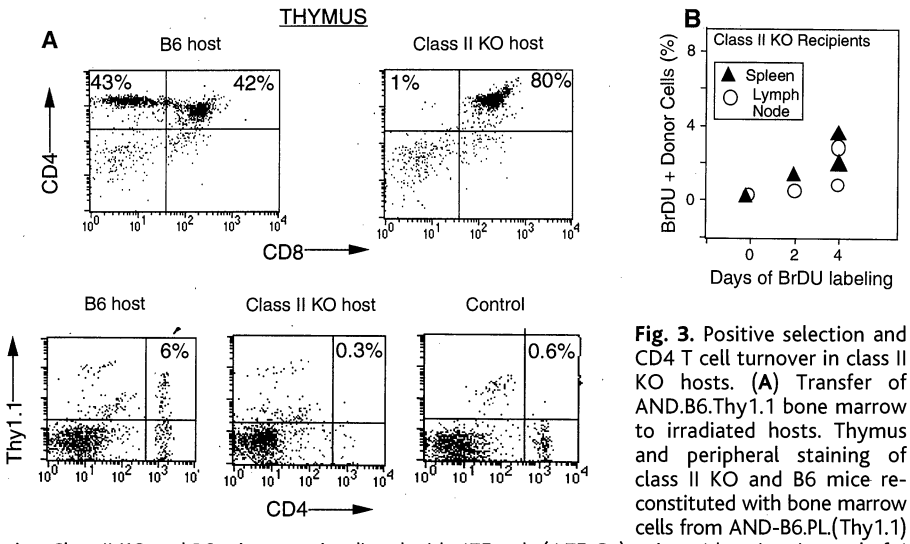


Fig. 3. Positive selection and CD4 T cell turnover in class II KO hosts. (**A**) Transfer of AND.B6.Thy1.1 bone marrow to irradiated hosts. Thymus and peripheral staining of class II KO and B6 mice reconstituted with bone marrow cells from AND-B6.PL.(Thy1.1) mice. Class II KO and B6 mice were irradiated with 475 rads (4.75 Gy) twice with a time interval of 4 hours. The irradiated mice were reconstituted with T cell–depleted bone marrow cells from AND-B6.PL mice. After 6 weeks, the thymocytes and total lymph node and spleen cells of reconstituted mice were stained with FITC-CD8α [or CD8β; (*24*)], PE-Vβ3, cychrome-CD4, and strepavidin-APC-Thy1.1. The thymus staining was gated on live Thy1.1$^+$ cells and peripheral staining were gated on live cells. (**B**) Memory cell turnover is very slow. BrdU staining of Th2 effectors after adoptive transfer in class II KO mice. Eight weeks after transfer, mice were fed with BrdU-containing water (0.8 mg/ml) for 2 or 4 days. BrdU staining was analyzed on Vβ3-, CD4-, and Thy1.1-positive cells of total lymph node or spleen cells.

(*22*). To evaluate the extent of turnover, BrdU was added to the drinking water of class II KO recipients, which had received T_H2 effectors 8 weeks previously. The fraction of donor BrdU$^+$ CD4 T cells in spleen and pooled lymph nodes was determined after 0, 2, and 4 days of labeling. Only 1 to 3% of the CD4 T cells were labeled, a very low rate (Fig. 3B). This lack of donor cell division in the class II KO mice indicates antigen stimulation is indeed negligible and that the cells in the memory population had an intermitotic time on the order of 100 days. Thus, individual memory cells, as well as the population, were long lived in the absence of both antigen and class II.

The antigen independence of the generation of memory from effectors is consistent with properties of T cell effectors. Effector cells restimulated with antigen rapidly divide and produce cytokines in large quantities (*2*), and most effectors undergo rapid activation-induced cell death (*12*). Antigen thus promotes continued activation, division, and death, none of which are compatible with development of small resting memory cells. Recent studies of CD8 T cell memory persistence, in a model where donor class I expression is minimized, suggest that CD8 memory cells, like the CD4 memory cells studied here, are antigen- and MHC-independent (*23*).

The transition from naïve to effector and memory cells thus includes a loss of dependence on interaction with self MHC for prolonged survival. With this transition, removal of antigen—not persistence in an immunogenic form—fosters memory generation and longevity.

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14. Effector populations are characterized in all experiments and they contain 99% CD4$^+$ T cells. We find that the few remaining contaminating cells found at day 4 include <0.02% class II$^+$ cells (either IA^b or IE^k, by staining with M5114 antibody). Moreover, the few class II$^+$ cells (≤0.01% in three experiments) are all CD19$^+$ (not found on professional APCs such as dendritic cells and macrophages), propidium iodide–binding cells, indicating they are dead B cells [Web figure 1 (*25*)] (H. Hu and S. L. Swain, unpublished data). We also cultured effectors for 6 to 7 days in vitro before transfer to class II KO hosts. After this time, no class II$^+$ cells were detected in the effector population, but generation of memory was retained (Web table 1). The effector populations responded vigorously to peptide in the presence of freshly added APCs, but addition of peptide to the effector population without fresh APCs resulted in no cytokine production [Web table 2 (*25*)], further supporting the lack of any functional APCs in the effector population.
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16. Transfer model: In each case, effectors were derived from highly purified naïve CD4 T cells from AND TCR Tg mice with a Vβ3/Vαll receptor specific for PCCF bound to I-E^k. Cells were cultured at 1.5 × 10^5 per milliliter with 0.5 × 10^5 per milliliter DCEK-ICAM APCs and 5 μM PCCF (fragment 88–104) in the presence of IL-2 (50 U/ml). T_H1 effectors were generated in the presence of IFN-γ (1000 U/ml) plus anti–IL-4 antibodies (11B11; 10 μg/ml), whereas T_H2 effectors were generated in the presence of IL-4 (200 U/ml) plus anti–IFN-γ antibodies (XMG1.2; 10 μg/ml). Effectors were harvested after 4 days of culture. Cells were washed and recovery of CD4 T cells was assessed by flow cytometry. Effectors were large cells expressing high levels of the transgenic TCR and CD44. Recipients received 10^7 effectors per mouse by intravenous injection. For analysis, recipients were killed at different times and their spleens and lymph node lymphocytes were analyzed by flow cytometry and by their capacity for cytokine production. For flow cytometry, recovered cells were stained with fluorescein isothiocyanate (FITC)–Vα11, phycoerythrin (PE)–Vβ3, cychrome-CD4, and strepavidin-APC-Thy1.1. The majority of cells that were positive for CD4 and for Thy1.1 donor marker were also positive for both chains of Tg TCR. Cells staining for all four markers were considered "donor cells." Donor cell recovery was the product of the fraction of donor cells in each population and in total cell recovery. The mean of results from individual animals and the standard error of that mean are shown in Fig. 1, B and C, whereas values from several individual mice are shown in Figs. 1A, 2A, 3A, and 3B.
17. Experimental animals: In all the transfer experiments, we used AND Tg mice on a C57BL/6 (B6) background derived by backcrossing the original AND mice (*11*) to B6 at least 10 times. For Thy1.1-positive donors, we used F_1 progeny of AND.B6 mice crossed to B6.PL.Thy mice, which are Thy1.1. The AND.RAG-2 KO mice were produced by crossing RAG-2 KO (male) mice on the B6 background to the AND.B6 female mice and selecting for Tg$^+$ RAG-2$^{+/-}$ progeny. These F_1 mice were mated to RAG-2 KO mice and Tg$^+$ RAG-2$^{-/-}$ progeny selected. Recipients were either B6 mice, thymectomized as adults, lethally irradiated and reconstitute with syngeneic, T-depleted bone marrow (*2*) (ATXBM) or Aβ$^{-/-}$ mice (class II KO) deficient in the β chain of class II on a B6 background. In transfers to such hosts we have never seen any evidence of either host-versus-graft or graft-versus-host response in more than 40 transfers for ATXBM and 10 transfers for class II KO recipients.
18. Conditions for ex vivo stimulation: Spleens and lymph nodes were harvested from recipients, cell suspensions prepared and enriched for CD4 lymphocytes following standard protocols for depletion of non-CD4 T cells (*2*). The percent Tg$^+$ and/or Thy1.1$^+$ cells was determined by flow cytometry. Cells were resuspended to 1.5 × 10^5 per milliliter and cultured with cells of the DCEK-ICAM APC line (0.5 × 10^5 per milliliter) (*1*) and PCCF (5 μM). After 40 hours of culture, supernatants were harvested and assayed for IL-4, IL-5, and IFN-γ by enzyme-linked immunosorbent assay and IL-2 by bioassay (*2*). Results are expressed as cytokine production per 10^5 Tg$^+$ cells.
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20. Naïve CD4 T cells from AND mice were labeled with carboxyfluorescein succinimidyl ester and transferred into ATXBM and class II KO hosts. The naïve cells had a short half-life in the class II KO hosts (11 days) compared to the ATXBM host (>35 days), consistent with a role of class II in prolonging naïve CD4 life span (H. Hu, J. Decker, G. Huston, S. Swain, unpublished data).
21. Experiments of identical design to those described in Figs. 1C and 2 have been done using Th1 effectors with equivalent results. Memory cells developed and persisted in both ATXBM and class II KO hosts at similar levels (S. Swain, unpublished data).
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References and Notes

[6] **Differential Requirements for Survival and Proliferation of CD8 Naïve or Memory T Cells**

Corinne Tanchot; François A. Lemonnier; Beatrice Pérarnau; Antonio A. Freitas; Benedita Rocha

Science, New Series, Vol. 276, No. 5321. (Jun. 27, 1997), pp. 2057-2062.

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[10] **Long-Term T Cell Memory Requires the Surface Expression of Self-Peptide / Major Histocompatibility Complex Molecules**

Mary A. Markiewicz; Cristina Girao; Joseph T. Opferman; Jiling Sun; Qinghui Hu; Alexander A. Agulnik; Colin E. Bishop; Craig B. Thompson; Philip G. Ashton-Rickardt

Proceedings of the National Academy of Sciences of the United States of America, Vol. 95, No. 6. (Mar. 17, 1998), pp. 3065-3070.

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[15] **Restoration of T Cell Development in RAG-2-Deficient Mice by Functional TCR Transgenes**

Yoichi Shinkai; Shigeo Koyasu; Kei-ichi Nakayama; Kenneth M. Murphy; Dennis Y. Loh; Ellis L. Reinherz; Frederick W. Alt

Science, New Series, Vol. 259, No. 5096. (Feb. 5, 1993), pp. 822-825.

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LINKED CITATIONS

[23] **Persistence of Memory CD8 T Cells in MHC Class I-Deficient Mice**

Kaja Murali-Krishna; Lisa L. Lau; Suryaprakash Sambhara; Francois Lemonnier; John Altman; Rafi Ahmed

Science, New Series, Vol. 286, No. 5443. (Nov. 12, 1999), pp. 1377-1381.

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