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Most microbe-specific naïve CD4+ T cells produce memory cells during infection

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Infection elicits CD4+ memory T lymphocytes that participate in protective immunity. Although memory cells are the progeny of naïve T cells, it is unclear that all naïve cells from a polyclonal repertoire have memory cell potential. Using a single-cell adoptive transfer and spleen biopsy method, we found that in mice, essentially all microbe-specific naïve cells produced memory cells during infection. Different clonal memory cell populations had different B cell or macrophage helper compositions that matched effector cell populations generated much earlier in the response. Thus, each microbe-specific naïve CD4+ T cell produces a distinctive ratio of effector cell types early in the immune response that is maintained as some cells in the clonal population become memory cells.

Infection in vertebrates elicits CD4+ memory T lymphocytes that participate in protective immunity (1, 2). The process begins when major histocompatibility complex class II (MHC II)–bound microbial peptides are displayed on host cells and recognized by T cell receptors (TCRs) on a few naïve CD4+ T cells from a vast repertoire. These cells proliferate and differentiate into distinct types of effector cells that help B cells or macrophages to eliminate the infection (3). About 90% of the cells then disappear, leaving a population of long-lived memory cells. Some naïve CD4+ T cells have been reported to make terminally differentiated effector cells, while others—perhaps those with the most avid TCRs (4, 5)—make memory cells (6). In contrast, other studies of one TCR showed that a single naïve cell can make both effector and memory cells (7, 8). Thus, the contribution of all naïve T cells in a polyclonal repertoire to the memory cell pool is unclear.

We addressed this issue by determining the fates of many single cells from the repertoire of naïve CD4+ T cells specific for an MHC II (I-A^b)–bound peptide (LLOp) from the listeriolysin O protein of *Listeria monocytogenes*. Using I-A^b tetramer–based cell enrichment and flow cytometry (9, 10), we confirmed that uninfected C57BL/6 (B6) mice contained about 80 CD4+ CD44^{low} LLOp:I-A^b tetramer–binding naïve cells (Fig. 1A) (10). Seven days after intravenous infection with the attenuated ActA-deficient *L. monocytogenes* strain (referred to hereafter as *L. monocytogenes*),

these cells proliferated to produce about 200,000 CD44^{high} effector cells (Fig. 1A) consisting of CXCR5$^-$ PD-1^{low} T helper 1 (T$_H$1) macrophage helpers (10) and two kinds of B cell helpers: CXCR5$^+$ PD-1$^-$ T follicular helper (T$_{FH}$) cells and CXCR5^{++} PD-1$^+$ germinal center T$_{FH}$ (GC-T$_{FH}$) cells (11) (Fig. 1B). The number of LLOp:I-A^b tetramer–binding cells then fell by about an order of magnitude 21 days after infection (10), and the surviving cells were CD44^{high} memory cells of two types: CXCR5$^-$ PD-1$^-$ T$_H$1 effector memory cells and CXCR5$^+$ PD-1$^-$ T$_{FH}$-like central memory cells (Fig. 1B) (10). Thus, acute systemic infection with *L. monocytogenes* caused a naïve T cell population to generate at least two long-lived memory cell populations that resemble earlier populations of effector cells.

A limiting dilution adoptive transfer strategy was then used to study the progeny of single naïve cells (12). CD4+ T cells from eight different uninfected congenic strains expressing various combinations of CD45.1, CD45.2, CD90.1, or CD90.2 were transferred together into B6 mice (CD45.2/2 CD90.2/2) at a number expected to contain on average less than one LLOp:I-A^b tetramer–binding naïve T cell from each donor population. Recipient mice were then infected with *L. monocytogenes*, and 8 days later, LLOp:I-A^b tetramer–enriched cells were stained with fluorochrome-labeled CD45.1, CD45.2, CD90.1, and CD90.2 antibodies and analyzed by flow cytometry. Cells expressing CD45.1 and/or CD45.2 were identified in the LLOp:I-A^b tetramer–binding population (Fig. 1C), and cells expressing CD90.1 and/or CD90.2 were then identified in those populations (Fig. 1D).

This strategy identified nine different LLOp:I-A^b tetramer–binding effector cell populations (Fig. 1D), one derived from the naïve cells of the recipients (CD45.2/2 CD90.2/2) and eight others from single cells from one of the donor populations (Fig. 1D). Each of the eight donor cell–

derived populations was detected in only 20 to 75% of the recipient mice and thus had an 83 to 98% chance of being derived from a single naïve cell (13). The earlier finding that all the cells from donor-derived populations like these had the identical *Tcrb-VDJ* sequence supports this contention (12).

The approach was then modified so that clonal effector and memory cells could be analyzed in the spleen from the same animal. This was accomplished by surgical removal of part of the spleen for analysis of effector cells, followed by the other part several months later for analysis of memory cells. This strategy was possible because more than 95% of secondary lymphoid organ–resident LLOp:I-A^b tetramer–binding CD4+ effector and memory T cells were in the spleen on days 8 and 62 after infection (Fig. 1E). In addition, limiting dilution transfer experiments revealed that clonal populations were in both halves of the spleen on day 8 after infection (Fig. 1F) and the clonal cells in each half had similar T$_H$1, T$_{FH}$, and GC-T$_{FH}$ ratios (Fig. 1G).

This strategy was then used to track the progeny of single naïve CD4+ T cells. Examples for two different recipient mice, each containing two donor-derived clonal populations (CD45.1/2 and CD45.1/1 for mouse 1; CD90.1/2 and CD90.1/1 for mouse 2), are shown in Fig. 2A. All four of these clonal effector cell populations generated memory cells 60 days after infection. In this experiment, 73 different clonal effector cell populations ranging from 30 to 6000 cells and averaging about 500 cells were detected in 31 mice on day 8 after infection (Fig. 2B). Sixty-seven of these populations (92%) yielded detectable memory cells on day 60 to 62. All but one of the clonal memory cell populations were smaller than their effector cell predecessor populations and on average had the same 15% survival rate as polyclonal cells of recipient origin but were more variable in this regard (Fig. 2C). Although large effector cell populations tended to have lower survival rates, as suggested in other studies (14), this weak trend was not statistically significant (Fig. 2D). On the contrary, the six populations that did not produce detectable memory cells all contained fewer than 120 cells on day 8. Although this result may indicate that naïve cells that produce few effector cells are less likely to make memory cells, it is possible that memory cells were produced from these small populations but fell below the limit of detection of the assay. Essentially identical results were obtained in a second set of experiments in which 87% (41 of 47) of day 8 clonal effector cell populations produced memory cells on day 30 after infection (fig. S1). Thus, although clonal effector cell populations undergo contraction, almost all produce memory cells.

The phenotypes of clonal LLOp:I-A^b tetramer–binding T cell populations were also assessed. Different clonal populations had different numbers and percentages of CXCR5$^-$ T$_H$1, CXCR5$^+$ PD-1$^-$ T$_{FH}$, and CXCR5^{++} PD-1$^+$ GC-T$_{FH}$ effector cells on day 8 (Fig. 3A and fig. S1), as previously described (12). The number of cells in each

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population decreased by day 60 to 62, but the surviving memory populations had about the same percentages of CXCR5$^-$ and CXCR5$^+$ cells as the parent effector cell populations (Fig. 3, A to C). However, CXCR5^{++} PD-1$^+$ cells, which were present in many effector populations, were absent in memory cell populations, whereas CXCR5$^+$ PD-1$^-$ cells were found at elevated frequencies (Fig. 3, A, C, and D). Adoptive transfer experiments demonstrated that all tetramer-binding and most tetramer-negative CXCR5^{++} PD-1$^+$ effector cells become CXCR5$^+$ PD-1$^-$ memory cells
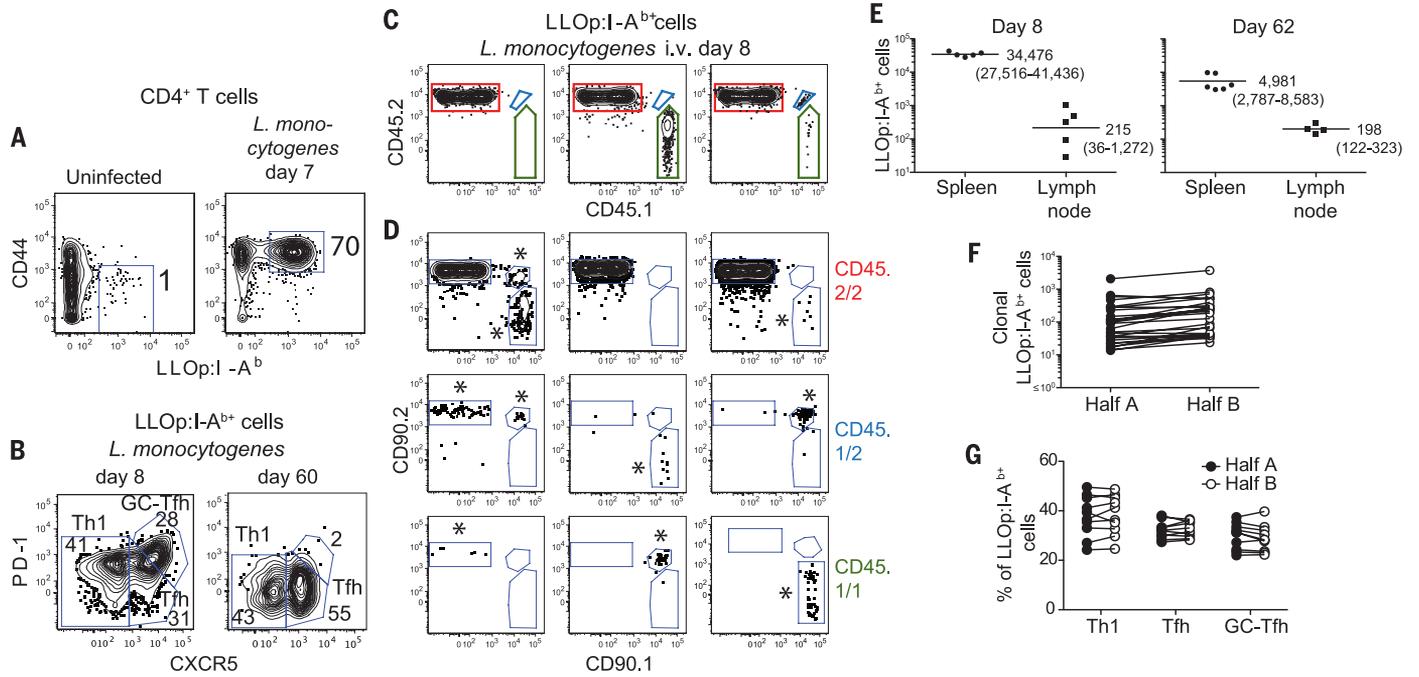

Fig. 1. Identification of clonal LLOp:I-A^b tetramer–binding T cells. (**A**) LLOp:I-A^b tetramer–enriched CD4$^+$ T cells from an uninfected mouse or a day 7 *L. monocytogenes*–infected B6 mouse with gates on CD44^{low} or CD44^{high} tetramer-binding cells. (**B**) Gates to identify LLOp:I-A^b tetramer–binding CXCR5$^-$ PD-1$^-$ T$_H$1, CXCR5$^+$ PD-1$^-$ T$_{FH}$, and CXCR5^{++} PD-1$^+$ GC-T$_{FH}$ effector cells (left) or memory cells (right). (**C**) CD45.1 versus CD45.2 expression by LLOp:I-A^b tetramer–binding cells from day 8 *L. monocytogenes*–infected B6 mice that received 7 × 10^5 CD4$^+$ T cells from eight unique CD45 and CD90 congenic strains. (**D**) CD90.1 versus CD90.2 expression on cells expressing CD45.2/2 (red), CD45.1/2 (blue), or CD45.1/1 (green) identified as in (C) from nine different mice. Cells of recipient origin were CD45.2/2 CD90.2/2. Donor-derived populations considered to be genuine (at least five events) are indicated with asterisks. (**E**) LLOp:I-A^b tetramer–binding CD4$^+$ T cells in the spleens or lymph nodes of B6 mice after *L. monocytogenes* infection. Each dot is a value from a single mouse. Geometric mean values are shown with 95% confidence intervals in parentheses. (**F**) Number of donor-derived LLOp: I-A^b tetramer–binding cells derived from single naïve cells in each half of the spleen 7 days after *L. monocytogenes* infection. Lines connect values for the same clonal population. (**G**) Frequencies of T$_H$1, T$_{FH}$, and GC-T$_{FH}$ cells [as defined in (B)] in clonal LLOp:I-A^b tetramer–binding cell populations in halves of the same spleens. In (F) and (G), lines connect populations derived from the same naïve cell. Representative data from single experiments are shown in (A) to (C). Pooled results from two independent experiments are shown in (E) to (G).

Fig. 2. Nearly all clonal effector cell populations produce memory cell populations. (**A**) LLOp:I-A^b tetramer–binding CD4$^+$ T cells from two different B6 mice that received 7 × 10^5 CD4$^+$ T cells from eight unique CD45 and CD90 congenic strains, identified by partial splenectomy and tetramer-based cell enrichment 8 days and 60 days after *L. monocytogenes* infection. Each mouse contained a recipient-derived polyclonal (CD45.2/2 for mouse 1 or CD90.2/2 for mouse 2) and two donor-derived clonal populations (mouse 1, CD45.1/2 and CD45.1/1; mouse 2, CD90.1/2 and CD90.1/1). (**B**) Number of cells produced by different single naïve cells at day 8 and day 60 or 62 after infection. (**C**) Percent survival between day 8 and day 60 or 62 after infection for different donor-derived clonal or recipient-derived polyclonal effector cell populations. Note that one clone, with a calculated percent survival of 149%, is not shown. (**D**) Number of effector cells produced by different single naïve cells at day 8 versus the percentage that survived on day 60 or 62. Statistical values in (B) and (D) were calculated with the Spearman correlation test and in (C) with Student's *t* test for unpaired samples or an F test of equality of variances. Pooled results from two independent experiments are shown.
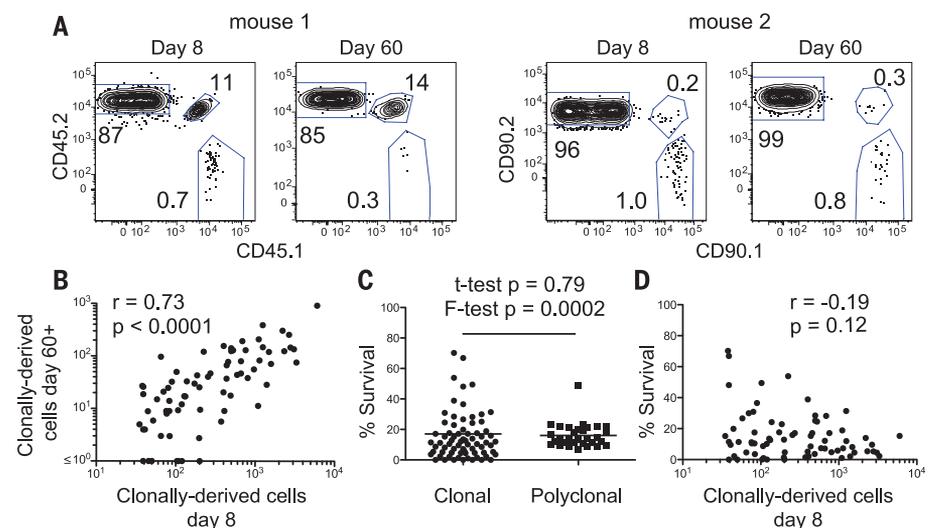
(fig. S2), as previously reported (15, 16). Thus, after contracting, memory cells retain the phenotype of their effector cell predecessors, except that CXCR5^{++} PD-1$^+$ cells lose PD-1.

We then examined the recall responses of clonal memory cell populations. Limiting numbers of CD4$^+$ T cells from uninfected congenic strains were transferred into B6 mice, which were then infected with *L. monocytogenes*. Sixty-five donor-derived clonal LLOp:I-A^b tetramer–binding memory cell populations, ranging from 6 to 1010 cells and averaging about 40 cells, were detected by partial splenectomy and cell enrichment 60 days later (Fig. 4, A and B). Seven days after another *L. monocytogenes* in-

fection, clonal memory cell populations expanded by a factor of about 15 (Fig. 4, A and B) to produce an average of about 600 effector cells (Fig. 4C), which tended to have the same CXCR5 phenotype as their memory cell predecessors (Fig. 4, C and D). Thus, clonal memory cell populations expanded during a recall infection, albeit less well than naïve cells, and produced effector cell subsets like themselves.

These results indicate that essentially all naïve T cells in a polyclonal repertoire that respond to a bacterial p:MHC II ligand by producing effector cells also produce memory cells. Although we have evidence that the tetramer used here detects many of the T cells with TCRs specific for

this ligand (17), it should be noted that some p:MHC II–specific T cells do not bind to the relevant tetramer, presumably due to expression of low-affinity TCRs (18). Thus, although all naïve CD4$^+$ T cells with TCRs that bind p:MHC II tetramer can become memory cells, it remains to be seen whether this conclusion applies to any p:MHC II–specific cells that do not.

Although the nature of the TCR signal experienced by individual naïve clones could influence their effector cell fate (12), the present results suggest that all TCRs can support memory cell formation, although some may be better at it than others (4–6). The observation that a clonal memory cell population was very likely to
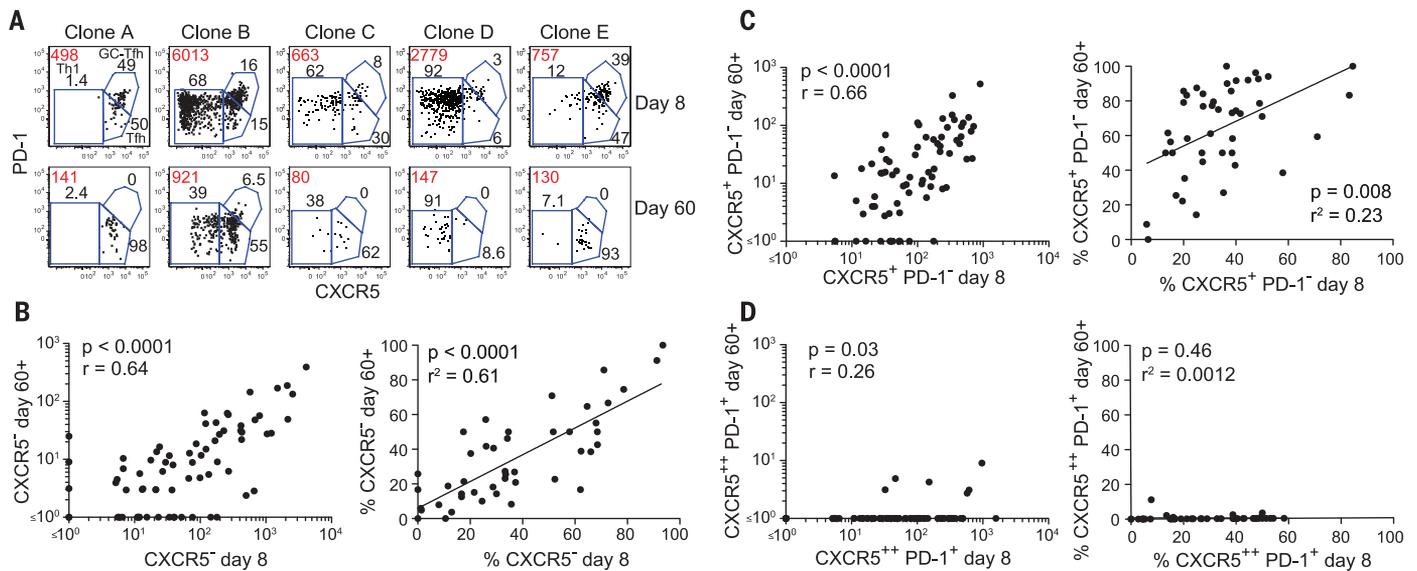

Fig. 3. Memory cells retain the CXCR5$^-$ and CXCR5$^+$ phenotype of effector cells. (**A**) Identification of T$_H$1, T$_{FH}$, and GC-T$_{FH}$ effector cells in different clonal populations of CD44^{high} LLOp:I-A^b tetramer–binding cells on day 8 and day 60 after infection. Red values indicate the absolute number of cells in each population; black values indicate the percentages of each subset. (**B** to **D**) Left graph: Numbers of CXCR5$^-$ (B), CXCR5$^+$ PD-1$^-$ (C), or CXCR5^{++} PD-1$^+$ (D) cells in clonal populations at day 8 and day 60 or 62 after infection. Statistical values were calculated with the Spearman correlation test. Right

graph: Percentages of cells in LLOp:I-A^b tetramer–binding clonal populations that were CXCR5$^-$ (B), CXCR5$^+$ (C), or CXCR5^{++} PD-1$^+$ (D) on day 8 plotted versus the percentages of those subpopulations 60 to 62 days after infection. Each dot represents a clonal population. Only populations in which five or more events were recovered at day 60 or 62 were included to optimize the meaningfulness of the percentage values. Statistical values and trend lines from linear regression analyses are shown. Each dot represents a single clonal population. Pooled results from two independent experiments are shown.
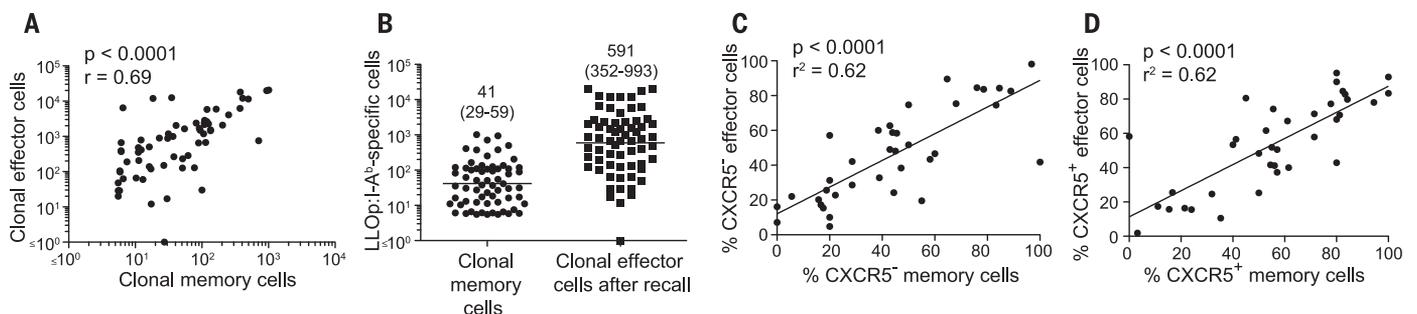

Fig. 4. Memory cells produce effector cells with a similar phenotype. (**A**) Numbers of LLOp:I-A^b tetramer–binding memory cells in 65 clonal populations plotted versus the number of effector cells produced from those populations 7 days after a second infection. (**B**) Numbers of LLOp:I-A^b tetramer–binding memory cells in 65 clonal populations before or 7 days after a second infection. Geometric mean values with 95% confidence

intervals in parentheses are shown. (**C** and **D**) Percentages of CXCR5$^-$ or CXCR5$^+$ memory cells in individual clonal populations plotted versus the percentages of CXCR5$^-$ or CXCR5$^+$ effector cells generated from those populations after secondary infection. Statistical values and trend lines from linear regression analyses are shown. Pooled results from three independent experiments are shown.

have the same helper cell subset ratio as its predecessor population is consistent with each effector cell in the population having the same chance of becoming a memory cell. This model is consistent with studies of CD8^{+} T cells indicating that memory cells arise from the effector cell pool by a TCR-independent stochastic process (14, 19–21).

Our finding that a clonal CD4^{+} memory T cell population tended to produce an effector cell population with the same subset composition after recall suggests that a CXCR5^{−} memory cell produces CXCR5^{−} effector cells and that a CXCR5^{+} memory cell produces CXCR5^{+} effector cells. Thus, although we (10) and others (15) have found that bulk CXCR5^{+} memory cells can produce CXCR5^{−} and CXCR5^{+} effector cells, the present results fit the suggestion that both CXCR5^{+} and CXCR5^{−} cells are relatively lineage-committed (15). An advantage of this process is that the helper cell subset diversity of the effector cell pool is carried into the memory cell pool and retained thereafter.

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SEX CHROMOSOME

Two genes substitute for the mouse Y chromosome for spermatogenesis and reproduction

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The mammalian Y chromosome is considered a symbol of maleness, as it encodes a gene driving male sex determination, Sry, as well as a battery of other genes important for male reproduction. We previously demonstrated in the mouse that successful assisted reproduction can be achieved when the Y gene contribution is limited to only two genes, Sry and spermatogonial proliferation factor Eif2s3y. Here, we replaced Sry by transgenic activation of its downstream target Sox9, and Eif2s3y, by transgenic overexpression of its X chromosome–encoded homolog Eif2s3x. The resulting males with no Y chromosome genes produced haploid male gametes and sired offspring after assisted reproduction. Our findings support the existence of functional redundancy between the Y chromosome genes and their homologs encoded on other chromosomes.

Many sexual characteristics are influenced by sex chromosome constitution, with mammalian females typically carrying XX and males XY. We recently reported that in the mouse, only two Y-chromosome genes—testis-determinant Sry and spermatogonial proliferation factor Eif2s3y—are needed for successful assisted reproduction (1). Here, we asked if these two genes could be replaced by transgenic activation of their homologs encoded on other chromosomes.

For Sry replacement, we chose Sox9 (Sry-related high-mobility–group box gene 9), a direct target of SRY (2). Prior work showed that transgenic overexpression of Sox9 driven by the Wt1 promoter results in female-to-male sex reversal in XX mice (3). We placed the Wt1-Sox9 transgene in the context of a single X chromosome carrying the Eif2s3y transgene (fig. S1A) (4) and found that it generated males (X[E]OSox9). In these males, the Y-chromosome gene contribution is limited to Eif2s3y (table S1).

XOSry males, which carry an autosomally encoded Sry transgene, develop testes containing spermatogonia that are unable to proliferate, which results in seminiferous tubules appearing empty when compared with those from males with an intact Y chromosome (XY) (Fig. 1, A and B). This defect can be overcome by transgenic Eif2s3y addition to the X chromosome (X[E]OSry) (table S1 and fig. S4A) (1, 5). To replace Eif2s3y, we transgenically overexpressed its X chromosome–encoded homolog, Eif2s3x (fig. S2). We then placed the Eif2s3x transgene in the context of XOSry (fig. S1B and supplementary text). The resulting XOSry,Eif2s3x males (carrying autosomally

encoded Sry and Eif2s3x transgenes) had the Y-chromosome contribution limited to Sry (table S1).

X[E]OSox9 and XOSry,Eif2s3x males had small testes (fig. S3), but spermatogenesis was initiated and progressed through meiosis and arrested at the round spermatid stage (fig. S4, B and C). Spermatogonia/Sertoli ratios in X[E]OSox9 and XOSry,Eif2s3x and spermatid/Sertoli ratio in X[E]OSox9 were comparable to X[E]OSry but lower than those in XY (Fig. 1, D and E). Round spermatids in XOSry,Eif2s3x were dramatically depleted; X[E]OSry and XY had 10 and 88 times as many, respectively (Fig. 1E and table S2). The spermatids from both X[E]OSox9 and XOSry,Eif2s3x males were functional in assisted fertilization, and live offspring were obtained after embryo transfer (Table 1).

We next tested whether spermatogenesis can take place in males with a complete absence of Y-chromosome genes. We used the same transgenes that were successful in single–Y gene substitutions (Wt1-Sox9 and Eif2s3x Tg1) to generate mice transgenic for Sox9 and Eif2s3x in the XO context (XOSox9,Eif2s3x) (fig. S1C and table S1). The majority (35 out of 48) of XOSox9,Eif2s3x males had testicular defects and essentially no germ cells (fig. S5 and supplementary text). In the remaining males, spermatogonial proliferation arrest was overcome (Fig. 1C and fig. S5), and spermatogenesis progression was comparable to that of XOSry,Eif2s3x (Fig. 1, D and E, and table S2). Using assisted reproduction [round spermatid injection (ROSI)], we injected oocytes with spermatids from 13 males and obtained zygotes with two well-developed pronuclei and normal two-cell embryos (fig. S6, A to C, and movie S1). Embryos from 11 males were used for transfer. Ten resulted in pregnancy, and nine yielded offspring (Table 1). Among the males that yielded progeny, there were F₁, F₂, and F₃ generation XOSox9,Eif2s3x ROSI males (fig. S6D). ROSI offspring from males with one or no Y-chromosome

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Science

Most microbe-specific naïve CD4+ T cells produce memory cells during infection

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All T cells can remember
One of the hallmarks of adaptive immunity is that T and B lymphocytes "remember" previous infections, protecting the host from subsequent infections. When T cells respond to a pathogen, they proliferate, and a fraction of their progeny goes on to form long-lived memory cells. It is not clear whether all of the T cell clones that respond to the initial infection have the potential to form memory T cells. Tubo *et al.* used a single-cell adoptive transfer model in mice to answer this question. Nearly all T cell clones produced memory cells, which suggests that breadth is probably an important component of immunological memory.

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