no acid position 9 in all *SD* strains examined, and a C-to-Y substitution at position 146 in only some *SD* strains. In addition, because the mutant cDNA is incomplete at the 5′ end, we have been unable to confirm that the distal dRanGAP transcript initiates at the equivalent point as the wild-type and proximal dRanGAP transcripts. If there is a difference here, it should affect only the 5′ untranslated leader and not the coding sequence.

13. A genomic fragment encoding amino acids 1 through 251 of the wild-type dRanGAP protein was cloned into the pQE30 expression vector (Qiagen) and expressed in *Escherichia coli*. The gel-purified polypeptide was injected into a rabbit, and subsequent bleeds were examined by protein immunoblot analysis for the ability to identify the wild-type and mutant dRanGAP proteins in *Drosophila* testes. The expressed protein was bound to nitrocellulose strips and incubated with crude antiserum to affinity purify the antibodies to RanGAP.
14. Ten independently isolated *SD* lines from the United States (*SD-MAD*, *SD-5*, *SD-72*, and *SD-Weymouth*), Italy (*SD-Roma*, *SD-Oviedo*, and *SD-VO17*), Spain (*SD-Los Arenos*), Japan (*SD-NH2*), and Australia (*SD-Armindale*) were examined by protein immunoblot analysis and found to express both the 66-kD and 40-kD proteins. Both proteins were present at consistent levels in testes, whole flies, carcasses (minus testes or ovaries), heads, larvae, and pupae. Because no somatic phenotypes are seen in *SD* heterozygotes or homozygotes, it is likely that some step in spermatogenesis, such as the high degree of chromatin compaction that takes place, is particularly sensitive to perturbations caused by expression of the truncated dRanGAP.
15. The apparent reduction in dRanGAP protein expression in the P[(w^+Sd)12A] transformant is likely due to the particular location of the insert on the *TM3* chromosome, because both increased and decreased levels of dRanGAP expression are observed when P[(w^+Sd)12A] is remobilized to new insertion sites (C. Merrill and B. Ganetzky, unpublished results).
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17. Although how the truncated dRanGAP is functionally altered remains unknown, it is worth noting that the truncation eliminates the lysine residue at position 533, which is the presumed target site for covalent linkage to the small ubiquitin-related modifier SUMO-1 [S. Saitoh, R. T. Pu, M. Dasso, *Trends Biochem. Sci.* **22**, 374 (1997); R. Mahajan, L. Gerace, F. Melchior, *J. Cell Biol.* **140**, 259 (1998)]. Because this modification is essential for targeting dRanGAP to nuclear pore structures, subcellular mislocalization of the truncated dRanGAP is one potential consequence.
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24 November 1998; accepted 5 February 1999

Linear Differentiation of Cytotoxic Effectors into Memory T Lymphocytes

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A central question in immunology is the origin of long-lived T cell memory that confers protection against recurrent infection. The differentiation of naïve T cell receptor transgenic CD8$^+$ cells into effector cytotoxic T lymphocytes (CTLs) and memory CD8$^+$ cells was studied. Memory CD8$^+$ cells that were generated after strong antigenic stimulation were the progeny of cytotoxic effectors and retained antigen-specific cytolytic activity 10 weeks after adoptive transfer to antigen-free recipient mice. Thus, potential vaccines based on CTL memory will require the differentiation of naïve cells into post-effector memory T cells.

The engagement of T cell receptors (TCRs) on CD8$^+$ T cells by antigen peptide–class I major histocompatibility complexes (pMHC) on the surface of cells leads to the proliferation and differentiation into CTLs, which lyse cells presenting antigen pMHC (*1*). After the effector phase, a period of death ensues during which activated T cells undergo apoptosis, known as activation-induced cell death (AICD) (*2*). The third phase of the T cell response is characterized by the appearance of memory cells that persist for many years. Accelerated T cell responses seen upon reexposure to antigen are due to increases in the frequency of antigen-specific T cells and to qualitative changes in memory cells that allow them to respond to antigen more efficiently than naïve cells (antigen hyperreactivity). However, the precise lineage by which naïve CD8$^+$ lymphocytes differentiate into memory cells is unclear. There are two models for the development of memory CD8$^+$ cells. The linear differentiation model predicts that memory T cells are the progeny of CTLs that escape AICD. Conversely, weak antigenic stimulation could result in memory T cells that are derived from a precursor that precedes CTLs and so differentiate through a lineage parallel to effectors (decreasing potential model) (*3*).

To address the issue of CD8$^+$ cell memory differentiation, we analyzed the development of transgenic memory CD8$^+$ cells that express a H-2D^b–restricted TCR (B6.2.16) specific for a male antigen (H-Y) (*4*). Activation of B6.2.16 CD8$^+$ cells with male cells (*5*) or H-Y peptide (*6*) gives rise to long-lived anti–H-Y memory CD8$^+$ cells that persist in the absence of antigen. In the absence of markers that distinguish between effector and memory CD8$^+$ cells, we followed the differentiation of naïve CD8$^+$ cells into effector and memory-precursor cells in vitro by "counting" the number of cell divisions. B6.2.16 CD8$^+$ cells were labeled with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE), then incubated with H-Y peptide (*7*). The proliferation of B6.2.16 CD8$^+$ cells was controlled by using various doses of H-Y peptide for 4 days of culture, and cell division was monitored by measuring 50% decreases in CFSE fluorescence (Fig. 1A). Staining of cells with the TCR-clonotypic monoclonal antibody (mAb) T3.70 (*8*) revealed that all of the divided CD8$^+$ cells in culture were B6.2.16 TCR-positive (*9*). Low doses of antigen resulted in little cell division and little detectable anti–H-Y cytolytic activity (0.1 nM H-Y peptide: <5% specific lysis). At 100 nM H-Y peptide we observed substantial cell division and a high degree of cytolytic activity (>50% specific lysis), demonstrating that antigen dose can control the differentiation of anti–H-Y CTLs (*10*).

At a fixed antigen concentration (100 nM) over 4 days in culture, B6.2.16 CD8$^+$ cells proliferated over the first five cell divisions (generation 1, 2×10^4 cells; generation 5, 29×10^4 cells) then decreased (generation 9, 6×10^4 cells) due to AICD (Fig. 1B). We observed an increase in anti–H-Y cytolytic activity with each generation (Fig. 1C). The cytolytic activity of CD8$^+$ cells remained constant until generation 7, after which it diminished, presumably because of AICD. The onset and maintenance of cytolytic activity over the course of cell division correlated with accumulation of cytoplasmic perforin, a molecule required for efficient target cell lysis by CTLs (*11*) (Fig. 1D), and by five divisions every CD8$^+$ cell was positive for intracellular perforin (Fig. 1E) (*12*). Because every B6.2.16 CD8$^+$ cell had cytolytic machinery after five cell divisions, all cells had differentiated into effectors.

To examine the effect of antigen dose on memory cell differentiation we quantitated the number of memory cells generated from B6.2.16 CD8$^+$ cells that had been cultured with various doses (Figs. 1A and 2A). Equal numbers of naïve and activated CFSE-labeled

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B6.2.16 CD8$^+$ cells were adoptively transferred to antigen-free mice deficient for the recombination-activating gene 1 (RAG-1) (*13*). After 21 or 70 days, we compared the ability of the two populations to give rise to anti–H-Y CTLs after in vitro challenge with H-Y peptide (*14*). Low antigen doses, which failed to drive the differentiation of cytolytic effectors, did not drive the differentiation of memory cells (Fig. 2A). However, cells incubated with 100 nM H-Y peptide underwent substantial cell division and gave rise to memory cells. Thus, strong antigen stimulation was required for the production of memory CD8$^+$ cells.

To determine if memory CD8$^+$ cells arose from differentiated effector CTLs or from progenitors that developed before effector CTLs, we measured the ability of post- and pre-effector B6.2.16 CD8$^+$ cells to give rise to memory cells after adoptive transfer. The progeny of pre-effector cells did not generate any more anti–H-Y CTLs than adoptively transferred naïve cells (Fig. 2, B and C) (*15*). Only cells derived from activated B6.2.16 CD8$^+$ cells that had divided more than five times in vitro gave rise to a greater number of H-Y CTLs than adoptively transferred naïve cells. Memory CD8$^+$ cells were smaller than activated cells (forward light scatter in arbitrary units: activated cells 497, memory cells 390 ± 2, n = 5) and remained CD44^{hi} (mean fluorescence intensity, MFI: 496 ± 23, n = 2) even 10 weeks after adoptive transfer, whereas naïve cells remained CD44^{lo} (MFI: 280 ± 8, n = 3). It is unlikely that the progeny of activated B6.2.16 CD8$^+$ cells were stimulated by persistent antigen from the adoptive transfer procedure. First, activated B6.2.16 CD8$^+$ cells were depleted of antigen-presenting cells (>95% pure) before adoptive transfer, and second, H-Y peptide–H-2D^b molecules on the surface of cells are short-lived (*6*). To exclude the possibility that memory CD8$^+$ cells were derived from the small amount (<5%) of undivided cells present after 4 days of culture, we adoptively transferred activated B6.2.16 CD8$^+$ cells that were depleted of undivided cells by fluorescence activated cell sorting (FACS). After 21 days, the number of anti–H-Y CTLs generated from the FACS-purified B6.2.16 CD8$^+$ cells [1.0 (± 0.6) × 10^3 per mouse, n = 3] was comparable with that of unsorted CD8$^+$ cells of similar generation number [median generation of division was 6; 2 (± 1) × 10^3 per mouse, n = 3]. Thus, the contribution of undivided B6.2.16 CD8$^+$ cells from day 4 cultures in generating anti–H-Y CTLs after adoptive transfer and antigen challenge was negligible, and therefore memory CD8$^+$ cells were the progeny of anti–H-Y CTLs.

The quantitation of CTL precursor frequency after antigen rechallenge may result in an underestimation of the number of memory CD8$^+$ cells, presumably because not all memory CD8$^+$ cells can give rise to CTLs (*16*). However, the progeny of post-effector cells gave rise to the greatest number of memory cells (Table 1) and anti–H-Y CTL precursors. Differences in the recovery of

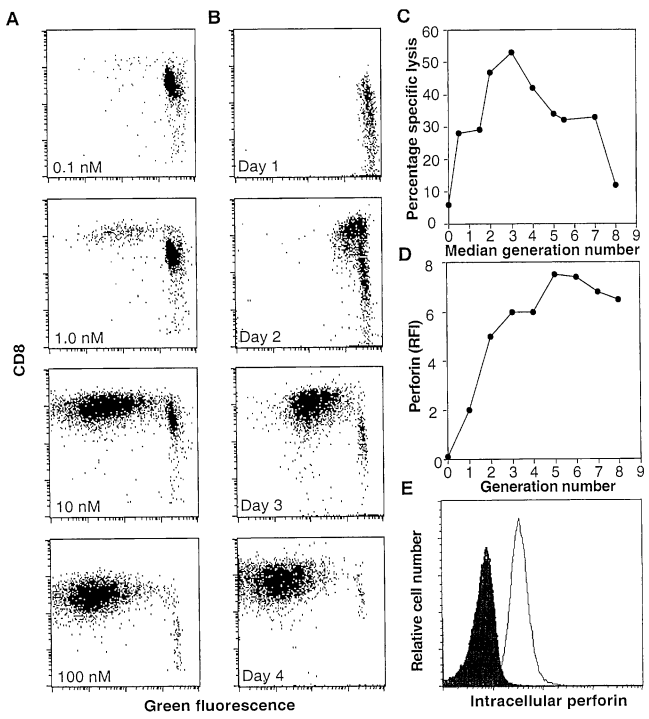

Fig. 1. Differentiation of anti–H-Y CTLs in vitro. Analysis of cell division by flow cytometry of CFSE-labeled CD8$^+$ cells from B6.2.16 transgenic mice activated (**A**) with various doses of H-Y peptide for 4 days or (**B**) with 100 nM H-Y peptide for up to 4 days. (**C**) Cytolytic ability of CFSE-labeled, activated B6.2.16 CD8$^+$ cells that had undergone cell divisions after culture with H-Y peptide at 100 nM. (**D**) Intracellular perforin staining on CFSE-labeled B6.2.16 CD8$^+$ cells is expressed as the times fluorescence intensity above the isotype control in each generation of cell division (relative fluorescence intensity, RFI). (**E**) Intracellular perforin staining (open histogram) and isotype control (closed histogram) of CFSE-labeled, activated B6.2.16 CD8$^+$ cells from generation number 5.

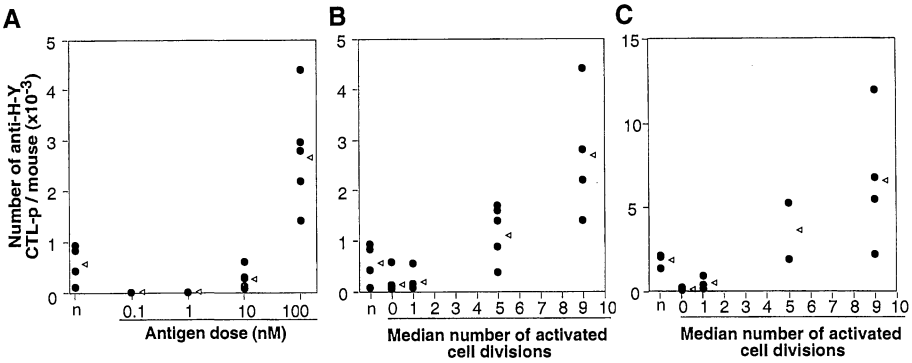

Fig. 2. Generation of memory CD8$^+$ cells requires at least five cell divisions. Numbers of anti–H-Y CTL precursors were quantitated by limiting dilution analysis from mice adoptively transferred with CFSE-labeled naïve (n) or activated B6.2.16 CD8$^+$ cells that had undergone a defined number of cell divisions after culture with 100 nM H-Y peptide. Each filled circle represents an individual animal, and the open triangle denotes the mean number of anti–H-Y CTL precursors (CTL-p) per mouse. (**A**) Twenty-one days after adoptive transfer, the number of anti–H-Y CTL precursors derived from CFSE-labeled B6.2.16 CD8$^+$ cells that were incubated with 100 nM H-Y peptide (mean = 3 × 10^3, n = 5) was greater than the number of CTL precursors derived from naïve cells (mean = 0.6 × 10^3, n = 4) ($0.01 > P > 0.005$) or activated cells incubated with H-Y peptide at 10 nM (mean = 0.3 × 10^3, n = 7) ($0.002 > P > 0.001$), 1 nM (mean < 0.1 × 10^3, n = 3) ($0.002 > P > 0.001$), or 0.1 nM (mean < 0.1 × 10^3, n = 2) ($0.05 > P > 0.02$). (**B**) After 21 days, the number of anti–H-Y CTL precursors derived from B6.2.16 CD8$^+$ cells of median generation number 9 (mean = 3 × 10^3, n = 4) was greater than the number of CTL precursors derived from naïve cells (mean = 0.6 × 10^3, n = 4) ($0.02 > P > 0.01$) or activated cells of median generation 0 (mean = 0.2 × 10^3, n = 7) ($P < 0.001$), 1 (mean = 0.2 × 10^3, n = 5) ($0.005 > P > 0.002$), or 5 (mean = 1 × 10^3, n = 6) ($0.05 > P > 0.02$). (**C**) After 70 days, the number of anti–H-Y CTL precursors derived from cells of median generation number 9 (mean = 7 × 10^3, n = 4) was greater than the number of CTL precursors derived from naïve cells (mean = 1.9 × 10^3, n = 3) ($0.05 > P > 0.02$), activated cells of median generation 0 (mean = 0.2 × 10^3, n = 3) ($0.05 > P > 0.02$), or 1 (mean = 0.5 × 10^3, n = 3) ($0.1 > P > 0.05$). The frequencies of anti–H-Y CTL precursors in mice injected with B6.2.16 CD8$^+$ cells incubated with 0.1 nM or 1 nM were below the level of detection, therefore they are nominally considered to have a number of less than 0.1 × 10^3 B6.2.16 CD8$^+$ cells per mouse.

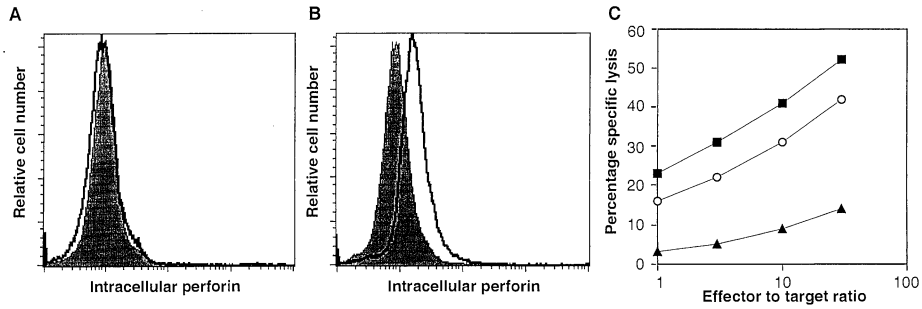

Fig. 3. Ex vivo cytolytic function of B6.2.16 CD8$^+$ memory cells. Intracellular perforin staining (open histogram) and isotype control (closed histogram) of B6.2.16 CD3$^+$ cells harvested from (**A**) naïve B6.2.16 transgenic mice and (**B**) recipient mice 70 days after adoptive transfer with activated B6.2.16 CD8$^+$ cells (4 days with 100 nM antigen). (**C**) Direct ex vivo cytolysis of antigen-labeled targets by naïve B6.2.16 CD8$^+$ cells (closed triangles), B6.2.16 CD8$^+$ cells activated in vitro for 2.5 days (open circles), and B6.2.16 CD8$^+$ memory cells 70 days after adoptive transfer to antigen-free hosts (closed boxes).

Table 1. Numbers of B6.2.16 CD8$^+$ cells recovered from RAG-1–deficient recipient mice. Naïve and activated B6.2.16 CD8$^+$ cells, adoptively transferred to recipient mice, were harvested from hosts after 21 and 70 days. Cell numbers were calculated by staining with T3.70 mAb clonotypic for B6.2.16 TCR expression and anti-CD8 mAb to determine the percentage of positive cells in each recipient animal. Values are the mean ± SEM from (*n*) recipient mice.

Median generation	10^4 recovered cells (*n*)	
	Day 21	Day 70
	Naïve	
–	6.7 ± 2.0 (4)	3.9 ± 0.1 (3)
	Activated	
0	0.30 ± 0.01 (4)	1.9 ± 0.2 (4)
1	1.3 ± 0.3 (5)	3.1 ± 1.0 (5)
5	3.4 ± 0.7 (6)	7.0 ± 3.7 (2)
9	5.8 ± 0.5 (4)	8.6 ± 1.7 (5)

CD8$^+$ memory cells may be due in part to differences in the ability of cells, at various stages of differentiation, to home to the spleen and lymph nodes. However, 10 weeks after adoptive transfer fewer post-effector cells (25 ± 5, $n = 4$) than naïve cells (75 ± 10, $n = 2$) were required to generate one anti–H-Y CTL precursor after challenge with antigen in a limiting dilution assay. Therefore, the progeny of anti–H-Y CTLs generated more long-lived memory cells that had antigen hyperreactivity.

The memory cells derived from activated B6.2.16 CD8$^+$ cells of median generation 9 retained the presence of intracellular perforin (activated cells: 1.6 ± 0.1 times that of the isotype control, $n = 6$; naïve cells, 1.1 ± 0.1 times more) up to 70 days in the absence of antigen (Fig. 3, A and B), as in human memory CD8$^+$ cells (*17*). We tested their ability to lyse antigen-labeled targets directly ex vivo (*18*). When compared with equal numbers of naïve cells, memory cells harvested after adoptive transfer had seven times higher specific lysis (Fig. 3C). This degree of lysis was slightly higher than B6.2.16 CD8$^+$ cells activated in vitro for 2.5 days with 100 nM H-Y peptide. Therefore, memory CD8$^+$ cells had cytolytic machinery and were capable of direct cytolysis after 10 weeks without antigen.

Our finding that memory CD8$^+$ cells are derived from the progeny of cytotoxic effectors supports a linear differentiation model of memory cell development. The progeny of cytotoxic effectors are prone to AICD (*19*), yet they are the precursors of anti–H-Y memory cells. Therefore, we propose that a selective mechanism allows a low proportion of post-effector cells to escape apoptosis and differentiate into memory cells. This is in contrast to an instructive mechanism proposed by the decreasing potential model, in which different signals transduced by the TCR and costimulatory molecules of naïve cells lead to the differentiation of effector and memory cells, which develop along separate pathway (*20*).

Although our results do not support the differentiation of effector and memory CD8$^+$ cells along separate lineages, the dichotomy of effector B cell and memory B cell differentiation is well established (*21*). Long-lived memory B lymphocytes produce anti-viral antibodies after pathogen rechallenge thus providing protective immunity (*22*). However, the highly desirable induction of protective CTL memory by vaccination has proved difficult (*23*). The linear differentiation of memory CD8$^+$ cells predicts that effective CTL memory can only be generated after complete effector cell differentiation. These findings may allow the development of vaccines that induce protective cytotoxic T cell memory.

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13. CFSE-labeled spleen and lymph node cells were activated, purified (*7*), and stained with T3.70-biotin (followed by streptavidin-PE) and anti-CD8–CyC and analyzed by FACS. Equal numbers of B6.2.16 CD8$^+$ cells (2 × 10^6 per mouse) that had undergone various degrees of cell division were washed and resuspended in sterile phosphate-buffered saline and adoptively transferred by lateral tail vein injection into 4- to 6-week-old female RAG-1–deficient mice (C57BL/6 × 129Sv, H-2^b) [P. Mombaerts *et al.*, *Cell* **68**, 869 (1992)].
14. After 21 or 70 days of adoptive transfer, mixed spleen and lymph node cell suspensions were prepared from recipient mice. To determine the number of B6.2.16 CD8$^+$ cells present in each mouse, a sample of 5 × 10^5 cells was stained with T3.70-biotin (then streptavidin-PE) and anti-CD8–allophycocyanin, and analyzed by three-color FACS. Limiting dilu-

tion analysis was performed to calculate the CTL precursor frequency (*6*).
15. Lymph node cells from B6.2.16 RAG-1–deficient mice (C57BL/6 × 129Sv, H-2^b) were labeled with CFSE and purified (*7*). The cells were analyzed by FACS (>95% B6.2.16 CD8$^+$ cells) and adoptively transferred to female RAG-1–deficient mice (*13*) and analyzed after 21 and 70 days by limiting dilution analysis (*6*).
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22 December 1998; accepted 9 February 1999

HLA and HIV-1: Heterozygote Advantage and *B*35-Cw*04* Disadvantage

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A selective advantage against infectious disease associated with increased heterozygosity at the human major histocompatibility complex [human leukocyte antigen (*HLA*) class I and class II] is believed to play a major role in maintaining the extraordinary allelic diversity of these genes. Maximum *HLA* heterozygosity of class I loci (*A*, *B*, and *C*) delayed acquired immunodeficiency syndrome (AIDS) onset among patients infected with human immunodeficiency virus–type 1 (HIV-1), whereas individuals who were homozygous for one or more loci progressed rapidly to AIDS and death. The *HLA* class I alleles *B*35* and *Cw*04* were consistently associated with rapid development of AIDS-defining conditions in Caucasians. The extended survival of 28 to 40 percent of HIV-1–infected Caucasian patients who avoided AIDS for ten or more years can be attributed to their being fully heterozygous at *HLA* class I loci, to their lacking the AIDS-associated alleles *B*35* and *Cw*04*, or to both.

HLA class I and class II loci located within the human major histocompatibility complex (MHC) comprise the most polymorphic set of genes known in humans (*1*–*3*). Products of these genes present antigenic peptide to T cells, initiating an immune response and clearance of the foreign material. Evolutionary and population studies have led to the general idea that the great diversity and even distribution of allelic frequencies observed in the class I and class II genes of the MHC (*HLA* in humans) are maintained through selective forces, such as infectious disease morbidity (*4*, *5*). The hypothesis of overdominant selection (heterozygote advantage) at the MHC proposes that individuals heterozygous at *HLA* loci are able to present a greater variety of antigenic peptides than are homozygotes, resulting in a more productive immune response to a diverse array of pathogens (*6*).

Compelling evidence for the selective maintenance of MHC diversity has come from analyses of the population distribution of *HLA* allele frequencies (*7*), the high incidence of nonsynonymous (codon altering) base substitutions among peptide-binding regions of *HLA* transcripts (*8*), persistence of numerous polymorphic MHC amino acid motifs for several million years through the emergence of multiple species (*9*), and a concordant increase in infectious disease sensitivity of species with increased MHC homozygosity (*10*). Several examples of *HLA* influence on human pathogen sensitivity have been described, particularly for interaction with malaria and hepatitis B (*11*).

The AIDS epidemic is characterized by extreme heterogeneity in the clinical course as well as in the incidence of HIV-1 infection among exposed individuals (*12*, *13*), which is probably a result of genetic variants among HIV-1 strains and of host genetic differences such as variants in chemokine and chemokine receptor structural genes (*14*, *15*). More than 50 reports examining a role for *HLA* variation in AIDS outcomes have appeared, however the reported associations have been difficult to generalize or to affirm in multiple cohorts (*12*, *16*). Potential explanations for this difficulty involve a paucity of patients, limitations in patient clinical descriptions, failure to correct for multiple comparisons, and a reliance on serological typings that can miss allele differences found by molecular typing. Nonetheless, concordant AIDS outcomes in sib pair analyses (*17*), the recurrent implication of two *HLA* haplotypes (*A1-Cw7-B8-DR3-DQ2* and *Cw4-B35-DR1-DQ1*) (*12*, *16*, *18*), plus the quasi-species pattern of HIV-1 change in infected patients (*19*) are strong indicators of *HLA* involvement in HIV pathogenesis.

We performed survival and genetic association analyses to address two hypotheses: (i) that overall or specific locus heterozygosity at the *HLA* class I loci confers relative resistance to AIDS progression and (ii) that individual alleles at the class I loci vary in their influence on progression to AIDS. *HLA* class I loci were molecularly typed with DNA from individuals enrolled in five AIDS cohorts: Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), Hemophilia Growth and Development Study (HGDS), San Francisco City Clinic Cohort (SFCC), and AIDS Linked to Intravenous Experience (ALIVE) Study (*20*, *21*). Survival analyses incorporated data derived from HIV-1–positive individuals with known dates of infection and

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