antisense GAD65.67 transgene, we ligated antisense GAD65 complete transcription unit, which had been isolated from RIP-AS-GAD65 by digestion with Sal I and Not I, at the Sal I site of RIP-AS-GAD67 to produce RIP-AS-GAD65.67. The 7.6-kb RIP-AS-GAD65.67 full-length transgene (Fig. 1A) was separated from pBluescriptIISK by digestion with Xho I and Not I.

8. The total protein extracts from the islets or brain tissue of H-, M-, and L-AS-GAD-NOD mice as well as H_k-, M_k- and L_k-AS-GAD-NOD mice and their respective transgene-negative littermates were prepared, and 20 μg of protein was separated by 10% SDS–polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane (Amersham), the membrane was reacted with a 1:3000 dilution of polyclonal rabbit antibody to GAD67 (Chemicon), and the GAD protein was detected by the biotin-streptavidin-peroxidase method, with a chemoluminescence system. As an internal control, the same membrane was probed with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon).
9. Paraffin blocks were prepared and sectioned [P. Gilon, M. Tappaz, C. Remacle, *Histochemistry* **96**, 355 (1991)], the sections were reacted with GAD1 monoclonal antibody (ATCC), and GAD expression was detected by the avidin-biotin-peroxidase complex method with a Vectastain Elite ABC kit (Vector Laboratories). Serial sections of the pancreas were also stained with guinea pig polyclonal antibody to insulin (Vector).
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11. The data are available at www.sciencemag.org/feature/data/986073.shl
12. To measure the generation of diabetogenic T cells in GAD-suppressed transgenic NOD mice, we did adoptive transfer [T. Kawamura, M. Nagata, T. Utsugi, J. W. Yoon, *J. Immunol.* **151**, 4362 (1993); M. Nagata, P. Santamaria, T. Utsugi, J. W. Yoon, *ibid.* **152**, 2042 (1994)]. Splenocytes isolated from 20-week-old nondiabetic female H-AS-GAD-NOD mice, H_k-AS-GAD-NOD mice, and their respective age-matched, transgene-negative littermates were transfused intravenously (1×10^7 cells per mouse) into 6- to 8-week-old NOD.*scid* mice. The animals were monitored three times per week for glycosuria (>+2) and hyperglycemia (>16.7 mM) [J. W. Yoon, M. M. Rodrigues, C. Currier, A. Notkins, *Nature* **296**, 566 (1982)]. To determine whether the GAD-suppressed β cells are protected from autoimmune attack by diabetogenic T cells, we transfused splenocytes (1×10^7 cells per mouse) from acutely diabetic NOD mice intravenously into 6-week-old, irradiated, male H-AS-GAD-NOD mice and age-matched transgene-negative control male NOD mice. The animals were monitored as described above.
13. Splenocytes were isolated from individual 8-, 12-, and 15-week-old H-AS-GAD-NOD mice or H_k-AS-GAD-NOD mice, their respective transgene-negative littermates, and control NOD mice, and a proliferation assay was performed as described previously (*2*, *4*, *5*). The splenocytes (1×10^6 cells per well) were plated in 200 μl of culture medium in triplicate and reacted with GAD peptide (mixed 17, 34, and 35 peptides; 7 μM) (*4*), recombinant human GAD65 protein (Syntax), HSP60 (StressGen), porcine insulin (Sigma), or ovalbumin (Sigma) at 20 μg/ml for 72 hours and pulsed with 1 μCi of [^{3}H]thymidine. The incorporation of [^{3}H]thymidine was measured.
14. Islets were isolated from 4-week-old male H-AS-GAD-NOD or H_k-AS-GAD-NOD mice or age- and sex-matched transgene-negative littermates and transplanted (400 islets per mouse) into the renal subcapsular region of acutely diabetic NOD mice [T. Utsugi, M. Nagata, T. Kawamura, J. W. Yoon, *Transplantation* **57**, 1799 (1994)]. The animals were monitored for glycosuria and hyperglycemia as described above. Sections of the kidney capsules containing the transplanted islets were stained with haematoxylin and eosin and examined for lymphocytic infiltration.
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17. The total RNA was isolated from the pancreas of H-, M-, and L-AS-GAD-NOD mice and transgene-negative littermates with Trizol (Gibco BRL). The total RNA (20 μg) was separated by agarose-formaldehyde gel electrophoresis, transferred to a nylon membrane, and probed with in vitro transcribed sense rGAD65 or rGAD67 RNA. As an internal control, the same membrane was probed with antisense GAPDH RNA.
18. We thank D. Hanahan for the plasmid RIP-DIPA/pXF3 containing the RIP, A. Tobin for the rat GAD65 and rat GAD67 cDNA, K. Clarke and A. Kyle for editorial assistance, and B. Pinder for artwork. Supported by grants from the Medical Research Council of Canada, the Juvenile Diabetes Foundation International, the NIH (DK 45735 and DK 53015-01), the Alberta Heritage Foundation for Medical Research, and Korea Green Cross.

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Inactivation of Misselected CD8 T Cells by CD8 Gene Methylation and Cell Death

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Misselected CD8 cells that express T cell receptors (TCRs) that do not recognize class I major histocompatibility complex (MHC) protein can emerge from thymic selection. A postthymic quality control mechanism that purges these cells from the repertoire is defined here. The failure of mature CD8 cells to simultaneously engage their TCR and CD8 coreceptor triggers an activation process that begins with inhibition of CD8 gene expression through remethylation and concludes with up-regulation of surface Fas and Fas ligand and cellular apoptosis. Thus, inhibition of a death signal through continued TCR-CD8 coengagement of MHC molecules is a key checkpoint for the continued survival of correctly selected T cells. Molecular defects that prevent delivery of the death signal to mistakenly selected T cells underlie the expansion of double-negative T cells, which is the cellular signature of a subset of systemic autoimmune diseases.

The selection process in the thymus that regulates the repertoire of T cell subsets is effective but not foolproof. Thymocytes that coexpress CD8 normally recognize MHC class I, but class II–reactive CD8 cells can mistakenly arise through interactions either with strong peptide agonists or with self-peptides that antagonize CD4 but not CD8 cell activation (*1*), perhaps reflecting reduced $p56^{lck}$ signaling (*2*) or up-regulation of Notch 1 (*3*). T cells bearing antigen receptors that do not functionally engage self-MHC products can also emerge from the thymus (*4*). Although CD8 cells that bear mismatched TCRs may be functionally active in vitro (*1*, *5*), they do not contribute substantially to normal immune responses (*6*). Thus, CD8 cells that express TCRs that fail to engage class I may normally be inactivated by a postthymic mechanism.

We first examined the fate of CD8 cells that express a TCR that does not interact with class I MHC. CD8 cells bearing the class II–restricted DO11.10 TCR transgene survive thymic selection and are exported in substantial numbers to peripheral tissues (*2*, *7*). However, our observations indicate that they do not express a stable CD8 phenotype. Within 2 to 3 days after the adoptive transfer of purified $DO11^+$ CD8 cells into syngeneic Balb/C (H-2^d, $\beta_2M^{+/+}$) or MHC-deficient (C57BL/6J $\beta_2M^{-/-} \times$ I-$A_\beta^{b-/-}$) hosts, these cells down-regulate CD8 coreceptor expression (Fig. 1A). The kinetics of CD8 down-regulation in syngeneic hosts and in hosts deficient in both class I and class II MHC are equivalent despite the availability in normal hosts of class I MHC for CD8 engagement and class II MHC for TCR engagement. In contrast, CD8 cells that express a TCR transgene specific for a class I–restricted antigen [histocompatibility-Y antigen (H-Y)] (*8*) continue to express the CD8 coreceptor after a similar period in adoptive syngeneic (female) hosts (Fig. 1). Thus, CD8 engagement seems necessary for stable CD8 expression.

To further test the hypothesis that continued expression of the CD8 receptor may require coengagement of CD8 and TCR by MHC class I peptide complexes in peripheral tissues, we tested the effects of class I deficiency on continued expression of CD8 by peripheral T cells. CD8 cells from $\beta_2M^{+/+}$ ($Thy1.1^+$) mice were infused into syngeneic $\beta_2M^{-/-}$ or $\beta_2M^{+/+}$ ($Thy\ 1.2^+$) hosts. Within 5 to 10 days, virtually all donor cells recov-

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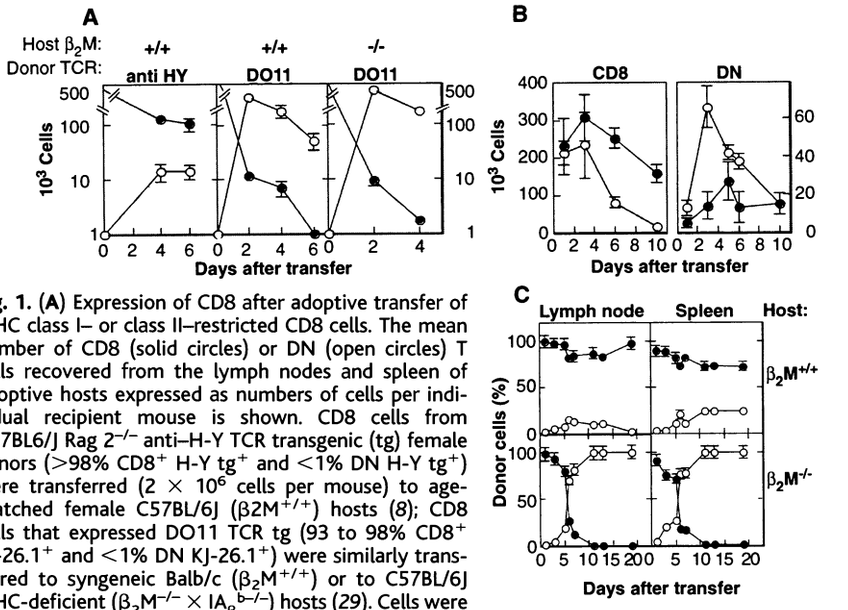
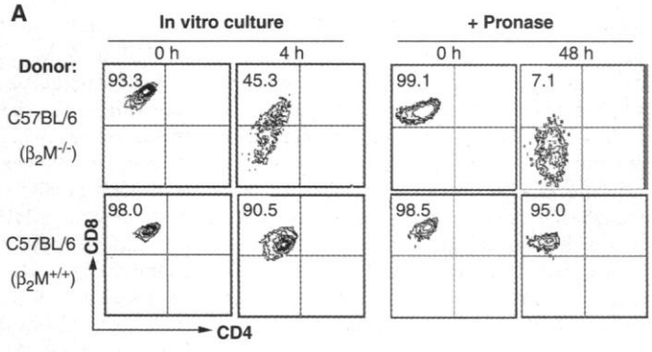


Fig. 1. (**A**) Expression of CD8 after adoptive transfer of MHC class I– or class II–restricted CD8 cells. The mean number of CD8 (solid circles) or DN (open circles) T cells recovered from the lymph nodes and spleen of adoptive hosts expressed as numbers of cells per individual recipient mouse is shown. CD8 cells from C57BL6/J Rag $2^{-/-}$ anti–H-Y TCR transgenic (tg) female donors (>98% $CD8^+$ H-Y tg^+ and <1% DN H-Y tg^+) were transferred (2×10^6 cells per mouse) to age-matched female C57BL/6J ($\beta 2M^{+/+}$) hosts (*8*); CD8 cells that expressed DO11 TCR tg (93 to 98% $CD8^+$ KJ-26.1$^+$ and <1% DN KJ-26.1$^+$) were similarly transferred to syngeneic Balb/c ($\beta_2M^{+/+}$) or to C57BL/6J MHC-deficient ($\beta_2M^{-/-} \times IA_\beta^{b-/-}$) hosts (*29*). Cells were recovered and enumerated after simultaneous staining with FITC-conjugated monoclonal antibodies (mAb's) specific for the clonotypic tg TCR [T3.70 for anti-H-Y TCR and KJ-26.1 for DO11, CD8 CYC, and CD4 PE at the indicated time points as described (*30*)]. Data shown represent three independent series of experiments ± SD (error bars). (**B**) Adoptive transfer of CD8 cells into syngeneic $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ hosts. Numbers of CD8 and DN T cells recovered from the lymph nodes and spleen of C57BL/6 $\beta_2M^{+/+}$ (solid circles) or C57BL/6 $\beta_2M^{-/-}$ (open circles) hosts are shown as the mean recovered number of cells per mouse. CD8 cells (3×10^6) purified from the lymph nodes of C57BL/6J Thy1.1 donors (*28*) were transferred to age- and sex-matched syngeneic C57BL/6J (Thy1.2) hosts; recovered; stained with mAb's specific for Thy1.1, CD8, and CD4; and enumerated at the indicated time points (*30*). Each point represents the mean of three to six experiments and 6 to 15 mice per group. Contaminant DN cells in donor CD8 T cell inocula was <1.0%. (**C**) The proportion (percentage) of CD8 cells (solid circles) and DN cells (open circles) within the donor population at the indicated time points after transfer are shown. Each point represents the mean of two to six experiments and 4 to 15 mice per group.



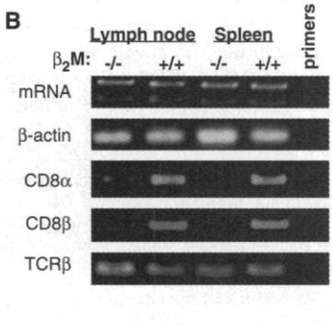


Fig. 2. Expression of CD8αβ RNA is down-regulated in CD8 cells from $\beta_2M^{-/-}$ mice. (**A**) In vitro culture FACS-sorted (*28*) CD8 cells from wild-type B6.$\beta_2M^{+/+}$ or B6.$\beta_2M^{-/-}$ (T cells, >98% CD8 and <3% DN) mice incubated for 4 hours in 5% fetal bovine serum Dulbecco's minimum essential medium and analyzed by flow cytometry for CD8 (clone 53.67, PE) and CD4 (RM4-5, CYC) expression. The number of apoptotic cells assayed by annexin V FITC reactivity was <3%. These cells were also treated with 0.04% pronase before incubation at 37°C for 48 hours and FACS analysis. Expression of surface CD8 and CD4 on viable (forward $scatter^{hi}$) cells is representative of one of three independent experiments. (**B**) Semiquantitative RT-PCR detection of CD8αβ gene expression. After isolation of RNA from CD8 cells (97% pure) from lymphoid tissues of 6-week-old C57BL/6J $\beta_2M^{+/+}$ or C57BL/6 $\beta_2M^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) through positive selection with anti-CD8α (clone 3.155)–conjugated magnetic beads (Dynal), cells were resuspended in Trizol for RNA extraction (Gibco-BRL), and equal amounts of RNA were analyzed by semiquantitative RT-PCR as described (*24*). The results shown are representative of four independent experiments.

ered from $\beta_2M^{-/-}$ mice expressed a $CD4^-$ $CD8^-$ [double negative (DN)] phenotype, similar to the fate of DO11 class II–specific CD8 cells in a normal MHC environment (Fig. 1B). In contrast, CD8 cells that were recovered from $\beta_2M^{+/+}$ hosts continued to express this coreceptor over the entire (3-week) period of observation (Fig. 1, B and C), similar to the fate of adoptively transferred CD8 cells expressing a class I–restricted TCR transgene (Fig. 1A). Thus, even correctly selected CD8 cells rapidly down-regulate their CD8 coreceptor when transferred to a lymphoid environment that is deficient in class I MHC products. Coreceptor down-regulation in an MHC-deficient environment is specific for the CD8 lineage, because purified CD4 cells transferred to MHC class II–deficient ($IA_\beta^{b-/-}$) hosts (or, for that matter, $\beta_2M^{-/-}$ hosts) maintained normal CD4 coreceptor expression (*9*).

Mice that carry the $\beta_2M^{-/-}$ mutation, although severely deficient in expression of class I MHC self-peptide complexes, harbor substantial numbers of CD8 cells in their peripheral lymphoid tissues (*10*). In view of the above results suggesting coreceptor down-regulation in class I–deficient environments, we tested whether CD8 expression on these cells reflected stable and active synthesis of the CD8 protein. CD8 cells from $\beta_2M^{-/-}$ but not $\beta_2M^{+/+}$ mice began to lose expression of surface CD8 protein within 4 hours of in vitro culture and did not resynthesize CD8 proteins after enzymatic removal (Fig. 2A), in contrast to the unchanged or elevated levels of CD8 on activated and memory T cells (*11*). An analysis of genes expressed by purified CD8 cells from $\beta_2M^{-/-}$ mice also showed that these cells express little or no CD8α or CD8β RNA (Fig. 2B), consistent with their "lame duck" status with respect to CD8 expression. These cells have also down-regulated expression of the granzyme B gene, which mediates cytotoxic effector activity of CD8 cells, whereas expression of housekeeping genes, such as actin and glyceraldehyde phosphate dehydrogenase (GAPDH), was unchanged (*12*). Additional analysis of gene expression in CD8 cells that were unable to engage class I MHC in peripheral tissues indicated reduced expression of the lung Kruppel-like factor (LKLF), which normally acts to inhibit Fas ligand (FasL) expression (*13*), and markedly elevated levels of FasL (*12*).

We next investigated molecular mechanisms that might account for down-regulation of CD8 gene expression in the absence of conventional class I MHC products. Demethylation of the CD8α gene accompanies CD8 expression, as judged by increased susceptibility to methylation-sensitive enzymes such as Hha I (*14*). A Bam HI 8-kb restriction fragment from the 5′ portion of the CD8α

gene was sensitive to Hha I digestion in CD8 cells but not in CD4 cells, yielding a single diagnostic 4.4-kb fragment (Fig. 3). We used this observation to determine the methylation status of CD8α in thymocytes at progressive stages of development. The CD8α Hha I site is fully demethylated in double-positive ($CD4^+8^+$) thymocytes from both $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice and remains fully demethylated in lineage-committed CD8 single-positive (SP) thymocytes from $\beta_2M^{+/+}$ mice (Fig. 3). In contrast, Hha I digestion of the CD8α gene in CD8 SP thymocytes from $\beta_2M^{-/-}$ mice yields about equal proportions of the 4.4-kb fragment and the 8-kb (Hha I–resistant) fragment, indicating partial remethylation. Finally, this region of the CD8α gene is completely remethylated in "mature" peripheral CD8 cells from $\beta_2M^{-/-}$ mice (yielding only a single 8-kb Hha I–resistant fragment) (Fig. 3), consistent with the virtual absence of CD8α mRNA expression in these cells (Fig. 2B).

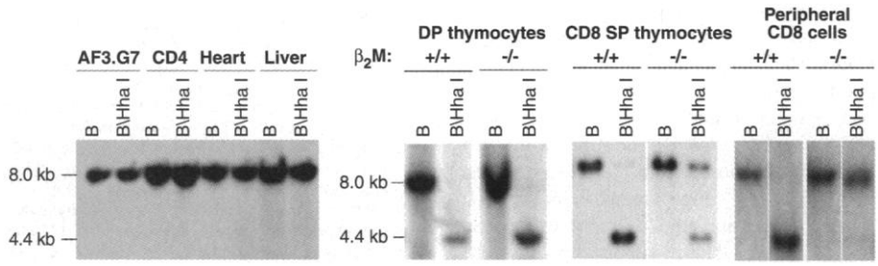


Fig. 3. Methylation patterns of the CD8α gene during CD8 development in $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice. Genomic DNA was extracted from FACS-sorted lymph node CD8 cells (98% pure) from C57BL/6 (B6.$\beta_2M^{+/+}$) and B6.$\beta_2M^{-/-}$ mice, AF3.G7 cells (a $CD4^+$ T cell hybridoma), B6.$\beta_2M^{+/+}$ CD4 cells (98% pure), and heart and liver tissue. Genomic DNA was also isolated from FACS-sorted thymocytes from B6.$\beta_2M^{+/+}$ or B6.$\beta_2M^{-/-}$ mice [FACS-sorted $CD4^+CD8^+$ (DP) thymocytes (98% pure)], and [CD8 SP thymocytes (97%)]. These DNA samples were digested with either Bam HI alone (B) or Bam HI and Hha I (B/Hha I) and analyzed by Southern blotting (*31*) after hybridization with a ^{32}P-labeled 800-bp murine CD8α cDNA (*14*) and autoradiography with BioMax x-ray film (Sigma) and lightning-plus intensifiers (Kodak). Data are representative of three independent experiments.

The decline in absolute numbers of CD8 cells after transfer into $\beta_2M^{+/+}$ hosts was greatly accelerated in $\beta_2M^{-/-}$ recipients (Fig. 1B) (*15*). Analysis of the surface phenotype of donor CD8 and DN cells recovered from

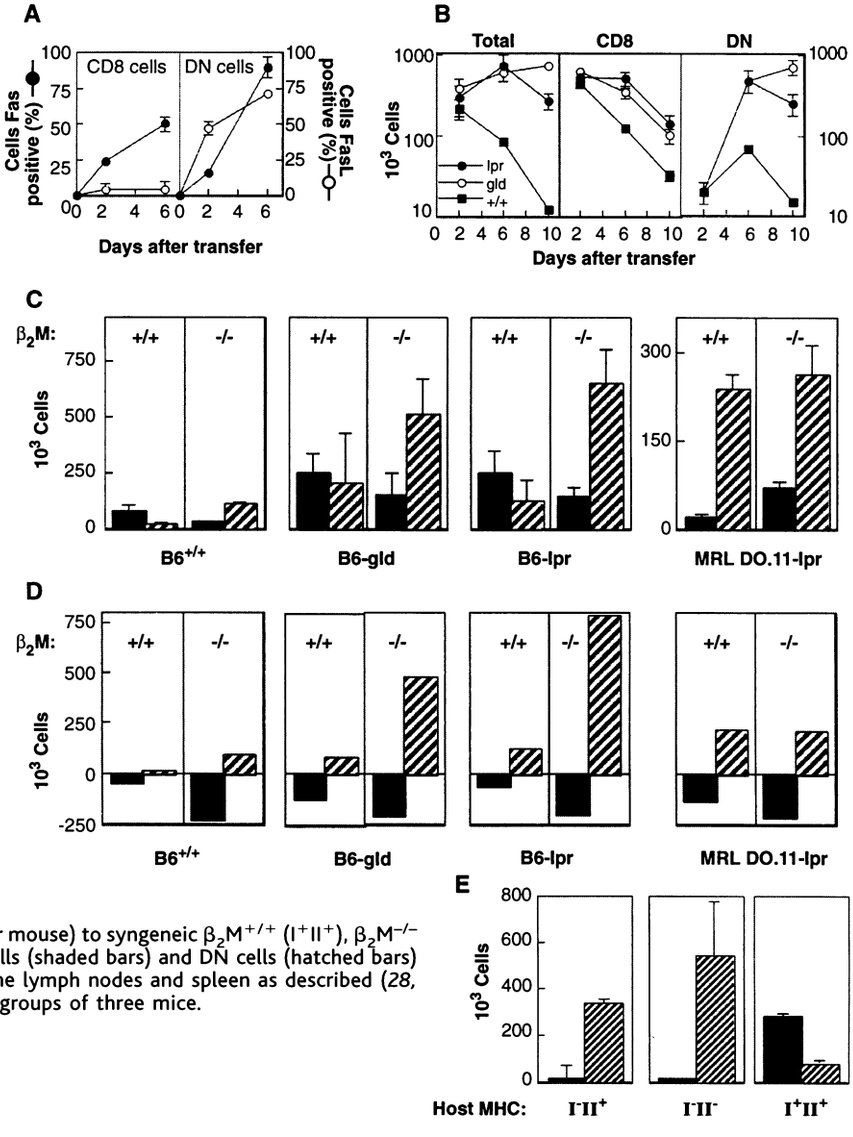


Fig. 4. (**A**) Induction of Fas and FasL expression on CD8 cells transferred to MHC-deficient hosts. Thy1.1$^+$ CD8–enriched cells (>98% pure) adoptively transferred (1×10^7 cells per mouse) to syngeneic C57BL/6J $\beta_2M^{-/-}$ hosts were recovered at the indicated points after transfer (*28, 30*). Donor cells expressing a CD8 or DN ($CD4^-/CD8^-$) phenotype were analyzed for Fas^{hi} (JO2) and Fas ligand (K10) expression. Data shown represent three independent experiments ± standard errors (error bars). (**B** through **E**) Effects of *lpr* and *gld* mutations on the developmental fate of CD8 cells in adoptive hosts. CD8 cells (>97% pure) from B6.$\beta_2M^{+/+}$, B6.$Fas^{lpr/lpr}$, B6.$FasL^{gld/gld}$, or MRL DO11 tg ($Fas^{lpr/lpr}$) were adoptively transferred (3×10^6 cells per mouse) to syngeneic $\beta_2M^{+/+}$ or $\beta_2M^{-/-}$ hosts, recovered, and analyzed as described (*28*). (B) Total donor cells and CD8 and DN cells in syngeneic B6.$\beta_2M^{-/-}$ hosts were identified according to differential expression of H-$2K^b$ (FITC-labeled clone AF6-88.5) at the time points indicated. Data represent three independent experiments ± standard errors. (C) CD8 cells (shaded bars) or DN cells (hatched bars) recovered on day 6 after transfer into B6.$\beta_2M^{+/+}$ (Thy 1.1$^+$) or B6.$\beta_2M^{-/-}$ hosts according to differential expression of Thy 1.2 (clone 53-2.1) and H-$2K^b$ (clone AF6-88.5). In MRL hosts, donor cells were defined according to DO11 TCR expression with the clonotypic mAb (KJ-26.1) as described in the caption for Fig. 1. Data represent three independent experiments with groups of three mice ± standard errors. (D) The change in the number of donor CD8 and DN T cells between days 2 and 6 after transfer into the indicated syngeneic hosts is shown. The increment in DN cells (hatched bars) during this 4-day interval is compared to the decline in CD8 cells (shaded bars) during the same period. Data are derived from (C) and represent three independent experiments with groups of three mice. (E) CD8 cells isolated from B6.$Fas^{lpr/lpr}$ expressing the Thy1.1 allele were adoptively transferred (3×10^6 cells per mouse) to syngeneic $\beta_2M^{+/+}$ (I^+II^+), $\beta_2M^{-/-}$ (I^-II^+), and $\beta_2M^{-/-} \times IA_\beta^{b-/-}$ (I^-II^-) hosts. CD8 cells (shaded bars) and DN cells (hatched bars) were recovered after 6 days and enumerated in the lymph nodes and spleen as described (*28, 30*). Data shown represent two experiments with groups of three mice.

$\beta_2M^{-/-}$ adoptive hosts suggested a potential mechanism for their short life-span in this environment: (i) up to 50% of the donor CD8 cells expressed elevated Fas but did not express FasL and (ii) ~75 to 90% of the DN progeny expressed both Fas and FasL (Fig. 4A). The small population of lame duck CD8 cells in $\beta_2M^{-/-}$ mice was about two-thirds $CD44^{hi}$, and about one-half had up-regulated Fas expression and underwent apoptosis at two to three times the rate of CD8 cells from $MHC^{+/+}$ mice (*16*). Thus, in the absence of peripheral class I MHC expression, down-regulation of the LKLF transcription factor precedes up-regulation of FasL expression and apoptosis.

We therefore asked whether defects in Fas or FasL expression might enhance the accumulation of DN cells from CD8 precursors. Expression of either the *lpr* (Fas) or *gld* (FasL) mutation (*17*) by transferred CD8 cells protected donor cells from attrition in $\beta_2M^{-/-}$ hosts (Fig. 4B) due to enhanced accumulation of DN progeny; CD8 cells from $B6^{lpr}$, $B6^{gld}$, and $B6^{+/+}$ donors declined at a similar rate in $\beta_2M^{-/-}$ hosts (Fig. 4B). The accumulation of DN cells from $CD8^{lpr}$ or $CD8^{gld}$ precursors was enhanced in $\beta_2M^{-/-}$ but not in $\beta_2M^{+/+}$ hosts, indicating that defective expression of Fas or FasL enhances the accumulation of newly generated DN cells in a $\beta_2M^{-/-}$ environment (Fig. 4C). To discount differentiative events already set in motion in *lpr* and *gld* donor mice, we quantitated DN cell generation beginning 2 days after CD8 cells had been placed in a $\beta_2M^{+/+}$ or $\beta_2M^{-/-}$ lymphoid environment (Fig. 4D). CD8 cells from $B6^{gld}$ or $B6^{lpr}$ donors generated 15 to 20 times more DN cells than CD8 cells from $B6^{+/+}$ donors during this interval in $\beta_2M^{-/-}$ hosts. Enhanced generation of DN cells in adoptive $\beta_2M^{-/-}$ hosts reflected the absence of TCR-CD8 ligation by class I MHC rather than an undefined property of the $\beta_2M^{-/-}$ environment because class II–specific CD8 cells from $DO11^{lpr}$ mice generated increased numbers of DN cells [compared to CD8 cells from $DO11^{+}$ ($Fas^{+/+}$) donors] in either $\beta_2M^{+/+}$ or $\beta_2M^{-/-}$ recipients (Fig. 4, C and D). Engagement of class II MHC products by CD8 cells was not responsible for enhanced generation of DN cells because CD8 cells gave rise to similar numbers of DN progeny in $\beta_2M^{-/-}$ (class I–deficient) and $\beta_2M^{-/-} \times IA_\beta{}^{b-/-}$ (MHC-deficient) recipients (Fig. 4E). Finally, analysis of $B6^{lpr}$ CD8 cells and DN progeny in adoptive $\beta_2M^{-/-}$ hosts showed that, although CD8 cells did not replicate during this process, about two-thirds of newly generated DN cells underwent at least one round of replication, as judged by 5-carboxyfluorescein diacetate succininyl ester (CFSE) dye dilution analysis (*18*).

We have identified a differentiative pathway taken by CD8 cells bearing receptors that cannot engage class I MHC self-peptide molecules, either because of incorrect thymic selection, defects in peripheral MHC class I expression, or antigen presentation. In any of these cases, failed CD8-TCR coengagement results in down-regulation of genes that account for specialized cytolytic T lymphocyte (CTL) function and resistance to cell death (CD8αβ, granzyme B, and LKLF) and up-regulation of the Fas and FasL death genes.

Thus, MHC engagement is required to inhibit expression and delivery of a death program rather than to supply a putative trophic stimulus for T cell survival. This view of the consequence of failed MHC engagement bears directly on the fate of CD8 cells in the context of tumor growth and autoimmune disease. "Tumor escape" by neoplastic cells that do not express appropriate class I MHC products has been ascribed to reduced susceptibility of tumor cells to killing by CTLs. Our findings suggest that diminished class I expression by tumor cells may also result in the inactivation and elimination of tumor-specific CTLs; preliminary experiments support this view (*19*).

Expansion of DN cells in animals that carry mutations in Fas or FasL has been attributed to a proliferative response of autoreactive T cells (*20*). We found that these cells actually represent the detritus of the CD8 repertoire that is unable to engage host MHC products but is spared elimination through mutations in Fas-related death molecules. These mistakenly selected CD8 cells include clones that are positively selected by class I self-peptide complexes (*21*) expressed in the thymus but not in the peripheral lymphoid tissues (*22*) and CD8 cells that are selected by thymic class II self-peptide complexes (*1*). Defects in delivery of the death signal to these cells underlie the explosive growth and accumulation of DN T cells (i) in animals bearing Fas or FasL mutations, (ii) in patients that carry inherited mutations of these genes, and (iii) in ~25% of systemic lupus erythematosus patients that display the cellular signature of defects in this mechanism of quality control of CD8 cells (*23*).

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12. Relative gene expression was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) (as described in the caption of Fig. 2B) for CD8 T cells recovered from the lymph nodes of 6-week-old C57BL/6J $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice. We noted decreased expression of CD8αβ, granzyme B, and LKLF transcripts in CD8 cells from $\beta_2M^{-/-}$ mice as compared to $\beta_2M^{+/+}$ mice [CD8α (0.16 ± 0.05 to 0.67 ± 0.3) and CD8β (0.006 ± 0.05 to 1.07 ± 0.2)], granzyme B (0.036 ± 0.01 to 0.46 ± 0.04), and LKLF (0.07 ± 0.07 to 0.48 ± 0.01). In contrast, the level of FasL expression was up-regulated in CD8 cells from $\beta_2M^{-/-}$ mice (0.32 ± 0.18 to 0.02 ± 0.03). The expression of GAPDH (0.4 ± 0.03 to 0.4 ± 0.01) and Thy1.2 (0.6 ± 0.02 to 0.6 ± 0.01) was virtually identical in CD8 cells from either strain of mouse, and transcripts for CD4 were always undetectable. RT-PCR was performed on equalized RNA samples from the respective cell samples, and densitometric scans were conducted on a digital imaging system (IS-1000, Alpha Innotech, San Leandro, CA) after the exposure of ethidium bromide–stained agarose gels to ultraviolet irradiation. Relative gene expression was determined with PCR products recovered from the linear phase of amplification as described (*24*). To ensure that comparisons were based on the same amount of RNA in each sample, we divided the area under the densitometric peak for each gene by the area under the β-actin densitometric peak for the same cellular RNA as previously described (*25*). The ratio of each gene product level to that of actin in the same sample is expressed in relative densitometric units. The data given are the means ± SD of three independent experiments. LKLF (*13*) was detected with CCCTCGTCCCCTGGAGCTGCT and GAACGTGCCACAGTCGCGCAT (376 bp) FasL (537 bp) [K. Benedikt *et al.*, *J. Immunol.* **161**, 6939 (1998)]; Thy1.2 was detected with TGACCAGCCTGACAGCCTGCC and TTGGAGGAGGGAGAGGGAAAGC (401 bp), granzyme B (319 bp) (*26*), and GAPDH (950 bp) (Clontech, Palo Alto, CA).
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16. CD8 cells from lymph nodes and spleens of $\beta_2M^{-/-}$ or $\beta_2M^{+/+}$ mice were sorted with a fluorescence-activated cell sorter (FACS) and assayed with annexin V [fluorescein isothiocyanate (FITC)] (PharMingen, San Diego, CA) reactivity for levels of spontaneous apoptosis after in vitro culture at 48 hours in three separate experiments; at 48 hours, mean apoptosis of CD8 cells from $\beta_2M^{-/-}$ mice was 70% in comparison with a mean of 20% for CD8 cells from $\beta_2M^{+/+}$ mice. Surface phenotypes of CD8 and DN cells [prepared as described in (*27*)] were obtained with fluorochrome-conjugated antibodies to Thy1.2 [biotinylated clone 53-2.1 and streptavidin-AMCA (Coulter, Miami, FL)], CD8α (53.67, APC), CD4 [H129.19, red 613 (Gibco-BRL)], R-phycoerythrin (PE)- or FITC-labeled antibodies to CD44 (IM7), CD62L (MEL-14), CD45RB (16A), CD19 (1D3), CD25 (3C7), CD45R/B220 (RA3-6B2), CD3ε (145-2C11), TCR β (H57-597), TCR γδ (GL3), FasR (JO2), or FasL (K10) in various five-color combinations and analyzed on an

Elite FACS (Coulter). Wavelengths utilized for fluorochrome emission were AMCA (450 nm), FITC (525 nm), PE (575 nm), red 613 (610 nm), and APC (675 nm). In addition to up-regulated CD44 expression, ~50% of Thy1$^+$/CD8$^+$/CD3$^+$/CD4$^-$ cells in $\beta_2M^{-/-}$ mice have also down-regulated L-selectin (CD62L); >97% of CD8 cells in wild-type mice express high levels of L-selectin. B220 expression was also increased in both CD8 cells and DN cells (36 and 81% positive, respectively) from $\beta_2M^{-/-}$ mice in comparison to cells from $\beta_2M^{+/+}$ controls (10 and 15%, respectively), and expression of CD45RB was decreased by ~50% in CD8 cells from $\beta_2M^{-/-}$ mice. Data represent the mean values of 10 individual mice analyzed in three separate experiments.

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18. CD8 cells were isolated as described (*28*) and labeled with CFSE (Molecular Probes, Eugene, OR) [M. Hertz *et al.*, *Nature* **398**, 292 (1998)] before the transfer of 2×10^6 cells per syngeneic B6.$\beta_2M^{+/+}$ or B6.$\beta_2M^{-/-}$ host and recovery at 2 and 4 days after transfer. CD8 and DN CFSE$^+$ cells were identified by FACS analysis in the lymph nodes and spleen of the adoptive hosts after staining with anti-CD8 cychrome (CYC) and anti-CD4 PE. Although two-thirds of DN cells generated from CD8 precursors in adoptive hosts underwent three rounds of replication in these experiments, <5% of DN cells purified from B6^{lpr} mice underwent replication in the same experiments, which is consistent with earlier findings that these cells, once formed, are quiescent [E. S. Sobel, V. N. Kakkanaiah, R. G. Rapoport, R. A. Eisenberg, P. L. Cohen, *Clin. Immunol. Immunopathol.* **74**, 177 (1995)].
19. S. Ferrone and F. M. Marincola, *Immunol. Today* **10**, 487 (1995). CD8 cells from anti–H-Y TCR transgenic mice immune to male splenocytes (2×10^7 cells per mouse) were incubated (10^5 cells per well) with irradiated (30 grays) RMA (H-2^b) lymphoma or the TAP-2–deficient/H-2^b–deficient variant RMA-S [H.-G. Ljunggren and K. Karre, *J. Exp. Med.* **162**, 1745 (1985)] at 5×10^5 cells per well for 72 hours before the addition of ^{51}Cr-labeled RMA tumor cells to each well. Anti–H-Y CD8 cells that had been incubated with RMA tumor cells for 3 days contained 48% apoptotic cells and lysed 90 to 100% of target (RMA) cells [effector:target (E:T) ratio, 5:1]; in contrast, anti–H-Y CD8 cells that had been incubated with (class I–deficient) RMA-S cells for 3 days contained 86% apoptotic cells and lysed 0 to 30% of tumor target cells at 5:1 or 10:1 E:T ratios.
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24. PCR primers were selected to span exons and to yield similarly sized single-band products. Sense and antisense primers, respectively, were as follows: β-actin, GACTACCTCATGAAGATCCT and CTAGAAGCACTTGCGGTGCAC (570 bp); CD8α, GCCAGAAGGTGGACCTGGTATGTG and GAGTGATGATCAAGGACAGCAGAAG (498 bp); CD8β, ATGCAGCCATGGCTCTGGCTGG and GCATGTCAGGCCCTTCTGGGTC (512 bp); and TCR Cβ, CCCACTATTTTTCTTCCTTCTGTTGCTGAA and TTTGTTGTTCTCATGTTTGACAATACA-ACT (255 bp). CD4 transcripts (615 bp) (Clontech) were always undetectable (*9*) [X. F. Yang, G. F. Weber, H. Cantor, *Immunity* **7**, 629 (1997)].
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28. Lymph nodes (cervical, axillary, inguinal, iliac, and mesenteric) (*27*) were used to prepare CD8 cells by magnetic selection (Dynal, Lake Success, NY) with antibodies to CD4 (GK1.5), B220 (RA3-6B2), Gr-1 (RB6-8C5), Mac-1 (M1/70), and NK1.1 (PK136) (PharMingen). In the experiments reported here, this procedure yielded T cells that were >97% CD8 cells and <3% DN cells from donor mice. Electronic cell sorting (FACS) was performed with fluorochrome-conjugated anti-CD8α (clone 53.76) on either a Becton-Dickinson FACS vantage flow cytometer or on a MoFlo (Cytomation, Fort Collins, CO); in these experiments, contamination by DN T cells was <1.0% of the sorted cells. Contaminant DN cells in the inocula of CD8 cells did not account for DN expansion in adoptive hosts because infusion of 2×10^6 purified DN cells from B6^{gld} donors (96% DN) into class I–deficient hosts yielded $<5 \times 10^4$ DN cells 6 days later, whereas infusion of CD8 cells from the same (*gld*) donors (<3% DN contamination) yielded ~5.1×10^7 DN cells.
29. Although C57BL/6 $\beta_2M^{-/-}$ mice can, in some instances, reject allogeneic tumors [S. Apasov and M. Sitkovsky, *J. Immunol.* **152**, 2087 (1994)], the similarity in kinetics of transferred CD8 cells in C57BL/6J ($\beta_2M^{-/-} \times$ I-A$_\beta^{b-/-}$) hosts to those of syngeneic Balb/C hosts (Fig. 1A) and the absence of a detectable change in the numbers or activation status of C57BL/6J ($\beta_2M^{-/-} \times$ I-A$_\beta^{b-/-}$) host spleen cells indicated that the potential MHC difference did not affect the response of the donor cells.
30. Cells from the lymph node and spleen of individual recipient mice were analyzed with FACS for 10^5 total events in each cell suspension. FITC-labeled (either clonotypic antibody or Thy1.1 antibody) donor cells in lymphoid tissues of host mice were monitored for CD8 expression. Surface expression of CD3, Thy1.1, T3.70, and KJ1-26.1 remained unchanged on donor cells in either $\beta_2M^{+/+}$ or $\beta_2M^{-/-}$ hosts at all times when tested after transfer. DN cells were defined as Thy1.1$^+$, TCR^{hi}, CD3$^+$, CD8$^-$, and CD4$^-$ cells. After sorting for CD8$^+$/KJ1-26$^+$ cells, in two independent experiments, 93 and 98% of sorted CD8 cells had this phenotype, and in both cell populations, <1% were DN/KJ1-26$^+$ before transfer into adoptive syngeneic hosts.
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