phorylated in anergic pGL10 cells and we detected no differences in expression of GAP between normal and anergic cells (*7*).

Although many aspects of TCR signal transduction remain unaffected in anergic T cells, we show clearly that a defect in Ras activation persists in these cells, and activation of this pathway is known to be required for IL-2 gene transcription (*22*). We postulate that an early signaling defect results in the inability to activate the Ras pathway upon T cell stimulation. This block in Ras activation may result in an inability to engage the transcription factors Fos and Jun, which drive transcription at AP-1 sites, thereby preventing IL-2 gene transcription in anergic T cells. The ability to regulate Ras activation in this way may be used by the immune system to specifically prevent IL-2 production while allowing other T cell functions to proceed. Determination of the precise target or targets in the TCR signaling cascade responsible for this block in Ras activation should provide important insight into the mechanisms involved in the maintenance of T cell tolerance.

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10. Cells were stimulated by soluble cross-linking of CD3 and CD4 with the use of mAbs GK1.5 and 145-2C11 (1:1 mixture) (*23*). After precoating of cells with this mAb cocktail, 0.5 ml of cross-linking antisera goat antiserum to hamster [(20 μg/ml) (Cappel)] was added and cells were stimulated while being mixed on a rotator at 37°C. This antibody bound to both GK1.5 and 145-2C11 efficiently (*7*). At various times, cells were pelleted by centrifugation (10,000*g* for 5 s) for lysis.
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Antigen Presentation and T Cell Development in H2-M–Deficient Mice

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HLA-DM (DM) facilitates peptide loading of major histocompatibility complex class II molecules in human cell lines. Mice lacking functional H2-M, the mouse equivalent of DM, have normal amounts of class II molecules at the cell surface, but most of these are associated with invariant chain–derived CLIP peptides. These mice contain large numbers of $CD4^+$ T cells, which is indicative of positive selection in the thymus. Their $CD4^+$ cells were unresponsive to self H2-M–deficient antigen-presenting cells (APCs) but were hyperreactive to wild-type APCs. H2-M–deficient APCs failed to elicit proliferative responses from wild-type T cells.

Experiments in vitro suggest that DM facilitates the exchange of major histocompatibility complex (MHC) class II–associated invariant chain peptides (CLIP) for antigenic peptides (*1*). The general importance of DM for peptide loading is debatable, however, because mouse class II molecules have been reported to be less dependent on DM (*2*) than human class II molecules (*3*). To evaluate the function of H2-M in vivo, we generated mice lacking H2-M_α (Fig. 1, A and B) (*4*). H2-M αβ heterodimer formation is necessary for H2-M function, and in the absence of H2-M_α H2-M_β has a reduced half-life and does not leave the endoplasmic reticulum (*5*, *6*).

Splenocytes from H2-$M^{+/+}$ (wild-type) and H2-$M^{-/-}$ (H2-M–deficient) mice were analyzed for H2-M expression with the use of indirect immunofluorescence (*7*). In H2-$M^{+/+}$ mice, H2-M staining was located in vesicular structures (Fig. 1C), as described previously (*6*), whereas no H2-M staining was detected in cells from H2-$M^{-/-}$ mice (Fig. 1D). Costaining with H2-A^b mono-

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clonal antibody (mAb) M5/114 showed no distinct staining differences between the two cell types (compare Fig. 1, C and D, green). The absence of normal H2-M protein in the mutant mice was confirmed by two-dimensional gel electrophoresis of immunoprecipitated H2-M from metabolically labeled splenocytes (8). Whereas precipitates from wild-type cells contained both H2-M_α and H2-M_β, no H2-M protein was detected in the precipitates from the H2-$M^{-/-}$ cells (Fig. 1E).

To determine the effect of H2-M on the cell surface expression of MHC class II molecules, we incubated lymph node cells from wild-type and H2-$M^{-/-}$ mice with a panel of H2-A^b–reactive mAbs and analyzed them by flow cytometry (fluorescence-activated cell sorting, or FACS) (9). Several of the mAbs (M5/114 in Fig. 2A and Y3P and AF6-120.1, not shown) stained wild-type and mutant cells with equal intensity, which indicates that the cell surface amounts of H2-A^b were comparable in the two types of cells. In contrast to the effects of these mAbs, differential staining was observed with two other H2-A^b mAbs: BP107 did not stain mutant cells at all, whereas KH74 stained mutant cells with reduced intensity (Fig. 2A). These findings suggest that the H2-A^b conformation on the H2-$M^{-/-}$ cells might be different from that of the wild-type control cells.

In view of the well-documented findings implicating DM in the removal of CLIP from class II molecules (*1*), the reduced binding of some H2-A^b mAbs to H2-$M^{-/-}$ cells could reflect a failure to exchange CLIP for other peptides. To examine this possibility, we stained mutant cells with mAb 30-2, which reacts with CLIP-associated H2-A^b (*10*). In contrast to its weak reactivity with wild-type cells, mAb 30-2 strongly stained H2-$M^{-/-}$ cells (Fig. 2A). Moreover, pre-incubation with mAb 30-2 completely blocked the reactivity of H2-A^b mAb KH74 with mutant cells, although the same treatment had no effect on KH74 staining of H2-$M^{+/+}$ control cells (Fig. 2A). Thus, virtually all class II molecules appeared to contain CLIP.

Immunohistochemical analysis of tissue sections from H2-$M^{-/-}$ mice confirmed the FACS analysis findings (*11*). Thus, H2-M expression in the lymphoid tissues of mutant mice was undetectable, whereas in wild-type mice H2-M expression was observed in B cells, macrophages, and dendritic cells in the spleen and lymph nodes. In normal thymus, H2-M was expressed in cortical epithelial cells and in the medulla but was completely undetectable in the H2-$M^{-/-}$ thymus (Fig. 2B). Class II expression in the thymuses of mutant mice was comparable to that in the wild-type controls when analyzed with mAb M5/114, whereas no staining was observed with mAb BP107 in the mutant thymuses (Fig. 2B). Similar to the class II molecules in lymph node B cells, the class II molecules in the H2-$M^{-/-}$ thymuses appeared to contain mainly CLIP, because both epithelial cells and bone marrow–derived APCs stained strongly with mAb 30-2. In contrast, this antibody stained only a few scattered cells in the medullas of the wild-type thymuses (Fig. 2B).

Under mildly denaturing conditions, class II molecules containing well-fitting peptides often migrate as dimers in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels (*12*), whereas class II molecules with poorly fitting peptides dissociate and migrate as single α and β chains. The SDS stability of H2-A^b molecules from wild-type or mutant mice was analyzed in a pulse chase experiment (8). After immunoprecipitation with M5/114, samples were analyzed by SDS-PAGE without boiling, thus leaving stable class II dimers intact. In splenocytes from H2-$M^{+/+}$ mice, SDS-stable dimers (αβ) were formed within 1 hour of incubation (chase) and were still prominent after 24 hours of chase (Fig. 2C). Only small amounts of SDS-unstable class II monomers were seen. Surprisingly, the H2-A^b molecules precipitated from H2-$M^{-/-}$ splenocytes also migrated as SDS-stable dimers, though their migration was slightly slower than the migration of dimers derived from wild-type cells (Fig. 2C, αβ*). Some class II monomers were also seen, and a low molecular weight band representing CLIP was prominent. H2-$M^{-/-}$–derived H2-A^b molecules appeared compact rather than floppy in nature (*13*), migrating as distinct bands in contrast to the diffuse dimer band seen in the wild-type precipitate.

These results suggest that a limited number of peptides, most likely CLIP peptides, were responsible for the dimer bands. SDS-stable DR1-CLIP complexes have been reported (*14*), and because CLIP binds strong-

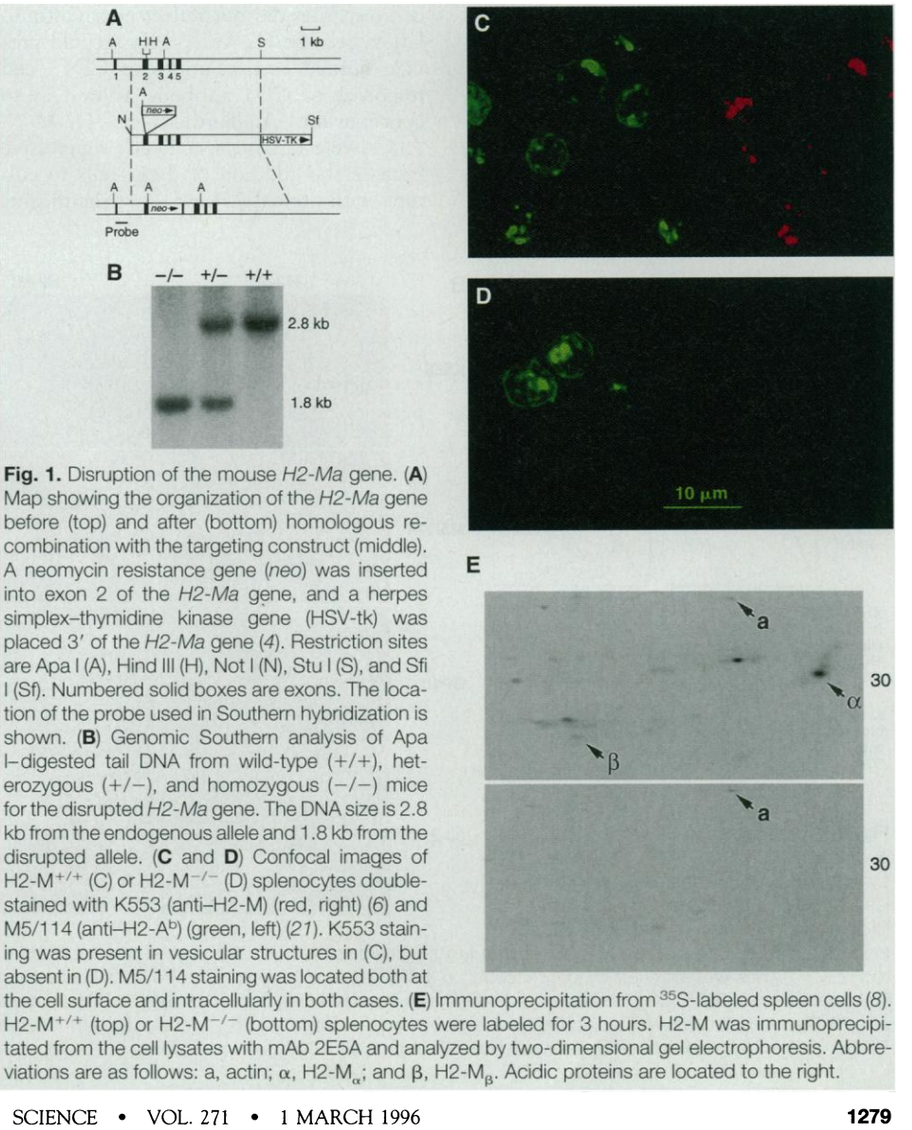

Fig. 1. Disruption of the mouse *H2-Ma* gene. (**A**) Map showing the organization of the *H2-Ma* gene before (top) and after (bottom) homologous recombination with the targeting construct (middle). A neomycin resistance gene (*neo*) was inserted into exon 2 of the *H2-Ma* gene, and a herpes simplex–thymidine kinase gene (HSV-tk) was placed 3′ of the *H2-Ma* gene (*4*). Restriction sites are Apa I (A), Hind III (H), Not I (N), Stu I (S), and Sfi I (Sf). Numbered solid boxes are exons. The location of the probe used in Southern hybridization is shown. (**B**) Genomic Southern analysis of Apa I–digested tail DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice for the disrupted *H2-Ma* gene. The DNA size is 2.8 kb from the endogenous allele and 1.8 kb from the disrupted allele. (**C** and **D**) Confocal images of H2-$M^{+/+}$ (C) or H2-$M^{-/-}$ (D) splenocytes double-stained with K553 (anti–H2-M) (red, right) (*6*) and M5/114 (anti–H2-A^b) (green, left) (*21*). K553 staining was present in vesicular structures in (C), but absent in (D). M5/114 staining was located both at the cell surface and intracellularly in both cases. (**E**) Immunoprecipitation from ^{35}S-labeled spleen cells (*8*). H2-$M^{+/+}$ (top) or H2-$M^{-/-}$ (bottom) splenocytes were labeled for 3 hours. H2-M was immunoprecipitated from the cell lysates with mAb 2E5A and analyzed by two-dimensional gel electrophoresis. Abbreviations are as follows: a, actin; α, H2-M_α; and β, H2-M_β. Acidic proteins are located to the right.

ly to H2-A^b (*15*), formation of SDS-stable dimers is possible, though unexpected. The intensity of the class II bands did not significantly decrease during the 24-hour chase period, indicating that the half-life of H2-A^b in the mutant mice is similar to its half-life in wild-type mice. In boiled (and reduced) samples (Fig. 2D), the class II molecules migrated as monomers, and the only distinct difference between the wild-type and mutant precipitates was the large amount of CLIP present in the mutant sample (*16*).

We next determined whether CLIP-associated class II molecules were able to mediate normal selection of $CD4^+$ T cells. The proportion of lymph node (and splenic; not shown in figure) $CD4^+$ cells (Fig. 3A) was reduced in H2-$M^{-/-}$ mice to approximately 30 to 50% of that in normal mice. This reduction in $CD4^+$ cells was also seen in the mutant thymuses, though to a lesser extent. Nonetheless, the lymphoid tissues appeared normal, and the finding that large numbers of $CD4^+$ cells did develop indicates that positive selection by means of H2-A^b molecules did occur in the H2-$M^{-/-}$ mice. The phenotype of the $CD4^+$ cells generated in these mice was similar to that from H2-$M^{+/+}$ mice. Thus, most of the extrathymic $CD4^+$ cells displayed a naïve phenotype (Fig. 3B), and analysis of V_β usage suggested that the cells were polyclonal (*17*).

To determine whether the H2-$M^{-/-}$ $CD4^+$ cells were functional, we analyzed their ability to proliferate in response to alloantigens. $CD4^+$ cells from the mutant mice failed to respond to their own splenic APCs, which is consistent with normal self-tolerance induction. In contrast, these cells reacted strongly to APCs from MHC-matched wild-type littermates (and to those from normal C57BL/6 mice). This hyperreactivity was apparent as early as day 2 in culture and was maximal by days 3 to 4. Titration of responder $CD4^+$ cells indicated that the H2-$M^{-/-}$ cells were 10- to 100-fold more responsive to H2-A^b than were $CD4^+$ cells from normal wild-type mice. An abnormally strong proliferative response was also seen after exposure of H2-$M^{-/-}$ $CD4^+$ cells to APCs from a variety of MHC allogeneic strains, including Bl0.D2 (H2-A^d), Bl0.BR (H2-A^k), and B6.bm12 (H2-A^{bm12}). Not surprisingly, in view of the limited peptide repertoire of the H2-$M^{-/-}$ class II molecules, the APCs from H2-$M^{-/-}$ mice failed to stimulate MHC allogeneic T cells (Fig. 4B). The inability to elicit proliferative T cell responses did not reflect poor costimulation because H2-$M^{-/-}$ APCs could provide normal costimulation for $CD4^+$ cell responses to CD3 antibody as well as to concanavalin A. Furthermore, H2-$M^{-/-}$ APCs were not nonspecifically suppressive because the addition of these cells to cultures with normal APCs did not significantly alter their ability to initiate a response.

These findings are consistent with the notion that a limited peptide repertoire (mainly consisting of CLIP) can support positive selection of large numbers of functional $CD4^+$ T cells. Nonetheless, the reduced number of $CD4^+$ cells in these mutant mice also suggests that a normal density of class II molecules on thymic epithelial cells is not sufficient to achieve maximal levels of positive selection and that peptide diversity contributes to the efficiency of this process. It is also not clear how diverse the selected T cell repertoire is, though the strong reactivity to both syngeneic and allogeneic APCs suggests that there is considerable diversity.

The H2-$M^{-/-}$ mice do not display overt autoimmunity, which indicates that their tolerance to CLIP-associated class II molecules is normal. The hyperreactivity of H2-$M^{-/-}$ $CD4^+$ cells to H2-A^b APCs from normal mice suggests, however, that the diversity of the class II–associated peptides is too limited in H2-$M^{-/-}$ mice to induce negative selection to self peptides other than CLIP. The three-dimensional structure of HLA-DR3-CLIP (*18*) suggests that class II–CLIP complexes may not be qualitatively different from other class II–peptide complexes. Therefore, the failure of allogeneic $CD4^+$ T cells to respond to H2-$M^{-/-}$ APCs is unlikely to reflect a conformational

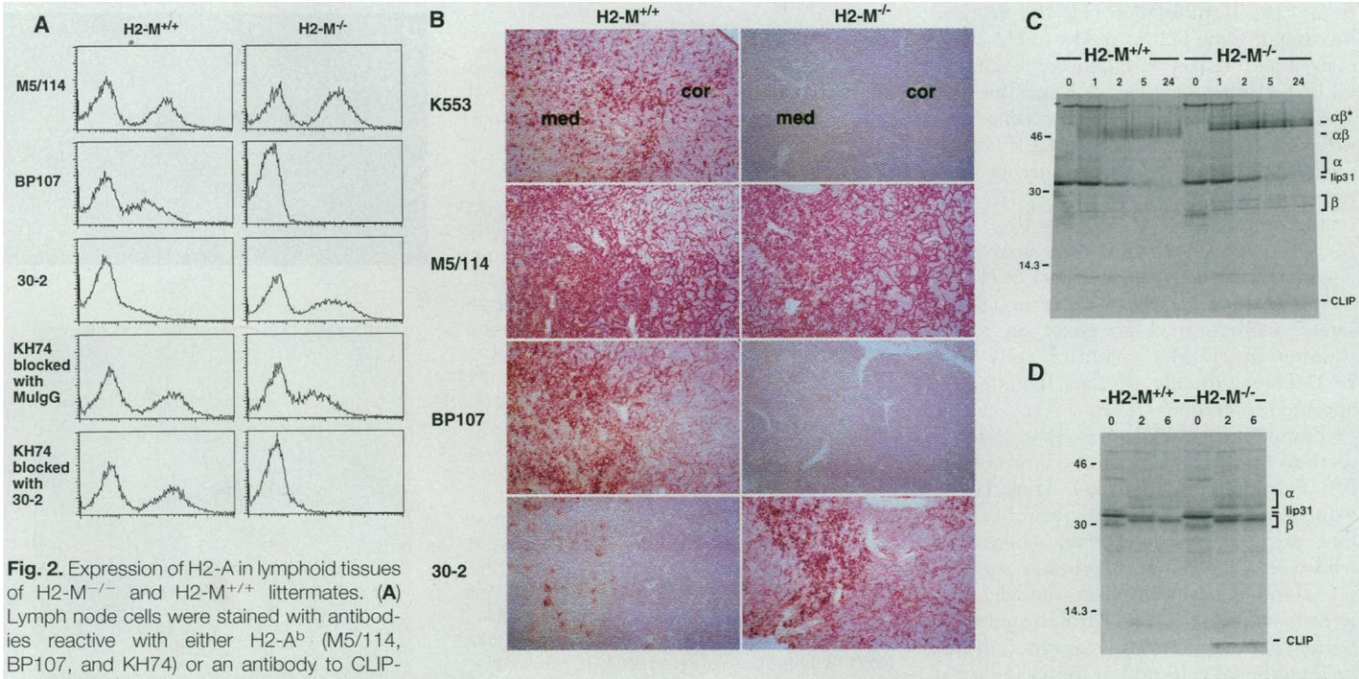

Fig. 2. Expression of H2-A in lymphoid tissues of H2-$M^{-/-}$ and H2-$M^{+/+}$ littermates. (**A**) Lymph node cells were stained with antibodies reactive with either H2-A^b (M5/114, BP107, and KH74) or an antibody to CLIP-loaded H2-A^b (30-2) and analyzed by flow cytometry (*9*). The binding of KH74 to H2-$M^{-/-}$ cells was blocked by prior incubation with 30-2 but not with irrelevant mouse IgG (MuIgG). Fluorescence intensity is measured along the *x* axis. (**B**) Thymus sections were stained with either K553 (which is reactive with H2-M), with H2-A^b–reactive mAbs (M5/114 and BP107), or with CLIP mAb (30-2); med, medulla; cor, cortex. (**C** and **D**) H2-$M^{+/+}$ and H2-$M^{-/-}$ splenocytes were labeled for 30 min and analyzed immediately (0) or after various periods of incubation with unlabeled medium as indicated (in hours). H2-A^b molecules were immunoprecipitated with M5/114. Nonboiled (C) or boiled (and reduced) (D) samples were analyzed by SDS-PAGE. Abbreviations are as follows: α, H2-A^b_α; β, H2-A^b_β; and αβ and αβ*, H2-A^b dimers. Size markers are in kilodaltons; lip31, invariant chain p31.

change in the class II molecules that would abolish T cell receptor binding. The lack of response is more likely a reflection of the fact that most class II molecules on H2-M$^{-/-}$ APCs contain CLIP. Despite the high density of this complex on the H2-M$^{-/-}$ APCs, the precursor frequency of T cells able to recognize a single class II peptide complex (that is, allogeneic MHC-CLIP) is presumably low and the reactivity of these cells undetectable in assays of proliferative response. The data presented here show that H2-M is essential for generating a normal repertoire of CD4$^+$ T cells as well as for the presentation of a normal array of peptide antigens.

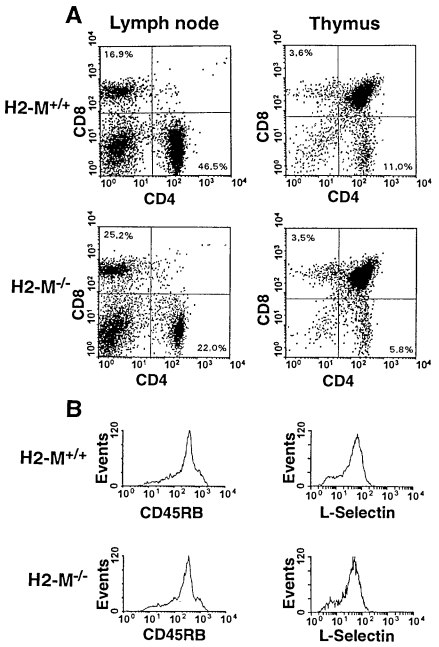

Fig. 3. Analysis of T cell markers in H2-M$^{-/-}$ and H2-M$^{+/+}$ mice. (**A**) Lymph node cells or thymocytes were stained with antibodies reactive with CD4 and CD8 and analyzed by flow cytometry. (**B**) Analysis of lymph node CD4$^+$ T cells for activation markers with the use of CD45RB (left) and L-selectin (right) indicated a naïve phenotype (*27*).

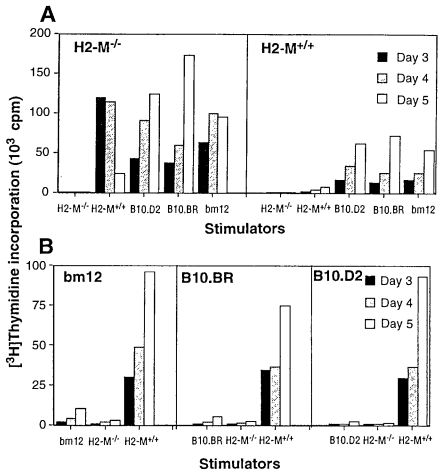

Fig. 4. CD4$^+$ T cell function and antigen-presenting capacity (*28*). (**A**) Reactivity of CD4$^+$ T cells from H2-M$^{-/-}$ (left) or H2-M$^{+/+}$ (right) mice to APCs from different mouse strains. (**B**) Ability of H2-M$^{-/-}$ and H2-M$^{+/+}$ APCs to stimulate allogeneic CD4$^+$ T cells. Responses were analyzed after 3, 4, and 5 days of culture.

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4. A 6.7-kb DNA fragment from a 129/Sv mouse genomic clone covering most of the *H2-Ma* gene except exon 1 and part of intron 1 was used in the targeting construct as a homologous region for recombination. A cassette containing a neomycin resistance gene (*neo*) was cloned into the second Hind III site in exon 2 of the *H2-Ma* gene. A deletion of 61 bp 5′ to the *neo* cassette insertion site in exon 2 was also made in the construct. An HSV-tk cassette (herpes simplex virus–thymidine kinase) was placed at the 3′ end of the construct. The DNA construct was introduced into E14 embryonic stem (ES) cells by electroporation. Cells were cultured in the presence of 400 μg/ml of G418 and 0.2 μM ganciclovir (*19*). ES cells with the targeted gene were detected by polymerase chain reaction with the use of the *H2-Ma* gene–specific oligonucleotide 5′-GGATTCCTGTCAGGAGTTTCAAAG-3′ and the *neo*-specific oligonucleotide 5′-AAGCGCATGCTCCAGACTGCCTT-3′. Mice that were +/+, +/−, and −/− for the disrupted *H2-Ma* gene were identified by genomic Southern (DNA) analysis with the use of a 0.6-kb DNA probe (shown in Fig. 1) to hybridize to Apa I–cut tail DNA. Homozygous H2-M$^{-/-}$ mice do not have any obvious signs of disease, and their organs appear macroscopically normal. H2-M$^{-/-}$ and H2-M$^{+/+}$ mice are H2^b and thus express only H2-A, not H2-E.
5. The H2-M locus contains two *H2-Mb* genes, *H2-Mb1* and *H2-Mb2*. In the spleen, 90% of H2-Mb transcripts are derived from H2-Mb2, resulting in a nonglycosylated H2-M$_\beta$ protein. It will here be referred to as H2-M$_\beta$.
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7. Spleen cells were cultured on cover slips coated with Cell-Tak (Collaborative Biomedical Research) before fixation with 4% formaldehyde–phosphate-buffered saline (PBS). After fixation, cells were washed with 50 mM NH_4Cl and PBS. Antibody incubations were made in PBS with 0.6% fish skin gelatin and 0.2% saponin for permeabilization. Texas-Red–labeled rabbit antibody to immunoglobulin G (IgG) (Molecular Probes) and fluorescein isothiocyanate (FITC)–labeled rat antibody to IgG (Cappel) were used as secondary reagents. Fluorescent cells were imaged with the use of a Bio-Rad confocal microscope.
8. Splenocytes were labeled with [^{35}S]methionine and [^{35}S]cysteine as described (*6*) before lysis in 1% Triton X-100, PBS, and complete proteinase inhibitor cocktail (Boehringer Mannheim). H2-M was immunoprecipitated with mAb 2E5A, which is reactive with H2-M αβ dimers (*20*), and H2-A^b was precipitated with M5/114 (*21*). Immunoprecipitates were harvested with protein G–sepharose, washed, and resuspended in isoelectric focusing (IEF) sample buffer (Fig. 1E) or SDS-PAGE sample buffer containing 2% SDS without (Fig. 2C) or with 10 mM dithiothreitol (Fig. 2D). Samples were left at room temperature for 20 min (Fig. 2C) or boiled for 5 min (Fig. 2D). Samples were separated on 7.5 to 12.5% polyacrylamide gels directly or after IEF (pH 5 to 7). The gels were then fixed, dried, and autoradiographed. Autoradiographs were scanned with an Agfa ArcusII scanner. Composites were printed on a Kodak XLS 8600 printer.
9. Lymph node cells (0.5 × 10^6) were incubated with biotinylated M5/114, BP107 (*22*), or KH74 (*23*) followed by FITC-streptavidin (Jackson ImmunoResearch). For staining with CLIP–H2-A^b antibodies, cells were incubated with 30-2, and the bound antibodies detected with biotinylated antibody to murine IgG (MulgG, γ chain–specific; Jackson ImmunoResearch) and FITC-streptavidin. For blocking KH74, cells were incubated with 10 μg/ml of 30-2 or MulgG for 1 hour before addition of biotinylated KH74. T cell and thymocyte stainings were done as described (*24*). KH74 does not immunoprecipitate CLIP-containing H2-A^b, which suggests that the binding affinity for this complex is low.
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11. Thymus cryostat sections were stained for H2-M with the use of rabbit antiserum K553 (*6*) followed by biotinylated rabbit antibody to IgG (Jackson ImmunoResearch); for H2-A^b, with the use of biotinylated M5/114 or BP107; and for CLIP, with the use of biotinylated 30-2 mAbs. Bound antibodies were detected with alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch) followed by colorimetric substrate as described (*25*).
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27. CD4$^+$ T cells from H2-M$^{-/-}$ and H2-M$^{+/+}$ mice were L-selectin^{hi}, CD44^{lo}, and CD45RB^{hi}, and few expressed markers associated with activation—for example, CD69 or interleukin-2 receptor.
28. Responder cell populations were pooled lymph node cells enriched for CD4$^+$ cells by treatment with a cocktail of antibodies specific for B cells, class II–expressing cells, and CD8$^+$ cells as described (*26*) together with complement. Spleen cells depleted of T cells by treatment with antibody to CD4 (RL172; anti-CD4), anti-CD8 (3.16.8), anti–Thy-1 (J1J), and complement were treated with mitomycin C and used as a source of APCs. Responder cells (1.5 × 10^5) were cultured with 5 × 10^5 APCs in a final volume of 200 μl. The wells were pulsed with 1 μCi of [^{3}H]thymidine for approximately 18 hours before harvesting and counting.

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