

# HLA-A2.1-restricted Education and Cytolytic Activity of CD8<sup>+</sup> T Lymphocytes from $\beta$ 2 Microglobulin ( $\beta$ 2m) HLA-A2.1 Monochain Transgenic H-2D<sup>b</sup> $\beta$ 2m Double Knockout Mice

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## Summary

Three different HLA-A2.1 monochains were engineered in which either the human or mouse  $\beta$ 2-microglobulin ( $\beta$ 2m) is covalently linked to the NH<sub>2</sub> terminus of the heavy chain by a 15-amino acid long peptide: HHH, entirely human, HHD, with the mouse H-2D<sup>b</sup>  $\alpha$ 3, transmembrane, and cytoplasmic domains, and MHD, homologous to HHD but linked to the mouse  $\beta$ 2m<sup>b</sup>. The cell surface expression and immunological capacities of the three monochains were compared with transfected cells, and the selected HHD construct was introduced by transgenesis in H-2D<sup>b</sup><sup>-/-</sup>  $\beta$ 2m<sup>-/-</sup> double knockout mice. Expression of this monochain restores a sizable peripheral CD8<sup>+</sup> T cell repertoire essentially educated on the transgenic human molecule. Consequently, infected HHD, H-2D<sup>b</sup><sup>-/-</sup>  $\beta$ 2m<sup>-/-</sup> mice generate only HLA-A2.1-restricted CD8<sup>+</sup> CTL responses against influenza A and vaccinia viruses. Interestingly, the CTL response to influenza A virus is mostly, if not exclusively, directed to the 58-66 matrix peptide which is the HLA-A2.1-restricted immunodominant epitope in humans. Such mice might constitute a versatile animal model for the study of HLA-A2.1-restricted CTL responses of vaccine interest.

Transgenic mice expressing unmodified HLA class I molecules have been derived in many laboratories to provide a suitable animal model for the study of HLA class I-restricted CTL responses (1). Despite a few reported successes (2–6), these attempts have been relatively disappointing; when virus infected or stimulated by other HLA class I alleles, these mice preferentially (most of the time exclusively) develop H-2-restricted CD8<sup>+</sup> CTL responses (7–10). Substitution of the HLA  $\alpha$ 3 by the homologous H-2 domain significantly improves the recognition and usage of some (A2.1, B27), however not all (i.e., B7.1, our unpublished observation), HLA class I molecules (11, 12). Similarly, we and others have established that recognition and usage of transgenic HLA class I molecules by mouse CD8<sup>+</sup> T lymphocytes can be promoted when H-2-restricted responses are controlled. More importantly, under such circumstances, a diversified V $\beta$  and V $\alpha$  TCR mouse repertoire is mobilized, suggesting a sufficient flexibility for an efficient usage of human class I molecules (13, 14). However, the experimental artifices (cross-tolerance, serial stimulations in vitro with appropriate antigen presenting cells) selected to favor the HLA-restricted CTL responses in transgenic mice are not of convenient usage. Therefore, we have derived mice expressing only HLA class I molecules to force the mouse CD8<sup>+</sup> T cell repertoire, both at the thymic and pe-

ripheral levels, to make use of the transgenic HLA class I molecules. These mice are H-2D<sup>b</sup> and mouse  $\beta$ 2 microglobulin ( $\beta$ 2m)<sup>1</sup> double knockouts and express  $\beta$ 2m-HLA-A2.1 monochains.

We report in vitro transfection experiments that resulted in the selection of a human  $\beta$ 2m-HLA-A2.1 ( $\alpha$ 1 $\alpha$ 2)-H-2D<sup>b</sup> ( $\alpha$ 3 transmembrane cytoplasmic) (HHD) monochain construct to derive transgenic animals. These HHD transgenic, H-2D<sup>b</sup><sup>-/-</sup>  $\beta$ 2m<sup>-/-</sup> double knockout mice are almost devoid of H-2 class I molecules. Phenotypic and functional analyses of their peripheral CD8<sup>+</sup> T cell repertoire indicate that HHD monochains support thymic positive selection of CD8<sup>+</sup> CTL and activate virus-specific HLA-A2.1-restricted CTL in the periphery.

## Materials and Methods

**Plasmids.** The 2.2-kb EcoRI-BglII fragment encompassing the promoter and the three first exons from the HLA-A2.1 gene (10) was subcloned in a modified BglII<sup>+</sup> pBluescript (Stratagene, La Jolla, CA) and site mutagenized to introduce a PstI site at the

<sup>1</sup>Abbreviations used in this paper:  $\beta$ 2m,  $\beta$ -2 microglobulin; HA, hemagglutinin; TAP, transporter associated with antigen presentation.

3' end of the first exon and a BamHI site at the 5' end of the second exon (HLA-A2.1 PB plasmid). BamHI and MscI restriction sites were introduced at the 5' and 3' ends of human  $\beta 2m$  and murine  $\beta 2m^b$  cDNA, respectively, corresponding to the mature form (without leader sequence) of the proteins. A three-partner ligation was performed between the 5' PstI (T4 polymerase blunt ended), 3' BamHI-digested HLA-A2.1 PB plasmid, the 5' BamHI (Klenow blunt ended), 3' MscI-digested human or murine  $\beta 2m$  cDNA, and a pair of complementary synthetic oligonucleotides (Genset, Paris, France) coding for a (Gly<sub>4</sub>Ser)<sub>3</sub> linker with 5' blunt and 3' BamHI cohesive end. Final constructs were verified by double-stranded DNA sequencing (Sequenase version 2.0; United States Biochemical, Cleveland, OH). In the final recombinant plasmids, super-exon I codes for the HLA-A2.1 leader sequence, the human or mouse  $\beta 2m$ , a 15 amino acid linker, and the  $\alpha 1$  domain of HLA-A2.1. A 5.1-kb BglII-BamHI or a 2.3-kb BamHI fragment containing the fourth to eighth exons and the 3' untranslated region of the HLA-A2.1 or H-2D<sup>b</sup> genes, respectively, was subsequently introduced in the BglII site of this construct. This provided the entirely human HHH, human  $\beta 2m$ -HLA-A2.1 ( $\alpha 1\alpha 2$ ) H-2D<sup>b</sup> ( $\alpha 3$  to COOH terminus) HHD, and mouse  $\beta 2m^b$ -HLA-A2.1 ( $\alpha 1\alpha 2$ ) H-2D<sup>b</sup> ( $\alpha 3$  to COOH terminus) MHD coding plasmids.

A control construct was also created introducing the BamHI 3' fragment of the H-2D<sup>b</sup> gene in the BglII site of the HLA-A2.1 gene (in wild-type configuration) providing a chimeric (HLA-A2.1  $\alpha 1\alpha 2$ , H-2D<sup>b</sup>  $\alpha 3$ , transmembrane and cytoplasmic) heavy chain.

**Cells and Transfectants.** RMA (transporter associated with antigen presentation [TAP] positive), RMA-S (TAP negative), EL4 ( $\beta 2m$  positive), and EL4 S3 Rob ( $\beta 2m$  negative) C57BL/6 (H-2<sup>b</sup>) lymphoma cells were maintained in RPMI, 10% FCS. Cells ( $5 \times 10^7$ ) in 500  $\mu$ l of PBS were electroporated with 50  $\mu$ g of plasmid constructs and 1  $\mu$ g of pMCS (15) linearized DNA at 250 V and either 1,500 (RMA, RMA-S) or 1,050 (EL4, EL4 S3 Rob)  $\mu$ F using an Easyject gene pulser (Eurogentec, Seraing, Belgium). After 24 h, cells were transferred in selective medium containing 1 mg/ml G418 (GIBCO BRL, Paisley, U.K.) and cloned by limiting dilution. Neomycin-resistant clones expressing the monochains were identified by indirect immunofluorescence using unlabeled B9.12.1 (anti-HLA class I, F(ab')<sub>2</sub>) and goat anti-mouse Ig (F(ab')<sub>2</sub>) FITC-conjugated antibodies and analyzed by flow cytofluorometry with a FACScan<sup>®</sup> (Becton Dickinson, San Jose, CA).

**Immunoprecipitations.** Cells ( $5 \times 10^6$ ) were washed in methionine- and cysteine-free RPMI medium (ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with 10% dialyzed FCS and incubated for 45 min at 37°C in 1 ml of the same medium before labeling for 15 min with 1 mCi of [<sup>35</sup>S] methionine-cystein mix (Pro-mix; Amersham, Buckinghamshire, U.K.). Pelleted cells were lysed in PBS containing 1% BSA and 1% NP-40 (BDH Chemicals, Ltd., Poole, U.K.). Lysates were precleared (2 h, 4°C with protein A-Sepharose beads) and then incubated overnight at 4°C with 10  $\mu$ g of purified anti-HLA-A2.1 (BB7.2) mAb. After addition of protein A-Sepharose beads and further incubation for 2 h at 4°C, beads were washed four times with 60 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholic acid, 5 mM EDTA, 1% NP-40, 0.1% SDS. Proteins were eluted, denatured, and separated by SDS-PAGE on 12% gels. Dried gels were exposed on a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and analyzed using Imagequant program (Molecular Dynamics).

**Generation of CTL and Cytolytic Assays.** HLA-A3 transgenic C57BL/6 mice were injected intraperitoneally with  $3 \times 10^7$  HLA-A2.1  $\times$  human  $\beta 2m$  transgenic C57BL/6 mouse splenocytes. 2 wk later, splenocytes ( $5 \times 10^7$ ) from immunized mice were restimulated in vitro with  $3 \times 10^7$  irradiated (2,000 rads) HLA-A2.1  $\times$

human  $\beta 2m$  transgenic splenocytes for 5 d. Human recombinant IL-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was added on day 3 of culture.

Influenza A-specific, HLA-A2.1-restricted HAM 42 CTL clone generated in HLA-A2.1 transgenic mice (8), was provided by Dr. V. Engelhard and maintained in vitro by weekly restimulation with HLA-A2.1-positive JY human lymphoblastoid cells, pulsed with  $10^{-6}$  M of influenza A matrix 58-66 synthetic peptide GILGFVFTL.

HHD<sup>+</sup> H-2D<sup>b</sup><sup>-/-</sup>  $\beta 2m$ <sup>-/-</sup> mice were intraperitoneally infected with either 1,000 hemagglutinin (HA) units (influenza A/PR/8/34, provided by Dr. J.-C. Manuguerra, Laboratoire de Genetique Molculaire des Virus Respiratoire Institut Pasteur, Paris, France) or  $10^7$  PFU (vaccinia) viruses. 2-4 wk later,  $2.5 \times 10^7$  (influenza A) or  $6 \times 10^7$  (vaccinia) splenocytes were restimulated in vitro with  $2.5 \times 10^7$  (influenza A) or  $3 \times 10^7$  (vaccinia) irradiated (3,000 rads) syngeneic red blood cell-depleted splenocytes infected for 1 h with influenza A (10 HA unit for  $10^6$  cells) or vaccinia (10 PFU/cell) viruses.

Responder mice were individually tested in a standard cytolytic assay 5 d later. In brief,  $10^6$  cells, uninfected or infected (1,000 HA units of influenza A or  $5 \times 10^7$  PFU of vaccinia viruses) for 1.5 h in medium without FCS and further incubated for 2 h in medium containing 5% FCS, were subsequently labeled with 100  $\mu$ Ci of sodium [<sup>51</sup>Cr]chromate for 1.5 h at 37°C and then washed three times. Cytolytic activity was determined in 4 h <sup>51</sup>Cr-release assays using V-bottom 96-well plates containing  $5 \times 10^3$  uninfected, influenza A matrix 58-66 peptide-pulsed ( $10^{-6}$  M) or virus-infected target cells/well in the presence of effector cells from bulk cultures. Effector/target ratios were as shown in Figs. 3 and 8. Results are the mean of triplicates calculated as  $100 \times [(\text{experimental} - \text{spontaneous release}) / (\text{total} - \text{spontaneous release})]$ , with maximal release being determined by lysis of target cells by 1 M HCl.

**Generation of H-2D<sup>b</sup><sup>-/-</sup> Knockout Mice.** A 10-kb HindIII fragment containing the whole H-2D<sup>b</sup> gene and a 1.9-kb PstI fragment encompassing the 3' part of the third exon, the fourth intron, and the 5' part of the fourth exon were cloned in pBluescript (Stratagene) vector. The H-2D<sup>b</sup> targeting construct was generated by inserting a 4-kb XbaI 5' fragment from the H-2D<sup>b</sup> gene and a 1.3-kb KpnI 3' fragment from the PstI subclone in the corresponding restriction sites of the pGNA vector polylinker (16). CGR-8 embryonic stem cells were cultured as described (17) in the absence of feeder cells in medium supplemented with murine differentiation inhibiting factor and/or leukemia inhibiting factor. To isolate homologous recombinants,  $10^8$  cells were electroporated in 900  $\mu$ l of PBS with 150  $\mu$ g of Spel-linearized plasmid DNA at 800 V and 3  $\mu$ F, using a Bio Rad Labs. (Hercules, CA) gene pulser and selected after 24 h in the presence of G418 (175  $\mu$ g/ml). 28 pools of 12 G418-resistant clones were screened by PCR, using a pGNA-specific 5' (CAGCAGAAACATACAAGCTGTC) and a H-2D<sup>b</sup> exon 5-specific 3' (AACGATCACCATGTAAGAGT-CAGT) pair of oligonucleotides, resulting in the amplification of the expected 1.6-kb fragment for 18 pools. The homologous recombination event, detected by hybridization of a 5.6-kb HindIII fragment with a 3' noncoding BamHI-HindIII probe, was confirmed in six colonies by Southern blot analysis. Two out of five clones injected into C57BL/6 blastocysts gave rise to germline transmission of the mutation. Germline chimeras were mated with C57BL/6 females and pups were typed by Southern blot analysis of tail DNA. Heterozygous offspring were backcrossed to C57BL/6 animals and then intercrossed at the N2 generation to give rise to two independent H-2D<sup>b</sup><sup>-/-</sup> homozygous strains. Inactivation of the H-2D<sup>b</sup> gene was confirmed by Southern blot analysis on tail DNA and immunofluorescence assay.

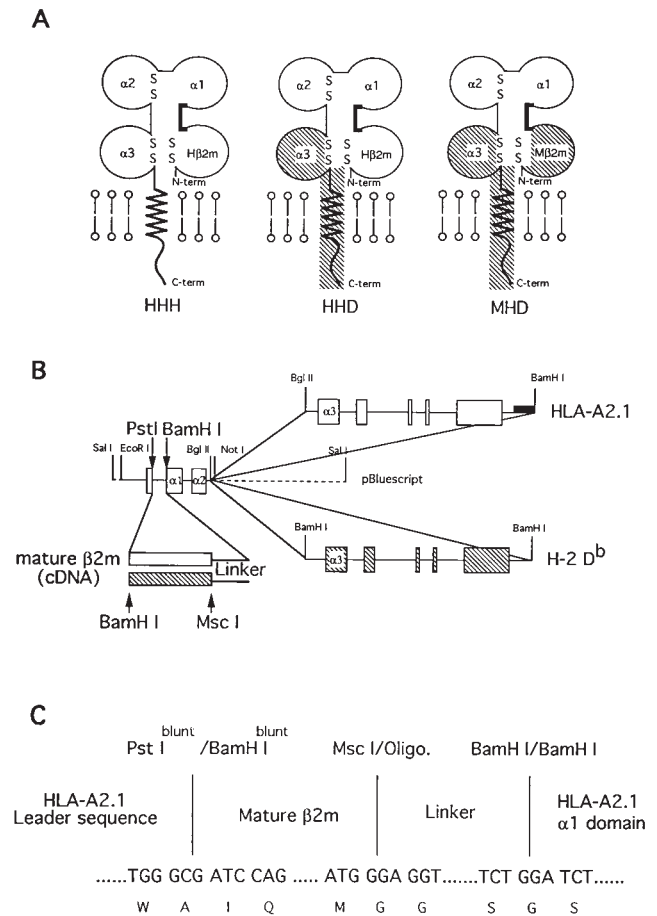
**Generation of HHD Transgenic Mice.** A 4-kb SalI–NotI fragment containing the HHD construct was injected into C57Bl/6 × SJL oocytes. Transgenic mice identified by Southern blot analysis of tail DNA and immunofluorescence assay were crossed with H-2D<sup>b</sup><sup>-/-</sup> β2m<sup>-/-</sup> double knockout mice.

**FACS<sup>®</sup> Analysis of the Peripheral T Cell Repertoire of HHD<sup>+</sup> H-2D<sup>b</sup><sup>-/-</sup> β2m<sup>-/-</sup> Knockout Mice.** Expression of the HHD monochain and lack of H-2D<sup>b</sup> and H-2K<sup>b</sup> were documented by indirect immunofluorescence analyses using B9.12.1 (anti-HLA class I), B22.249.R.19 (anti-H-2D<sup>b</sup>), and 20.8.4S unlabeled mAb, detected with F(ab)<sup>'</sup>2 FITC-conjugated goat anti-mouse IgG. Percentages of single CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were determined by double staining using phycoerythrin-labeled anti-mouse CD4 (CALTAG Labs., South San Francisco, CA) and biotinylated anti-mouse CD8 (CALTAG Labs.) detected with streptavidin–Perc-P (CALTAG Labs.). Expression of the different Vβ TCR were similarly analyzed using phycoerythrin-labeled anti-CD8 mAb (PharMingen, San Diego, CA) and purified, FITC-labeled Vβ2 (B.20.6), Vβ3 (KJ.25), Vβ4 (KT.10.4), Vβ5.1,2 (MR.9.4), Vβ6 (44.22), Vβ7 (TR 130), Vβ8.1,2,3 (F.23.1), Vβ9 (MR. 10.2), Vβ10 (B.21.5), Vβ11 (RR.3.15), Vβ13 (MR.12.4), and Vβ17 (KJ.23.1)-specific mAb. Splenocytes from three individual D<sup>b</sup><sup>-/-</sup>, β2m<sup>-/-</sup>, HHD<sup>+</sup>, or HHD<sup>-</sup> mice were red blood cell depleted and enriched in T lymphocytes by wheat germ agglutinin (Sigma Chemical Co., St Louis, MO) precipitation of B lymphocytes and NK cells as described (18). Staining of 10<sup>6</sup> cells was performed in 100 μl of PBS with 0.02% sodium azide for 30 min on ice. Purified mAb or F(ab)<sup>'</sup>2 were used at 10 μg/ml and F(ab)<sup>'</sup>2 FITC-conjugated goat anti-mouse IgG was used 1:100 diluted. A total of 25,000 1% paraformaldehyde-fixed cells per sample was subjected to one- or two-color analysis on FACScan<sup>®</sup>.

## Results

**Design of HLA-A2.1 Monochains.** Recombinant genes encoding HLA-A2.1 monochains were engineered, as illustrated in Fig. 1, by introducing between the first and second exon of the genomic HLA-A2.1 gene a β2m cDNA (deprived of from the nucleotides corresponding to the leader sequence) and a pair of synthetic oligonucleotides encoding a 15 residue (Gly<sub>4</sub>Ser)<sub>3</sub> peptide linker, as already described (19, 20). The first intron of the HLA-A2.1 gene was therefore deleted resulting in a chimeric exon 1 that codes for the leader sequence of HLA-A2.1, the β2m domain, the peptide linker, and the HLA-A2.1 α1 domain. Three different HLA-A2.1 monochains were engineered: HHH, fully human; HHD, containing the mouse H-2D<sup>b</sup> α3, transmembrane, and cytoplasmic domains; and MHD, homologous to HHD, with the mouse instead of the human β2m. All constructs, verified by sequencing, encode monochains in which the amino acid sequence of the leader, mature β2m, and α1 domains are totally conserved.

**Expression in RMA Cells.** By indirect immunofluorescence analysis of RMA lymphoma transfected cells (Fig. 2 A), cell surface expression of HHH, HHD, and MHD monochains was compared to the cell surface expression of their heterodimeric counterparts. Despite selection of transfectants expressing similar amounts of monochain transcripts (as judged by semiquantitative PCR, data not shown) cell surface expression reaches the level of control cells only for

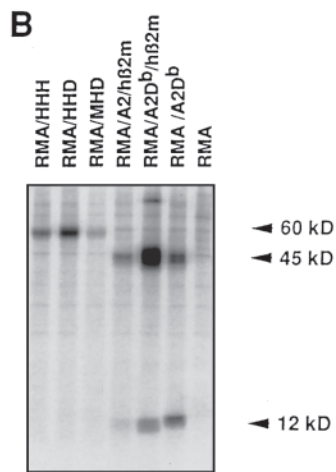
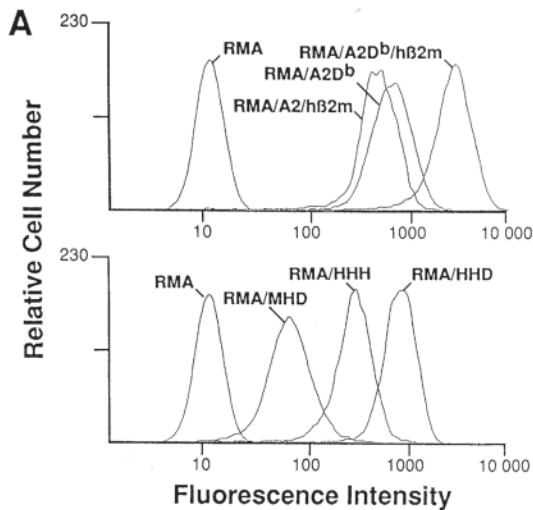


**Figure 1.** Design of the monochains. (A) Schematic representation of the monochains. Open area, human origin; hatched area, mouse origin. The thicker line corresponds to the 15 amino acid linker. (B) Linear representation of the final constructs. Open boxes, coding sequence of human origin; hatched boxes, coding sequences of mouse origin; closed box, 1.6-kb EcoRI–BamHI fragment from pBR328 plasmid. (C) Nucleotide and amino acid sequences of the created junctional regions.

HHH and HHD monochains. Surtransfection of MHD-expressing cells with the human β2m gene corrects the defect of cell surface expression of this monochain (data not shown), suggesting, in spite of their covalent linkage, poor interaction between the mouse β2m and the HLA-A2.1 α1 and α2 domains (21, 22). Tested with a panel of nine HLA-A2.1-specific mAb (data not shown), the three monochains exhibited normal serological reactivities, indicating that the peptide linker does not markedly alter the overall three-dimensional structure of the HLA-A2.1 molecule.

HLA-A2.1 monochains were exclusively detected as 60-kD proteins by immunoprecipitation using HLA-A2.1-specific BB7.2 mAb and PAGE analysis, suggesting that the fused molecules are not proteolytically separated after synthesis (Fig. 2 B).

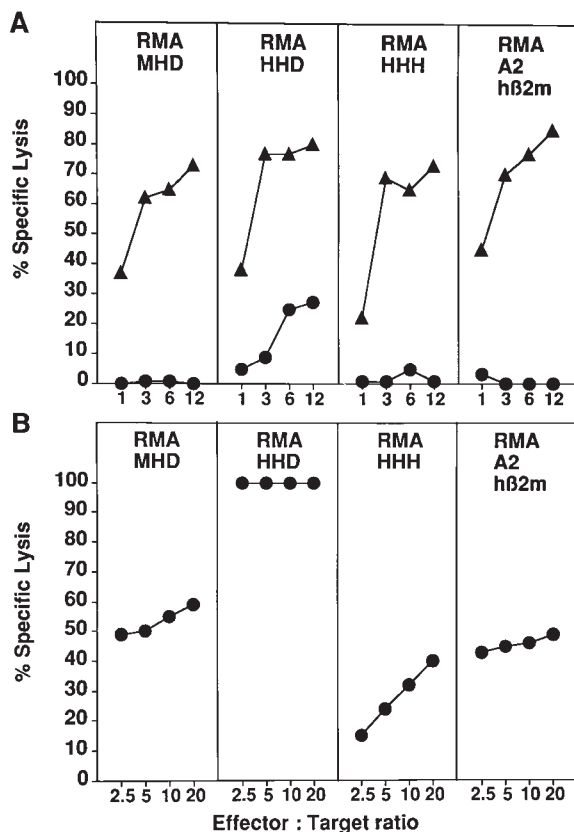
Finally, recognition by mouse CTL of the monochains was evaluated (Fig. 3). An influenza A matrix-specific, HLA-A2.1-restricted CTL clone (HAM 42; 14) was first tested on target cells pulsed with synthetic 58–66 influenza matrix pep-



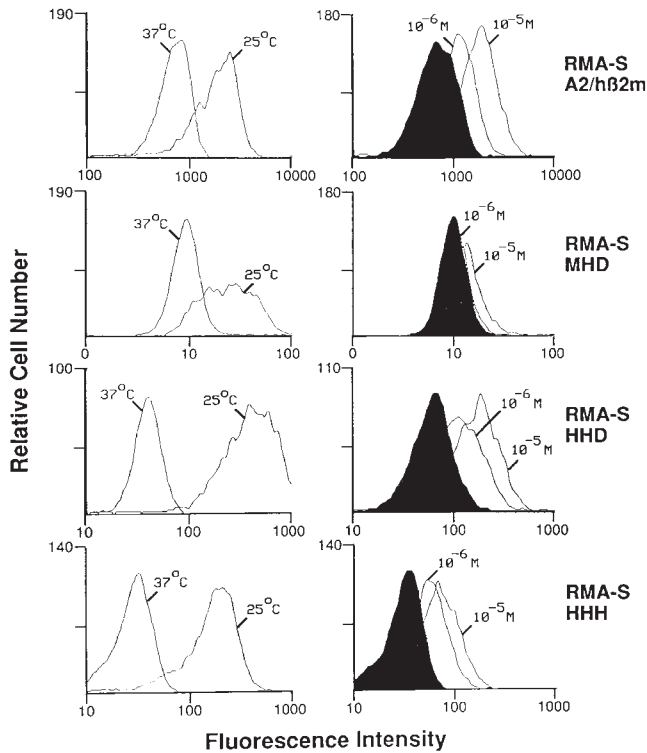
**Figure 2.** Monochain expression in RMA cells. (A) Flow cytometric analysis of RMA cells expressing HLA-A2.1 monochains or their heterodimeric counterpart, either wild-type (HLA-A2.1, A2) or chimeric (HLA-A2.1  $\alpha 1\alpha 2$ , H-2D<sup>b</sup>  $\alpha 3$ , transmembrane, cytoplasmic, A2D<sup>b</sup>) class I heavy chains associated with either human (RMA/A2/h $\beta 2m$ , RMA/A2D<sup>b</sup>/h $\beta 2m$ ) or mouse (RMA/A2D<sup>b</sup>)  $\beta 2m$ . HLA class I-specific B9.12.1 unlabeled mAb was detected with F(ab)'2 FITC-conjugated goat anti-mouse IgG. Negative control was RMA untransfected cells. Results are expressed in fluorescence intensity (*x-axis*, log scale) and relative cell number (*y-axis*). (B) Untransfected and transfected RMA cells were [<sup>35</sup>S]methionine labeled as described in Materials and Methods, and immunoprecipitations were carried out with HLA-A2-Aw69-specific BB7.2 mAb.

tides. This CTL clone killed HHH-, HHD-, and MHD-RMA monochain transfectants and RMA cells expressing heterodimeric HLA-A2.1  $\times$  human  $\beta 2m$  molecules with approximately the same efficiency (Fig. 3 A). Expression by the MHD and HHD monochains of a mouse H-2D<sup>b</sup>  $\alpha 3$

domain that should facilitate their interaction with mouse CD8 molecules did not result in more efficient lysis, under our experimental conditions, by HAM 42 clone. CD8-independent recognition of target cells has been observed for some CTL clones, possibly related to the expression by these clones of TCR of high affinity (23). Therefore, to more precisely evaluate the impact of the mouse  $\alpha 3$  domain on the recognition by mouse CTL of the HLA-A2.1 monochains, the same target cells were tested with polyclonally activated, HLA-A2.1-specific CTL, raised by immunization of C57BL/6 HLA-A3 transgenic mice with splenocytes of C57BL/6 HLA-A2.1  $\times$  human  $\beta 2m$  double transgenic mice. Such polyclonally activated CTL specifically lyse HLA-A2.1-positive human cell line (JY), HLA-A2.1 transfected P815(H-2<sup>d</sup>), and RMA(H-2<sup>b</sup>) murine cell lines (data not shown), as well as the three monochain transfectants. However, MHD and, more strikingly, HHD monochain transfectants were more efficiently recognized (Fig. 3 B). Thus, as already documented for heterodimeric HLA-A2.1 molecules (12), recognition by mouse CTL of the HLA-A2.1 monochains is facilitated by the introduction of a mouse  $\alpha 3$  domain.



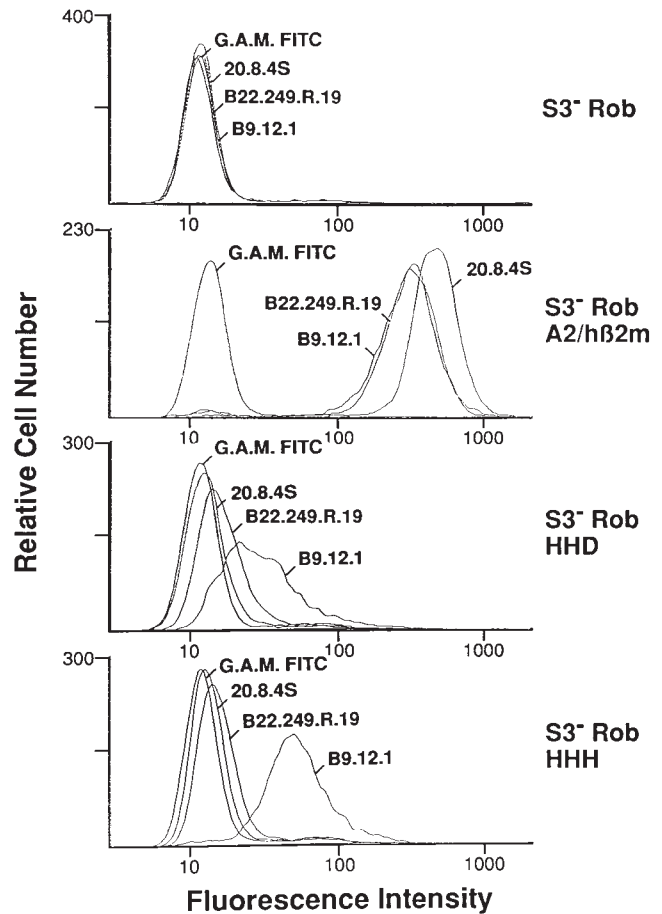
**Figure 3.** Recognition of the monochains by allospecific or influenza A matrix-specific CTL. <sup>51</sup>Cr-release assays were performed as described in Materials and Methods. Spontaneous release was <15% for all targets. (A) Influenza A matrix-specific, HLA-A2.1-restricted CTL clone HAM 42 was tested against MHD, HHD, HHH monochains and heterodimeric HLA-A2.1  $\times$  human  $\beta 2m$ -transfected RMA target cells, without peptide (circles) or loaded (triangles) with 10<sup>-6</sup> M synthetic peptide (amino acid 58-66 from the influenza A matrix protein). (B) HLA-A2.1 allospecific CTL from HLA-A3 transgenic mice isolated as described in Materials and Methods were similarly tested against the same target cells. Background lysis of nontransfected target cells was <3% at all E/T ratios (data not shown).



**Figure 4.** Peptide dependency of stable cell surface expression of the monochains. Cell surface-expressed monochains were stained with HLA class I-specific B9.12.1 unlabeled F(ab)'2 mAb detected with F(ab)'2 FITC-conjugated goat anti-mouse IgG. Results are expressed in fluorescence intensity (*x*-axis, log scale) and relative cell number (*y*-axis). (*Left*) Cell surface expression of monochains after overnight culture at 37 or 25°C. (*Right*) Stabilization of cell surface expression of HLA-A2.1 monochains after overnight culture at 25°C, addition of  $10^{-5}$  M,  $10^{-6}$  M, or no synthetic peptide (amino acid 58-66 from influenza A virus matrix protein; closed histograms) and subsequent incubation for 4 h at 37°C.

Altogether, these *in vitro* studies of transfected cells show that the three HLA-A2.1 monochains are cell-surface expressed, serologically not altered, bind exogenous peptides, and are recognized by CTL. This suggests that they have a three-dimensional structure similar to wild-type heterodimeric molecules. They further argue for the selection of HHD molecules that are efficiently cell-surface expressed and that interact with the mouse CD8 molecules.

**Peptide-dependency of Cell Surface Expression.** Monochain constructs were introduced in TAP-deficient RMA-S cells to test whether cell surface expression of HLA-A2.1 monochains would be promoted at 25°C and stabilized at 37°C by the fixation of exogenous peptides (24). The results of these experiments are illustrated in Fig. 4. Cultivating transfected RMA-S cells at 25°C resulted in enhanced cell-surface expression of HHH, HHD, and, to a lesser extent, MHD monochains, which were stabilized at 37°C by the fixation of the 58-66 influenza matrix peptide. Thus, HLA-A2.1 monochains exhibited the same peptide dependency for stabilization as their heterodimeric counterparts. Moreover, at the surface of RMA-S transfectants, a relatively high basal expression of HLA-A2.1 monochains was observed at



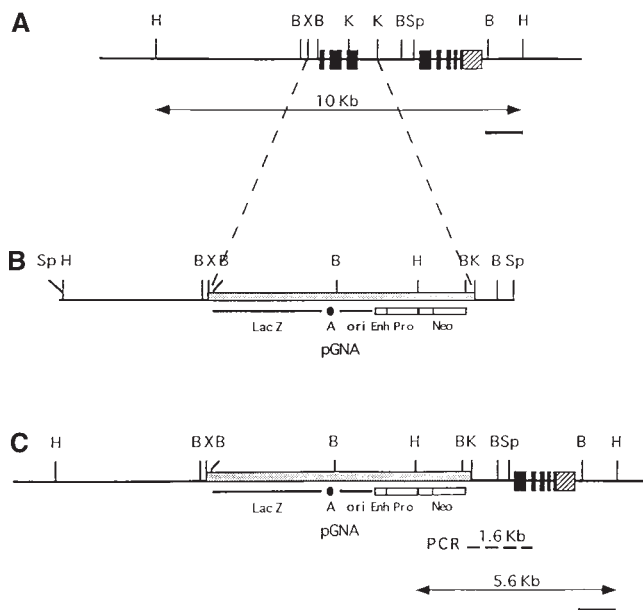
**Figure 5.** Expression of the monochains in  $\beta 2m$ -deficient cells. Untransfected, HLA-A2.1  $\times$  human  $\beta 2m$ -, HHD-, or HHH-transfected  $\beta 2m^{-}$  EL4 S3 Rob cells, cultured in medium containing 3% FCS and fully depleted in bovine  $\beta 2m$  by immunoadsorption on a bovine  $\beta 2m$ -specific (CAB.297) mAb column, were stained with B22.249.R.19 (H-2D<sup>b</sup> $\alpha 1\alpha 2$ -specific), 20.8.4S (H-2K<sup>b</sup> $\alpha 1\alpha 2$ -specific), or B9.12.1 (HLA class I-specific) unlabeled mAb, detected with F(ab)'2 FITC-conjugated goat anti-mouse IgG (goat anti-mouse FITC). Negative controls were with no first mAb. Results are expressed in fluorescence intensity (*x*-axis, log scale) and relative cell number (*y*-axis).

37°C in the absence of exogenous peptides, suggesting, as established for wild-type heterodimeric HLA-A2 (25, 26), that HLA-A2.1 monochains in TAP-deficient cells bind a significant amount of hydrophobic leader peptides in the endoplasmic reticulum.

**Monochain Expression in  $\beta 2m$ -deficient Cells.**  $\beta 2m$ -deficient EL4 S3 Rob cells (27) were stably transfected with the monochain constructs to precisely evaluate the possibility that heavy chain-linked  $\beta 2m$  could promote the reexpression of endogenous H-2 class I mouse heavy chains. To be in a situation analogous to that of  $\beta 2m$  knockout mice, selected clones of transfectants were cultured in medium supplemented with FCS deprived of bovine  $\beta 2m$  by extensive immunoadsorption on a bovine  $\beta 2m$ -specific, CAB.297 mAb column (28).

As illustrated in Fig. 5, we found limited, however repeatedly observed, reexpression of H-2D<sup>b</sup>, a conclusion

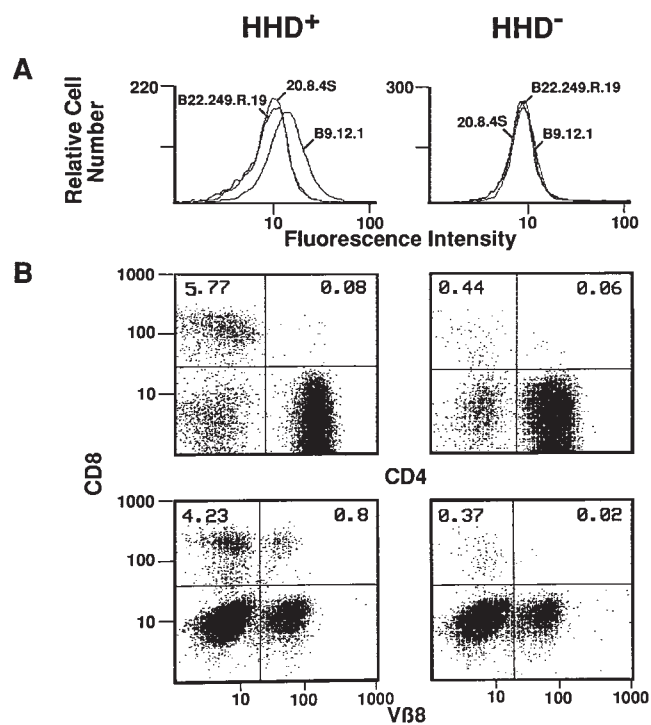




**Figure 6.** Disruption of the H-2D<sup>b</sup> gene. (A) Genomic structure of the H-2D<sup>b</sup> gene. Black box, coding exon; hatched box, noncoding exons. The 3' noncoding BamHI–HindIII probe used for Southern blot analysis and the length of the wild-type HindIII fragment are shown. (B) Restriction map of the SpeI-linearized targeting vector, which contains a 4-kb HindIII–XbaI 5' and a 1-kb KpnI–SpeI 3' fragment of the H-2D<sup>b</sup> gene. (C) Predicted structure of the targeted H-2D<sup>b</sup> gene, in which the first three exons are replaced by the whole plasmid sequence; the 1.6-kb PCR-amplified diagnostic fragment and the length of the diagnostic 5.6-kb HindIII fragment generated after recombination are shown. H, HindIII; B, BamHI; X, XbaI; K, KpnI; Sp, SpeI.

based on the fact that the B22.249.R.19 mAb reacts with the  $\alpha 1\alpha 2$  H-2D<sup>b</sup> domains. Since H-2D<sup>b</sup> molecules are furthermore susceptible to reach cell surfaces in the absence of endogenous  $\beta 2m$  (29), we concluded that the production of HLA-A2.1 monochain transgenic mice should be associated with the destruction of both mouse  $\beta 2m$  and H-2D<sup>b</sup> genes, by homologous recombination. Altogether, these *in vitro* studies using transfected cells show the integrity and the functional capacities of HLA-A2.1 monochains and led us to select the HHD construct for the production of the HLA-A2.1 monochain transgenic mice.

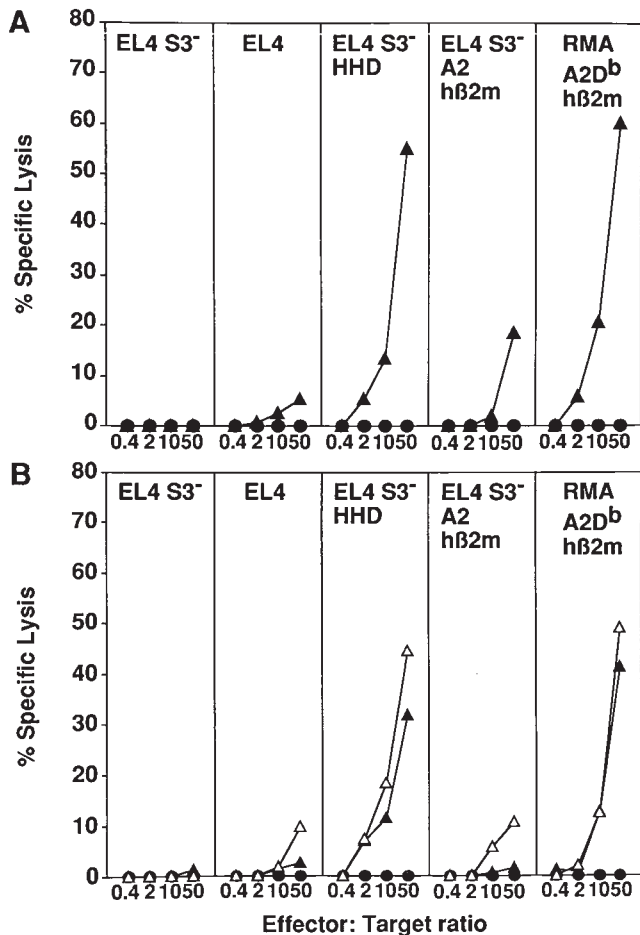
**Expression of HHD Monochains in H-2D<sup>b</sup>–/– Mouse  $\beta 2m$ –/– Double Knockout Mice.** A targeting construct in which exons 1, 2, and 3 of the H-2D<sup>b</sup> gene were replaced by a plasmid conferring resistance to G418, was electroporated in CGR-8 embryonic stem cells (30), and homologous recombinants were identified at the clonal level by PCR and Southern blot analyses (Fig. 6). After blastocyst injection and reimplantation, chimeric mice were obtained, which gave germline transmission of the targeted gene. H-2D<sup>b</sup>–/– homozygous animals were then produced. These animals show no profound quantitative and qualitative modifications of their CD8<sup>+</sup> T cell repertoire (data not shown, Pernau et al., manuscript in preparation). These H-2D<sup>b</sup>–/– mice were crossed with  $\beta 2m$ –/– knockout mice (31) to derive H-2D<sup>b</sup>–/–  $\beta 2m$ –/– double mutants. Since initial at-



**Figure 7.** Phenotypic characterization of H-2D<sup>b</sup>–/–,  $\beta 2m$ –/–, HHD transgenic mice. Flow cytometric analyses were performed on spleen T lymphocytes from H-2D<sup>b</sup>–/–,  $\beta 2m$ –/–, HHD<sup>+</sup> (left), or HHD<sup>–</sup> (right) mice. (A) Expression of H-2K<sup>b</sup>, H-2D<sup>b</sup>, and HHD molecules detected with 20.8.4S, B22.249.R.19, or B9.12.1 mAb, respectively and F(ab)<sup>2</sup> FITC-conjugated goat anti-mouse IgG, negative controls with no first mAb. Results are expressed in fluorescence intensity (*x*-axis, log scale) and relative cell number (*y*-axis). (B, top) Partial restoration of the peripheral pool of CD8<sup>+</sup> T lymphocytes. Double staining was performed with phycoerythrin-labeled anti-CD4 (*x*-axis, log scale) and biotinylated anti-CD8 detected with streptavidin–Perc-P (*y*-axis, log scale). (Bottom) Peripheral CD8<sup>+</sup> TCR repertoire. As illustrated for V $\beta$ 8.1.2.3, double staining was performed with FITC-labeled anti-V $\beta$ 2 (B.20.6), –V $\beta$ 3 (KJ.25), –V $\beta$ 4 (KT.10.4), –V $\beta$ 5.1,2 (MR.9.4), –V $\beta$ 6 (44.22), –V $\beta$ 7 (TR 130), –V $\beta$ 8.1,2,3 (F.23.1), –V $\beta$ 9 (MR.10.2), –V $\beta$ 10 (B.21.5), –V $\beta$ 11 (RR.3.15), –V $\beta$ 13 (MR.12.4), and –V $\beta$ 17 (KJ.23.1)-specific mAb (*x*-axis, log scale) and phycoerythrin-labeled anti-CD8 mAb (*y*-axis, log scale).

tempts to use these mice for transgenesis failed, C57BL/6  $\times$  SJL were used as recipients. Transgenic animals identified by Southern blotting and serological analyses were crossed with H-2D<sup>b</sup>–/–  $\beta 2m$ –/– knockout mice to derive HHD (heterozygous) H-2D<sup>b</sup>–/–  $\beta 2m$ –/– experimental animals.

Weak (compared to endogenous H-2 class I molecules in normal mice), but significant, expression of the transgenic HHD molecules on unactivated peripheral T lymphocytes was documented by indirect immunofluorescence and FACS<sup>®</sup> analysis. Under similar conditions, no H-2K<sup>b</sup> and H-2D<sup>b</sup> molecules could be detected (Fig. 7 A). This expression of HHD monochains partially restores the peripheral pool of CD8<sup>+</sup> T lymphocytes (5.5% of the T lymphocytes instead of 20–30% in normal mice). More importantly, testing the 10 available anti-V $\beta$  mAb, it appeared, as illustrated in Fig. 7 B for V $\beta$ 8, that these CD8<sup>+</sup> T lymphocytes exhibited a diversified TCR repertoire. By comparison with H-2D<sup>b</sup>–/–  $\beta 2m$ –/– double knockout mice, which are almost com-



**Figure 8.** Virus-specific, HLA-A2.1-restricted CTL responses of H-2D<sup>b</sup><sup>-/-</sup>,  $\beta$ 2m<sup>-/-</sup> HHD transgenic mice. 2–4 wk after immunization, CTL were restimulated in vitro for 5 d and <sup>51</sup>Cr-release assays were performed. Targets were  $\beta$ 2m-deficient (EL4 S3<sup>-</sup>), wild type (EL4), HHD, or HLA-A2.1  $\times$  human  $\beta$ 2m-transfected EL4 S3<sup>-</sup> and HLA-A2A2D<sup>b</sup>  $\times$  human  $\beta$ 2m-transfected RMA cells either uninfected (A and B, closed circles), vaccinia (A, closed triangles), influenza A (B, closed triangles) infected, or influenza A matrix (58–66) peptide pulsed (B, open triangles). Spontaneous release was <15% for all target cells.

pletely deprived of peripheral CD8<sup>+</sup> T lymphocytes, these results suggest that HHD monochains promote CD8<sup>+</sup> T cell positive education in the thymus.

**CTL Responses of HHD H-2D<sup>b</sup><sup>-/-</sup>  $\beta$ 2m<sup>-/-</sup> Mice.** HHD (heterozygous) H-2D<sup>b</sup><sup>-/-</sup>  $\beta$ 2m<sup>-/-</sup> animals were intraperitoneally infected with either vaccinia or influenza A viruses. 2–4 wk later, splenocytes were restimulated in vitro with virus-infected syngeneic irradiated splenocytes and tested 5 d later in a classical <sup>51</sup>Cr-release assay.

All animals tested (four out of four for each virus) have developed virus-specific, HHD-restricted CTL responses allowing the in vitro killing of virally infected HHD-transfected cells (Fig. 8). No significant lysis was observed testing the effector cells on vaccinia- or influenza A-infected EL4 cells, indicating that the CTL generated were HLA-A2.1 restricted. Two additional points are worth mentioning. First, these CTL recognized uninfected HHD-trans-

fected cells loaded with the 58–66 influenza A matrix peptide with the same efficiency as infected cells (Fig. 8 B). Second, the CTL responses appeared strongly CD8 dependent, HHD-transfected cells (as well as their heterodimeric counterparts) being much more efficiently recognized than transfectants expressing a fully human molecule.

## Discussion

Comparative analyses of the cell surface expression and CTL recognition of HHH, HHD, and MHD monochains led to the selection of the HHD construct for the development of HLA-A2.1 transgenic mice. Cell-surface expression of MHD monochains has constantly been found to be 5–10 times lower by FACS<sup>®</sup> analysis. We know, from pulse-chase and endoglycosidase H digestion experiments, that MHD monochains egress slowly from the endoplasmic reticulum. HHH and HHD monochains, by contrast, leave this cellular compartment as rapidly as HLA-A2.1  $\times$  human  $\beta$ 2m heterodimers (data not shown). Better interaction with mouse CD8 molecules has been the key element for the selection of the HHD construct. A similar observation has already been made analyzing the CTL responses of transgenic mice expressing, as heterodimers, chimeric HLA-A2.1  $\alpha$ 1 $\alpha$ 2  $\times$  H-2K<sup>b</sup>  $\alpha$ 3 transmembrane and cytoplasmic domain heavy chains (12). Further improvement of this interaction might be expected, particularly for some other HLA class I alleles (i.e., HLA-B7), by site-directed mutagenesis of the  $\alpha$ 2 residues implicated in the CD8 binding site (32). Alternatively, one might consider the possibility of introducing the human CD8  $\alpha$  and  $\beta$  chains by transgenesis.

Profound reduction in cell surface expression of H-2 class I molecules has been documented serologically after destruction of mouse  $\beta$ 2m gene by homologous recombination (31, 33). However, studying both  $\beta$ 2m<sup>-</sup> tumor cells and  $\beta$ 2m knockout mice, residual expression of H-2D<sup>b</sup> molecules has been described, indicating that a fraction of functionally conformed H-2D<sup>b</sup> heavy chains reaches the cell surface in the absence of  $\beta$ 2m (34). Since it was observed ex vivo that HHD monochains promote, to a certain extent, H-2D<sup>b</sup> molecule cell surface export, it was of interest to have HHD molecules expressed in H-2D<sup>b</sup><sup>-/-</sup> and  $\beta$ 2m<sup>-/-</sup> double knockout mice. Nevertheless, such mice cannot be considered as completely devoid of cell surface-expressed classical H-2 class I molecules. In fetal thymic organ cultures from  $\beta$ 2m<sup>-/-</sup> mice, H-2K<sup>b</sup>-restricted CTL can be positively selected in the presence of exogenously added  $\beta$ 2m and peptides (35). Additionally, H-2K<sup>b</sup>-specific CTL have been generated against  $\beta$ 2m<sup>-</sup> cells, and H-2K<sup>b</sup> molecules can be detected at the surface of Con A-stimulated  $\beta$ 2m<sup>-/-</sup> splenocytes, implying residual expression of  $\beta$ 2m-free, H-2K<sup>b</sup> heavy chains (36–38). This residual expression might account for the small percentage (0.4%) of CD8<sup>+</sup> T lymphocytes observed in H-2D<sup>b</sup>  $\beta$ 2m double knockout mice (Fig. 7 B). Even if it is generally assumed that  $\beta$ 2m-free H-2K<sup>b</sup> heavy chains are expressed in lower

amounts than  $\beta 2m$ -free H-2D<sup>b</sup> heavy chains, it may be of interest to derive H-2K<sup>b</sup>, H-2D<sup>b</sup>,  $\beta 2m$  triple knockout mice.

Despite the residual expression of  $\beta 2m$ -free H-2K<sup>b</sup> heavy chains and the relatively low expression level (for which we do not have definitive explanation) of HHD monochains by HHD (heterozygous) transgenic H-2D<sup>b</sup>  $\beta 2m$  double knockout mice, the results reported indicate efficient usage of the HLA-A2.1 monochain both at the educational and effector levels by the mouse CD8<sup>+</sup> T lymphocytes. Expression of the monochain restores a sizable and diversified CD8<sup>+</sup> T cell repertoire, supporting the notion that no significant species bias prevents interactions between mouse TCR and HLA class I molecules (8, 13). More complete restoration is anticipated once animals homozygous for the transgene will be isolated. It might be of importance to reach expression levels similar to those observed at the surface of human cells to study CTL responses against peptides that interact or are recognized with rela-

tively low affinity. In the absence of both H-2D<sup>b</sup> and mouse  $\beta 2m$ , it must be assumed that most peripheral CD8<sup>+</sup> T lymphocytes have been educated in the thymus on HHD monochains. This should facilitate the study of HLA class I-restricted responses compared to classical transgenic mice. One might hope that the information gained with these animals will be of human relevance. Two recently reported studies have indicated significant overlap between HLA-A2.1 transgenic mice and human CTL responses assaying a large panel of hepatitis C- and hepatitis B virus-derived T cell epitopes (39, 40). The development in HHD animals of potent CTL responses against the immunodominant (in HLA-A2.1 individuals) matrix peptide, documented in this report, also support such possibility. We are planning to use these animals for a comparative study of the vaccine potential of the HLA-A2.1-restricted T cell epitopes already characterized in various human diseases and a comparison of the different vaccine strategies.

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The authors are grateful to Drs. T. Meo and C. Babinet for helpful discussion, Drs. E. Mottet, J.-P. Abastado, V. Engelhard, J.-C. Manuguerra, and G. Hämmerling for providing  $\beta 2m$  cDNA, cells, and viruses, L. Anderson and A. Jeske for H-2D<sup>b</sup><sup>-/-</sup> mouse husbandry, P. Marchand for producing HHD transgenic animals, and Drs. M. Cochet, S. Dethlefs, and S. Wain-Hobson for careful reading of the manuscript.

During her postdoctoral stay at the Centre for Genome Research (Edinburgh, U.K.), B. Pérarnau was a recipient of a fellowship from the Human Frontier Science Program Organisation. The Centre for Genome Research is supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom. This work was funded by the Institut Pasteur and by grants from the Association pour la Recherche contre le Cancer, the Ligue contre le Cancer, and the Pasteur-Weizmann committees.

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Received for publication 19 February 1997.

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