HLA-A2.1–restricted Education and Cytolytic Activity of CD8⁺ T Lymphocytes from β2 Microglobulin (β2m) HLA-A2.1 Monochain Transgenic H-2Db β2m Double Knockout Mice

By Steve Pascolo,* Nathalie Bervas,* Jan M. Ure,‡ Austin G. Smith,‡ François A. Lemonnier,* and Béatrice Pérarnau*

From the *Institut Pasteur, Département SIDA-Rétrovirus, Unité d’Immunité Cellulaire Antivirale, 75724 Paris Cedex 15, France; and ‡Gene Targeting Laboratory, Centre for Genome Research, University of Edinburgh, Edinburgh EH9 3JQ, United Kingdom

Summary

Three different HLA-A2.1 monochains were engineered in which either the human or mouse β2-microglobulin (β2m) is covalently linked to the NH₂ terminus of the heavy chain by a 15–amino acid long peptide: HHH, entirely human, HHD, with the mouse H-2Db α3, transmembrane, and cytoplasmic domains, and MHD, homologous to HHD but linked to the mouse β2m. 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-2Db⁻/⁻ β2m⁻/⁻ double knockout mice. Expression of this monochain restores a sizable peripheral CD8⁺ T cell repertoire essentially educated on the transgenic human molecule. Consequently, infected HHD, H-2Db⁻/⁻ β2m⁻/⁻ mice generate only HLA-A2.1–restricted CD8⁺ 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⁺ CTL responses (7–10). Substitution of the HLA α3 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H-2 domain significantly improves the recognition and usage of some (A2.1, B27), however not all (i.e., B7.1, our unpublished observation), HLA class I molecules (11, 12).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⁺ T lymphocytes can be promoted when H-2–restricted responses are controlled. More importantly, under such circumstances, a diversified Vβ and Vα TCR mouse repertoire is mobilized, suggesting a sufficient flexibility for an efficient usage of human class I molecules (13, 14). However, the experimental artifacts (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⁺ T cell repertoire, both at the thymic and peripheral levels, to make use of the transgenic HLA class I molecules. These mice are H-2Db and mouse β2 microglobulin (β2m)¹ double knockouts and express β2m–HLA-A2.1 monochains.

We report in vitro transfection experiments that resulted in the selection of a human β2m–HLA-A2.1 (α1α2)–H-2Db (α3 transmembrane cytoplasmic) (HHD) monochain construct to derive transgenic animals. These HHD transgenic, H-2Db⁻/⁻ β2m⁻/⁻ double knockout mice are almost devoid of H-2 class I molecules. Phenotypic and functional analyses of their peripheral CD8⁺ T cell repertoire indicate that HHD monochains support thymic positive selection of CD8⁺ 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 BglII¹ pBluescript (Stratagene, La Jolla, CA) and site mutagenized to introduce a PstI site at the

¹Abbreviations used in this paper: β2m, β-2 microglobulin; HA, hemagglutinin; TAP, transporter associated with antigen presentation.
C57Bl/6 mice were injected intraperitoneally with 3 μg/ml G418 (GIBCO BRL, Paisley, U.K.) and cloned by limiting dilution. Cells (5 × 10⁶) were transferred in selective medium containing 1 μg/ml G418 (GIBCO BRL, Paisley, U.K.) and incubated overnight at 4°C. Eluate (Pro-mix; Amersham, Buckinghamshire, U.K.) containing cells was washed three times. Cytolytic activity was determined in 4 h ⁵¹Cr-release assays using V-bottom 96-well plates containing 5 × 10⁵ uninfected, influenza A matrix 58-66 peptide-pulsed (10⁻⁸ 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 at 100 × [(experimental – spontaneous release) / (total – spontaneous release)], with maximal release being determined by lysis of target cells by 1 M HCl.

**Generation of H-2Db⁻/⁻ Knockout Mice.** A 10-kb HindIII fragment containing the whole H-2Db gene and a 1.9-kb PsiI 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-2Db targeting construct was generated by inserting a 4-kb XbaI 5′ fragment from the H-2Db gene and a 1.3-kb Kpnl 3′ fragment from the PsiI 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, 10⁶ cells were electroporated in 900 μl of PBS with 150 μg of Spel-linearized plasmid DNA at 800 V and 3 μF, using a BioRad Labs. (Hercules, CA) gene pulser and selected after 24 h in the presence of G418 (175 μg/ml). 28 pools of 12 G418-resistant clones were screened by PCR, using a pGNA-specific 5′ (CAGCAGAACACATACAGCTGTC) and a H-2Db exon 5′–specific 3′ (AACCAGTCACATGTAAAGT-CAGT) pair of oligonucleotides, resulting in the amplification of the expected 1.6-kb fragment for 18 pools. The homologous combination 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 germine transmission of the mutation. Germine 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-2Db⁻/⁻ homozygous strains. Inactivation of the H-2Db gene was confirmed by Southern blot analysis on tail DNA and immunofluorescence assay.

**Cells and Transfectants.** RMA (transporter associated with antigen presentation [TAP] positive), RMA-S (TAP negative), EL-4 (β2m negative), and EL-4 S3 Rob (β2m negative) C57Bl/6 (H-2ª) H-2Db-negative, RMA-S (TAP negative), EL-4 (β2m negative) C57Bl/6 (H-2ª) H-2Db-negative, and EL-4 S3 Rob (β2m negative) C57Bl/6 (H-2ª) H-2Db-negative C57Bl/6 animals and then intercrossed at the N2 generation to give rise to two independent H-2Db⁻/⁻ homozygous strains. Inactivation of the H-2Db gene was confirmed by Southern blot analysis on tail DNA and immunofluorescence assay.

**Immunoprecipitations.** Cells (5 × 10⁶) 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 μCi of [³⁵S] methionine–cysteine mix (Pro-nix; 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 pre cleared (2 h, 4°C with protein A-Sepharose beads) and then incubated overnight at 4°C. After treatment with 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. Dry 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 × 10⁵ HLA-A2.1 × human β2m transgenic C57BL/6 mouse splenocytes. 2 wk later, splenocytes (5 × 10⁶) from immunized mice were restimulated in vitro with 3 × 10⁵ irradiated (2,000 rads) HLA-A2.1 × human β2m 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⁺ H-2Db⁻/⁻ β2m⁻/⁻ mice were intraperitoneally infected with either 1,000 hemagglutinin (HA) units (influenza A/PR/8/34, provided by Dr. J.-C. Manuguerra, Laboratoire de Genetiq Moleculair des Virus Respiratoir Institut Pasteur, Paris, France) or 10⁷ PFU (vaccinia) viruses. 2–4 wk later, 2.5 × 10⁵ (influenza A) or 6 × 10⁴ (vaccinia) splenocytes were restimulated in vitro with 2.5 × 10⁵ (influenza A) or 3 × 10⁵ (vaccinia) irradiated (3,000 rads) syngeneic red blood cell–depleted splenocytes infected for 1 h with influenza A (10 HA unit for 10⁵ cells) or vaccinia (10 PFU/cell) viruses.

Responder mice were individually tested in a standard cytotoxic assay 5 d later. In brief, 10⁶ cells, uninfected or infected (1,000 HA units of influenza A or 5 × 10⁵ 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 μCi of sodium [¹¹⁰]Cr. Labeled cells were washed 3 times. Cytolytic activity was determined in 4 h [¹¹⁰]Cr-release assays using V-bottom 96-well plates containing 5 × 10⁵ uninfected, influenza A matrix 58-66 peptide-pulsed (10⁻⁸ 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 at 100 × [(experimental – spontaneous release) / (total – spontaneous release)], with maximal release being determined by lysis of target cells by 1 M HCl.

**Generation of H-2Db⁻/⁻ Knockout Mice.** A 10-kb HindIII fragment containing the whole H-2Db gene and a 1.9-kb PsiI 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-2Db targeting construct was generated by inserting a 4-kb XbaI 5′ fragment from the H-2Db gene and a 1.3-kb Kpnl 3′ fragment from the PsiI 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, 10⁶ cells were electroporated in 900 μl of PBS with 150 μg of Spel-linearized plasmid DNA at 800 V and 3 μF, using a Bio Rad Labs. (Hercules, CA) gene pulser and selected after 24 h in the presence of G418 (175 μg/ml). 28 pools of 12 G418-resistant clones were screened by PCR, using a pGNA-specific 5′ (CAGCAGAACACATACAGCTGTC) and a H-2Db exon 5′–specific 3′ (AACCAGTCACATGTAAAGT-CAGT) pair of oligonucleotides, resulting in the amplification of the expected 1.6-kb fragment for 18 pools. The homologous combination 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 germine transmission of the mutation. Germine 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-2Db⁻/⁻ homozygous strains. Inactivation of the H-2Db gene was confirmed by Southern blot analysis on tail DNA and immunofluorescence assay.
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 (Gly4Ser)3 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-2Dβα3, 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 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-
This CTL clone killed HHH-, HHD-, and MHD-RMA monochain transfectants and RMA cells expressing heterodimeric HLA-A2.1 × human β2m molecules with approximately the same efficiency (Fig. 3 A). Expression by the MHD and HHD monochains of a mouse H-2Dα 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 α3 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 × human β2m double transgenic mice. Such polyclonally activated CTL specifically lyse HLA-A2.1–positive human cell line (JY), HLA-A2.1 transfected P815(H-2b), and RMA(H-2b) 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 α3 domain.

**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 α1α2, H-2Dα α3, transmembrane, cytoplasmic, A2Dα) class I heavy chains associated with either human (RMA/A2/h2, RMA/A2/h2m, RMA/A2/h2m) or mouse (RMA/A2/h2m) β2m. 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 [35S]methionine labeled as described in Materials and Methods, and immunoprecipitations were carried out with HLA-A2-Aw69–specific BB7.2 mAb.

**Figure 3.** Recognition of the monochains by allo-specific or influenza A matrix-specific CTL. 51Cr–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 × human β2m–transfected RMA target cells, without peptide (circles) or loaded (triangles) with 10−6 M synthetic peptide (amino acid 58-66 from the influenza A matrix protein). (B) HLA-A2.1 allo-specific CTL from HLA-A3 transgenic mice isolated as described in Materials and Methods, and immunoprecipitations were carried out with HLA-A2-Aw69–specific BB7.2 mAb.
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 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 β2m-deficient Cells. β2m-deficient EL4 S3 Rob cells (27) were stably transfected with the monochain constructs to precisely evaluate the possibility that heavy chain–linked β2m could promote the reexpression of endogenous H-2 class I mouse heavy chains. To be in a situation analogous to that of β2m knockout mice, selected clones of transfected cells were cultured in medium supplemented with FCS deprived of bovine β2m by immunoabsorption on a bovine β2m-specific (CAB.297) mAb column (28).

As illustrated in Fig. 5, we found limited, however repeatedly observed, reexpression of H-2Dβ, a conclusion
H-2Db gene.

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-2Db gene. (C) Predicted structure of the targeted H-2Db 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.

Figure 6. Disruption of the H-2Db gene. (A) Genomic structure of the H-2Db 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-2Db gene. (C) Predicted structure of the targeted H-2Db 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.

Figure 7. Phenotypical characterization of H-2Db^h-/^, β2m^-/-, HHD transgenic mice. Flow cytometric analyses were performed on spleen T lymphocytes from H-2Db^h/-, β2m^-/-, HHD^+ (left), or HHD^+ (right) mice. (A) Expression of H-2K^d, H-2D^d, and HHD molecules detected with 20.8.4S, B22.249.R.19, or B9.12.1 mAb, respectively and F(ab)’2 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^+ T lymphocytes. Double staining was performed with phycoerythrin-labeled anti-CD4 (x-axis, log scale) and biotinylated anti-CD8 detected with streptavidin–PerCP (y-axis, log scale). (Bottom) Peripheral CD8^+ TCR repertoire. Double staining was performed with FITC-labeled anti–CD8 (B2.20.6), -V^8.1.2.3, double staining was performed with FITC-labeled anti–CD4 (x-axis, log scale) and phycoerythrin-labeled anti-CD8 (y-axis, log scale).

Expression of HHD Monochains in H-2Db^-/- Mouse β2m^-/- Double Knockout Mice. A targeting construct in which exons 1, 2, and 3 of the H-2Db gene were replaced by a plasmid conferring resistance to G418, was electroporated in C57BL/6 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-2Db^-/- homozygous animals were then produced. These animals show no profound quantitative and qualitative modifications of their CD8^+ T cell repertoire (data not shown, Perrarnau et al., manuscript in preparation). These H-2Db^-/- mice were crossed with β2m^-/- knockout mice (31) to derive H-2Db^h/-/β2m^-/- double mutants. Since initial attempts to use these mice for transgenesis failed, C57BL/6 × SJL were used as recipients. Transgenic animals identified by Southern blotting and serological analyses were crossed with H-2Db^h/-/β2m^-/- knockout mice to derive HHD (heterozygous) H-2Db^h/-/β2m^-/- experimental animals.

These results show that the B22.249.R.19 mAb reacts with the α/β2 H-2Db domains. Since H-2Db molecules are further susceptible to reach cell surfaces in the absence of endogenous β2m (29), we concluded that the production of HLA-A2.1 monochain transgenic mice should be associated with the destruction of both mouse β2m and H-2Db 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 transgenics mice.

Expression of HHD Monochains in H-2Db^-/- Mouse β2m^-/- Double Knockout Mice. A targeting construct in which exons 1, 2, and 3 of the H-2Db gene were replaced by a plasmid conferring resistance to G418, was electroporated in C57BL/6 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-2Db^-/- homozygous animals were then produced. These animals show no profound quantitative and qualitative modifications of their CD8^+ T cell repertoire (data not shown, Perrarnau et al., manuscript in preparation). These H-2Db^-/- mice were crossed with β2m^-/- knockout mice (31) to derive H-2Db^h/-/β2m^-/- double mutants. Since initial attempts to use these mice for transgenesis failed, C57BL/6 × SJL were used as recipients. Transgenic animals identified by Southern blotting and serological analyses were crossed with H-2Db^h/-/β2m^-/- knockout mice to derive HHD (heterozygous) H-2Db^h/-/β2m^-/- experimental animals.
Cytolytic T lymphocytes (CTLs) were restimulated in vitro for 5 d and 51Cr-release assays were performed. Targets were β2m-deficient (EL4 S3*), wild type (EL4), HHD, or HLA-A2.1 × human β2m-transfected EL4 S3* and HLA-A2A2DβA2Db × human β2m-transfected RMA cells either uninfected (A and B, closed circles), vaccinia (A, closed triangle), influenza A (B, closed triangle) infected, or influenza A matrix (58-66) peptide pulsed (B, open triangles). Spontaneous release was <1.5% for all target cells.

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-A2A1 transgenic mice. Cell-surface expression of MHD monochains has constantly been found to be 5–10 times lower by FACS® 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 × human β2m 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 α1α2 × H-2Kb α3 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 α2 residues implicated in the CD8 binding site (32). Alternatively, one might consider the possibility of introducing the human CD8α and β chains by transgenesis.

Profound reduction in cell surface expression of H-2 class I molecules has been documented serologically after destruction of mouse β2m gene by homologous recombination (31, 33). However, studying both β2m−/− tumor cells and β2m knockout mice, residual expression of H-2Dβ molecules has been described, indicating that a fraction of functionally conformed H-2Dβ heavy chains reaches the cell surface in the absence of β2m (34). Since it was observed ex vivo that HHD monochains promote, to a certain extent, H-2Dβ molecule cell surface export, it was of interest to have HHD molecules expressed in H-2Dβ−/− and β2m−/− 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 β2m−/− mice, H-2Kb-restricted CTL can be positively selected in the presence of exogenously added β2m and peptides (35). Additionally, H-2Kb-specific CTL have been generated against β2m−/− cells, and H-2Kb molecules can be detected at the surface of Con A-stimulated β2m−/− splenocytes, implying residual expression of β2m-free, H-2Kb heavy chains (36–38). This residual expression might account for the small percentage (0.4%) of CD8+ T lymphocytes observed in H-2Dβ β2m double knockout mice (Fig. 7 B). Even if it is generally assumed that β2m-free H-2Kb heavy chains are expressed in lower

Figure 8. Virus-specific, HLA-A2.1–restricted CTL responses of H-2Dβ−/−, β2m−/− HHD transgenic mice. 2–4 wk after immunization, CTL were restimulated in vitro for 5 d and 51Cr-release assays were performed. Targets were β2m-deficient (EL4 S3*), wild type (EL4), HHD, or HLA-A2.1 × human β2m-transfected EL4 S3* and HLA-A2A2Dβ × human β2m-transfected RMA cells either uninfected (A and B, closed circles), vaccinia (A, closed triangle), influenza A (B, closed triangle) infected, or influenza A matrix (58-66) peptide pulsed (B, open triangles). Spontaneous release was <1.5% for all target cells.
amounts than β2m-free H-2D\(^b\) heavy chains, it may be of interest to derive H-2K\(^b\), H-2D\(^b\), β2m triple knockout mice.

Despite the residual expression of β2m-free H-2K\(^b\) 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\(^b\) β2m 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\(^+\) T lymphocytes. Expression of the monochain restores a sizable and diversified CD8\(^+\) 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 relatively low affinity. In the absence of both H-2D\(^b\) and mouse β2m, it must be assumed that most peripheral CD8\(^+\) 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.

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 β2m cDNA, cells, and viruses, L. Anderson and A. Jeske for H-2D\(^{b,\sim}\) 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.

Address correspondence to Béatrice Pérarnau, Institut Pasteur, Département SIDA-Rétrovirus, Unité d’Immunité Cellulaire Antivirale, 28 rue du Dr Roux, 75724 Paris Cedex 15, France. The present address of S. Pascolo is IMBB, Forth-Hellas P.O. Box 1527, Vasilika Vouton, Heraklion 711 10, Crete, Greece.

Received for publication 19 February 1997.

References

1. Arnold, B., and G.J. Hämmerling. 1991. MHC class-I transgenic mice. *Annu. Rev. Immunol.* 9:297–322.
2. Dill, O., F. Kievits, S. Koch, P. Ivanyi, and G.J. Hämmerling. 1988. Immunological function of HLA-C antigens in HLA-Cw3 transgenic mice. *Proc. Natl. Acad. Sci. USA.* 85:5664–5668.
3. Kievits, F., P. Ivanyi, P. Krinpenfort, A. Berns, and H.L. Ploegh. 1987. HLA-restricted recognition of viral antigens in HLA transgenic mice. *Nature (Lond.)*. 329:447–449.
4. Kievits, F., J. Wijffels, W. Lokhorst, and P. Ivanyi. 1989. Recognition of xeno-(HLA, SLA) major histocompatibility complex antigens by mouse cytotoxic T cells is not H-2 restricted: a study with transgenic mice. *Proc. Natl. Acad. Sci. USA.* 86:617–620.
5. Kievits, F., W. Lokhorst, and P. Ivanyi. 1990. Abnormal anti-viral immune response in mice is corrected in HLA-B27.2-transgenic mice. *Eur. J. Immunol.* 20:1189–1192.
6. Kievits, F., W.J. Boerenkamp, W. Lokhorst, and P. Ivanyi. 1990. Specificity and frequency of primary anti-HLA cytotoxic T lymphocytes in normal and HLA-B27.2, HLA-B27.5, and HLA-Cw3-transgenic mice. *J. Immunol.* 144:4513–4519.
7. Barra, C., B. Pérarnau, P. Gerlinger, M. Lemeur, A. Gillet, P. Gibier, and F. Lemonnier. 1989. Analysis of the anti-HLA-Cw3 CTL response of HLA-B7 x human β2m double transgenic mice. *J. Immunol.* 143:3117–3124.
8. Engelhard, V.H., E. Lacy, and J.P. Ridge. 1991. Influenza A–specific, HLA-A2.1 restricted cytotoxic T lymphocytes from HLA-A2.1 transgenic mice recognize fragments of the M1 protein. *J. Immunol.* 146:1226–1232.
9. Epstein, H., R. Hardy, J.S. May, M.H. Johnson, and N. Holmes. 1989. Expression and function of HLA-A2.1 in transgenic mice. *Eur. J. Immunol.* 19:1575–1583.
10. Le, A.-X.T., E.J. Bernhard, M.J. Holterman, S. Strub, E. Lacy, and V. Engelhard. 1989. Cytotoxic T cell responses in HLA-A2.1 transgenic mice: recognition of HLA alloantigens and utilization of HLA-A2.1 as a restriction element. *J. Immunol.* 142:1366–1371.

2050 HHHD Transgenic H-2D\(^{b,\sim}\)/β2m\(^{-\sim}\) Double Knockout Mice
11. Kalinke, U., B. Arnold, and G. Hämmerling. 1990. Strong xenogeneic HLA response in transgenic mice after introducing an α3 domain into HLA-B27. Nature (Lond.). 348:642–644.

12. Vitiello, A., D. Marchesini, J. Furze, L.A. Sherman, and R.W. Chesnutt. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human–mouse class I major histocompatibility complex. J. Exp. Med. 173:1007–1015.

13. Barra, C., H. Gournier, Z. Garcia, P.N. Marche, E. Jouvin-Marche, P. Briand, P. Filippi, and F.A. Lemonnier. 1993. Abrogation of H-2 restricted responses and efficient recognition of HLA-A3 molecules in DBA/2 HLA-A24 responder mice. J. Immunol. 150:3681–3689.

14. Man, S., J.P. Ridge, and V. Engelhard. 1994. Diversity and dominance among TCR recognizing HLA-A2.1 influenza matrix peptide in human class I transgenic mice. J. Immunol. 153:4458–4467.

15. Grosveld, F.G., T. Lund, E.J. Murray, A.L. Mellor, H.H.W. Dahl, and R.A. Flavell. 1982. The construction of cosmid libraries which can be used to transform eukaryotic cells. Nucl. Acids Res. 10:6715–6732.

16. Le Mouellic, H., Y. Lallemand, and P. Brulet. 1990. Targeted replacement of the homeobox gene Hox-3.1 by the Escherichia coli lacZ in mouse chimeric embryos. Proc. Natl. Acad. Sci. USA. 87:4712–4716.

17. Smith, A.G. 1991. Culture and differentiation of embryonic stem cells. J. Tissue Cult. Methods. 13:89–94.

18. Saron, M., P. Truffa-Bachi, and J.-C. Guillon. 1989. Rapid enrichment of mouse natural killer cells by use of wheat germ agglutinin. J. Immunol. Methods. 129:151–158.

19. Lee, L., L. McHugh, R. Ribaudo, S. Kozlowski, D. Margulis, and M. Mage. 1994. Functional cell surface expression by a recombinant single-chain class I major histocompatibility complex molecule with a cis-active β2-microglobulin domain. Eur. J. Immunol. 24:2633–2639.

20. Toskitani, K., V. Braud, M.J. Browning, N. Murray, A.J. McMichael, and W.F. Bodmer. 1996. Expression of a single-chain HLA class I molecule in a human cell line: presentation of exogenous peptide and processed antigen to cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA. 93:236–240.

21. Krimpenfort, P., G. Rudenko, F. Hochstenbach, D. Guesdon, A. Berns, and H. Ploegh. 1987. Crosses of two independently derived transgenic mice demonstrate functional complementation of the genes encoding heavy (HLA-B27) and light (β2-microglobulin) chains of HLA class I antigens. EMBO (Eur. Mol. Biol. Organ.) J. 6:1673–1676.

22. Péramau, B., A. Gillet, R. Hakem, M. Barad, and F.A. Lemonnier. 1988. Human β2-microglobulin specifically enhances cell surface expression of HLA class I molecules in transfected murine cells. J. Immunol. 141:1383–1389.

23. MacDonald, H.R., A.L. Glasebrook, C. Cron, A. Kelso, and J.-C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes (CTL): possible implications for the affinity of CTL antigen receptors. Immunol. Rev. 68:89–115.

24. Ljunggren, H.G., N.J. Stam, C. Öhlén, J.J. Neefjes, P. Höglund, M.T. Heemels, J. Bastin, T.N.M. Schumacher, A. Townsend, and K. Kärre. 1990. Empty MHC class I molecules come out in the cold. Nature (Lond.). 346:476–480.

25. Hunt, D.F., R.A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevill, A.L. Cox, E. Appella, and V.H. Engelhard. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science (Wash. DC). 255:1261–1263.

26. Wei, M.L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature (Lond.). 356:443–446.

27. Sturmhofel, K., and G. Hämmerling. 1990. Reconstitution of H-2 class I expression by gene transfection decrease susceptibility to natural killer cells of an EL4 class I variant. Eur. J. Immunol. 20:171–177.

28. Rocca, A., A. Opolski, A. Samaan, B. Frangoulis, L. Degos, and M. Pla. 1992. Localization of the conformational alteration of MHC molecules induced by the association of mouse class I heavy chain with a xenogeneic β2-microglobulin. Mol. Immunol. 29:481–487.

29. Allen, H., J. Fraser, D. Flyer, S. Calvin, and R. Flavell. 1986. β2-microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-2Dβ or of a truncated H-2Dβ. Proc. Natl. Acad. Sci. USA. 83:7447–7451.

30. Mountford, P., B. Zevnik, A. Düwel, J. Nichols, M. Li, C. Dani, M. Robertson, I. Chambers, and A. Smith. 1994. Distinct targeting constructs: reporters and modifiers of mammalian gene expression. Proc. Natl. Acad. Sci. USA. 91: 4303–4307.

31. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mhc deficient in β2-m; MHC class I proteins, and CD8+ T cells. Science (Wash. DC). 248: 1227–1230.

32. Sun, J., D.J. Leahy, and P.B. Kavathas. 1995. Interaction between CD8 and major histocompatibility complex (MHC) class I mediated by multiple contact surfaces that include the α2 and α3 domains of MHC class I. J. Exp. Med. 182:1275–1280.

33. Zijlstra, M., M. Bix, N. Simister, J. Loring, D. Raulet, and R. Janisch. 1990. β2-microglobulin deficient mice lack CD4+8+ cytolytic T cells. Nature (Lond.). 344:742–746.

34. Bix, M., and D. Raulet. 1992. Functionally conformed free class I heavy chains exist on the surface of β2-microglobulin negative cells. J. Exp. Med. 176:829–834.

35. Hoggquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. Cell. 76:17–27.

36. Glas, R., L. Franksson, C. Öhlén, P. Höglund, B. Koller, H. Ljunggren, and K. Kärre. 1992. Major histocompatibility complex I-specific and -restricted killing of β2-microglobulin-deficient cells by CD8+ cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA. 89:11381–11385.

37. Glas, R., C. Öhlén, P. Höglund, and K. Kärre. 1994. The CD8+ T cell repertoire in β2-microglobulin-deficient mice is biased towards reactivity against self-major histocompatibility class I. J. Exp. Med. 179:661–672.

38. Machold, R.P., S. André, L. Van Kaer, H.-G. Ljunggren, and H.L. Ploegh. 1995. Peptide influences the folding and intracellular transport of free major histocompatibility complex I heavy chains. J. Exp. Med. 181:1111–1122.

39. Shirai, M., T. Aritchi, M. Nishioka, T. Nomura, K. Ikeda, K. Kawanishi, V.H. Engelhard, S.M. Feinstein, and J.A. Berzofsky. 1995. CTL responses of HLA-A2.1—transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1. J. Immunol. 154:2733–2742.

40. Wentworth, P., A. Vitiello, J. Sidney, E. Keogh, R. Chesnut, H. Grey, and A. Sette. 1996. Differences and similarities in the A2.1—restricted cytotoxic T cell repertoire in humans and human leukocyte antigen—transgenic mice. Eur. J. Immunol. 26:97–101.
Double Knockout Mice