Direct recognition by $\alpha\beta$ cytolytic T cells of Hfe, a MHC class Ib molecule without antigen-presenting function

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Crystallographic analysis of human Hfe has documented an overall structure similar to classical (class Ia) MHC molecules with a peptide binding groove deprived of ligand. Thus, to address the question of whether $\alpha\beta$ T cells could recognize MHC molecules independently of bound ligands, we studied human and mouse Hfe interactions with T lymphocytes. We provide formal evidence of direct cytolytic recognition of human Hfe by mouse $\alpha\beta$ T cell receptors (TCR) in HLA-A*0201 transgenic mice and that this interaction results in ZAP-70 phosphorylation. Furthermore, direct recognition of mouse Hfe molecules by cytotoxic T lymphocytes (CTLs) was demonstrated in DBA/2 Hfe knockout mice. These CTLs express predominantly two T cell antigen receptor α variable gene segments (AV6.1 and AV6.6). Interestingly, in wild-type mice we identified a subset of CD8$^+$ T cells positively selected by Hfe that expresses the AV6.1/AV6.6 gene segments. T cell antigen receptor recognition of MHC molecules independently of bound ligand has potential general implications in alloreactivity and identifies in the Hfe case a cognitive link supporting the concept that the immune system could be involved in the control of iron metabolism.

T cell receptor

Most histocompatibility class I molecules have an open groove in their $\alpha1\alpha2$-domains in which they bind peptides (HLA and H-2 class Ia molecules) less often than glycolipids (CD1 molecules) (1). The composite structures are recognized through their T cell antigen receptors (TCRs) by subsets of T lymphocytes [CD8 T lymphocytes, natural killer T cells (2), and mucosal associated invariant T cells (3)], resulting in cytolysis and/or cytokine secretion, depending on the subset activated. Several TCR/peptide/MHC crystal structures have shown that the $\alpha1$- and $\alpha2$-helices of the MHC molecules are contacted by the TCR α- and β-chain complementarity-determining regions (CDR)1/CDR2, the bound peptides interacting essentially with the hypervariable CDR3s (4). The dual selectivity of some alloreactive CTL clones for a given allogeneic molecule and a given peptide presented by this molecule might suggest that effective TCR/MHC molecule interaction always requires a direct participation of the bound ligand (5, 6). However, less stringent peptide selectivity has been documented for other CTL clones (7, 8), suggesting in those cases predominant TCR interaction with the $\alpha1\alpha2$-helices of their allogeneic MHC molecular targets.

Hfe is a nonclassical MHC class Ib molecule sharing 37% amino acid identity with HLA-A*0201. Hfe is formed, like classical MHC class I, by the noncovalent association of a heavy α and a $\beta2$-microglobulin ($\beta2$m) light chain (9). The 2.8-Å crystallographic structure of the human Hfe (hHfe) molecule has been published (10). Whereas its overall structure is similar to MHC class Ia molecules, Hfe has a narrow and empty version of the classical MHC class I peptide groove. To determine whether cytotoxic $\alpha\beta$ T cells are able to directly recognize the hHfe or mouse Hfe (mHfe), which are MHC class I molecules without antigen-presenting function, two strategies were used. We first immunized HHD (HLA-A*0201) mice (11) with mouse $\beta2$m-deficient EL4S3-Rob cells expressing human $\beta2$m/hHfe single-chain molecules. Under such conditions, only the human $\beta2$m/hHfe single chains reach the cell surface, thus excluding indirect presentation of Hfe-derived peptides by other MHC class I molecules. CD8$^+$ TCR$\alpha\beta$ CTL recognizing directly hHfe were generated. We then demonstrated that mHfe-specific CTL could be produced in DBA/2 *mHfe* knockout (KO) mice. Furthermore, we established that mHfe direct recognition resulted from the mobilization of a subset of CD8$^+$ T cells expressing preferentially certain TCR AV segments. Interestingly, we provide evidence that mHfe is able to positively select these AV segments in wild-type mice. hHfe- and mHfe-specific mAbs that were concurrently isolated proved essential tools for the development of the reported work.

Materials and Methods

Hfe Monochains. Human and mouse $\beta2$m/Hfe monochains have a $\beta2$m C terminus linked by a 15-aa (GGGGSx3) sequence to the N terminus of the mature hHfe or mHfe heavy chain. hHfe and mHfe cDNAs, isolated from HepG2 and C57BL/6 liver cells, respectively, were PCR-amplified. The 5′ extension introduced a BamHI restriction site and reconstituted the 3′ end of the linker, and the 3′ extension introduced a NsiI restriction site downstream of the Hfe stop codon. BamHI–NsiI restricted hHfe and mHfe PCR products were introduced in BamHI–PstI restricted HHD (human $\beta2$m) and HMD (mouse $\beta2$m^b) monochain plasmids, respectively (11). The transcription of these chimeric genes is controlled by the *HLA-A*0201* promoter.

TCR Characterization. The TCR AV and BV families used by the T cell clones were identified by the RT-PCR-based immuno-

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Abbreviations: CTL, cytotoxic T lymphocyte; TCR, T cell antigen receptor; $\beta2$m, $\beta2$-microglobulin; hHfe, human Hfe; mHfe, mouse Hfe; KO, knock out; CDR, complementarity-determining regions.

See Commentary on page 12649.

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scope technique and sequenced (12) (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). Full-length α- and β-chain cDNAs of CTL clone 6 were individually transferred into a lentiviral DNA-flap$^+$ expression vector (13). Quantitative PCR were performed in the GeneAmp 5700 sequence detection system (Applied Biosystems) by using an SYBR green PCR kit (Applied Biosystems) with the following primers: AV6.1 segment (5′-AGTGACCC-TCTCAGAAGACG-3′ and 5′-TCATATGTAGCTTCAAAA-CCTCTGCTG-3′) and AV6.6 segment (5′-TCCGTGACTCA-AACAGAAGG 3′ and 5′-TTGTATGTGGCTTCAAACCC-TCTGCT-3′). Samples were compared by the Ct method as described by the manufacturer. The AV usages were calculated as $2^{-\Delta\Delta CT}$.

Mice. *HHD* mice express a HLA-A*0201 (α1 and α2 domains) hybrid (α3 to cytoplasmic H-2D^b domains) transgenic class I molecule in a monochain configuration (covalent amino acid linkage of the C terminus of human β2m to the heavy chain N terminus) (11). Because the *H-2D^b* and mouse *β2m* genes have been disrupted, HHD is the only MHC class I molecular species expressed by HHD mice. DBA/2 *Hfe* KO mice were derived by backcrossing *Hfe* KO mice [initially on a 129/Ola × C57BL/6 background kindly provided by S. Bahram (Centre de Recherche d'Immunologie et d'Hémetologie, Strasbourg, France) (14)] on DBA/2 mice 10 times (15). Protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations of Animal Welfare.

Cell Lines. EL4S3-Rob (β2m$^-$ mouse thymoma of C57BL/6 origin) cells were electroporated with human β2m/hHfe monochain plasmids and selected with anti-human β2m B2.62.2 mAb. P815 HTR (high transfection rate mouse mastocytoma of DBA/2 origin) were similarly transfected with mouse β2m/mHfe monochain plasmids. P815 transfectants were selected by using a DBA/2 *Hfe* KO immune serum against mHfe. *HHD*-transfected EL4S3-Rob and human *β2m/hHfe*-cotransfected VAD1279 mouse fibroblasts are described in refs. 11 and 16. CD8$^+$ BW51.47 mouse T cells were fused with hHfe-specific CTL clone 6 from mouse 8 (Table 2, which is published as supporting information on the PNAS web site), and HAT-resistant T cell hybridomas selected for TCR cell surface expression. A derivative of the CD8$^+$ BW51.47 cell (54ζ) constitutively expressing the mouse TCR ζ-chain was cotransduced with lentiviral α and β TCR chain recombinant vectors. The T8.1 T cell hybridoma, specific for tetanus toxoid peptide 830–843 and restricted by HLA-DR1102, is described in ref. 17. T cell hybridomas and transductants (5×10^4) were cocultured with 5×10^4 γ-irradiated (50 gray) stimulating cells in flat-bottomed, 96-well plates for 24 h, and the IL-2 content of supernatants was evaluated in a CTLL2 proliferation assay.

mAbs and Immunofluorescence Assays. mAb against hHfe (G2.3, IgM) was raised in *HHD* mice immunized with human *β2m/hHfe*-transfected EL4S3-Rob cells. G2.3 mAb reacts with hHfe in monochain or heterodimeric configuration, but not with mHfe or HLA-A, HLA-B, HLA-C, HLA-G, or HLA-E-expressing cells. mAb against mHfe (25.4; IgG2b) was raised in DBA/2 *Hfe* KO mice immunized with mouse *β2m/mHfe*-transfected P815 cells. G2.3 and 25.4 mAb binding was revealed with FITC-conjugated goat F(ab)′2 anti-mouse Ig Abs (Caltag, Burlingame, CA). FITC-conjugated rat anti-mouse CD4 (RM4-5, Caltag) and phycoerythrin-conjugated rat anti-mouse CD8-β (CT-CD8b, Caltag) were used to characterize the CTL clones. For TCR AV quantitative assessment, splenocytes were stained by using anti-TCRβ-phycoerythrin, anti-CD8α-allophycocyanin, and anti-CD4-FITC. TCRβ^+CD4$^+$CD8$^-$, CD8$^+$CD4$^-$, and CD4$^-$CD8$^-$ populations were separated (purity ≥97%) by using a MOFLO device (Cytomation, Fort Collins, CO).

CTL Induction. *HHD* mice were injected i.p. with 5×10^6 human *β2m/hHfe*-transfected EL4S3-Rob cells. Splenocytes were restimulated 10 days later *in vitro* with the same γ-irradiated (50 gray) transfected cells in RPMI medium 1640 supplemented with 10% FCS/200 units/ml penicillin/200 μg/ml streptomycin/50 μM 2-mercaptoethanol. Six days later, cells were tested for cytolytic activity against ^{51}Cr-labeled human *β2m/hHfe*- and *HHD*-transfected EL4S3-Rob cells. CTL clones were isolated under limiting dilution culture conditions. mHfe-specific CTL lines and clones were similarly induced in DBA/2 *mHfe* KO mice by i.p. injection of mouse *β2m/mHfe*-transfected, γ-irradiated P815 HTR cells or 500 ng of a lentiviral DNA-flap-positive vector encoding mouse β2m/mHfe molecule. The CTLs were then restimulated *in vitro* with γ-irradiated wild-type DBA/2 splenocytes, which include a subset of dendritic and monocytic cells expressing detectable amounts of mHfe molecules on their cell surface (Fig. 6, which is published as supporting information on the PNAS web site).

HIV1-Derived Lentiviral Vectors. The murine *β2m/mHfe* monochain was cloned into the pSIN.PW HIV-1-derived vector as an EcoRV–BsrGI fragment (New England Biolabs) to generate the pSIN.PW.*mHfe* vector (18). Lentiviral pSIN.PW.*mHfe* transfer vector particles were produced by cotransfection of human 293 T cells with the packaging plasmid (pCMVR8.74) providing gag, pol, tat, and rev helper viral genes and the envelop plasmid (pMD.G) encoding the vesicular stomatitis virus glycoprotein. Lentiviral particles encoding the TRAV14 and TRBV4 were similarly produced.

TCR Complex Phosphorylation Studies. The BW51.47×CTL clone 6 T cell hybridoma and the *hHfe*-transfected VAD1279 cells were washed and coincubated at a 3:1 ratio for 2 min in serum-free medium. For activation with mAb, cells (5×10^7 per ml) were incubated with 10 μg/ml anti-CD3ε 145-2C11 mAb at 4°C for 30 min, washed, and further incubated with 10 μg/ml goat anti-hamster IgG at 37°C for 2 min. T cells (8×10^6) were then pelleted and lysed for 15 min on ice. ZAP-70 immunoprecipitation and immunoblotting analyzes were performed by enhanced chemiluminescence (17, 19).

Results

Induction of CTL Against hHfe in HHD Mice. *HHD* mice which do not express mouse MHC class I molecules were used as responders (11). These animals were injected with human *β2m/hHfe* monochain-transfected mouse *β2m*-deficient EL4-S3-Rob cells (Fig. 1). Such a combination, in which responder mice and stimulating cells express and differ only by a single species of MHC class I molecules, was expected to induce hHfe-specific, H-2-unrestricted CTLs. After two i.p. injections of human β2m/hHfe expressing EL4-S3-Rob cells, five of 16 mice developed hHfe-specific CTL responses (Fig. 2*a*; see also Fig. 7, which is published as supporting information on the PNAS web site), which were specifically inhibited by anti-hHfe mAb (Fig. 2*b*), suggesting a direct T cell recognition of hHfe.

To study the involvement of the CTL TCR in hHfe recognition, we isolated CD8$^+$ T cell clones from one of these mice. All clones expressed the same AV14-BV2 TCR (see Table 2) and specifically lysed human β2m/hHfe monochain-expressing EL4-S3-Rob cells. This lysis was inhibited by anti-hHfe mAb. T cell hybridomas were derived from the fusion of one T cell clone with the BW51.47 CD8$^+$ cell line. Coincubation of these hybridomas with human β2m/hHfe$^+$ EL4S3-Rob cells resulted in IL-2 production not observed with control *HHD*-transfected EL4S3-Rob and specifically abolished by anti-hHfe mAb (Fig. 2*c*).

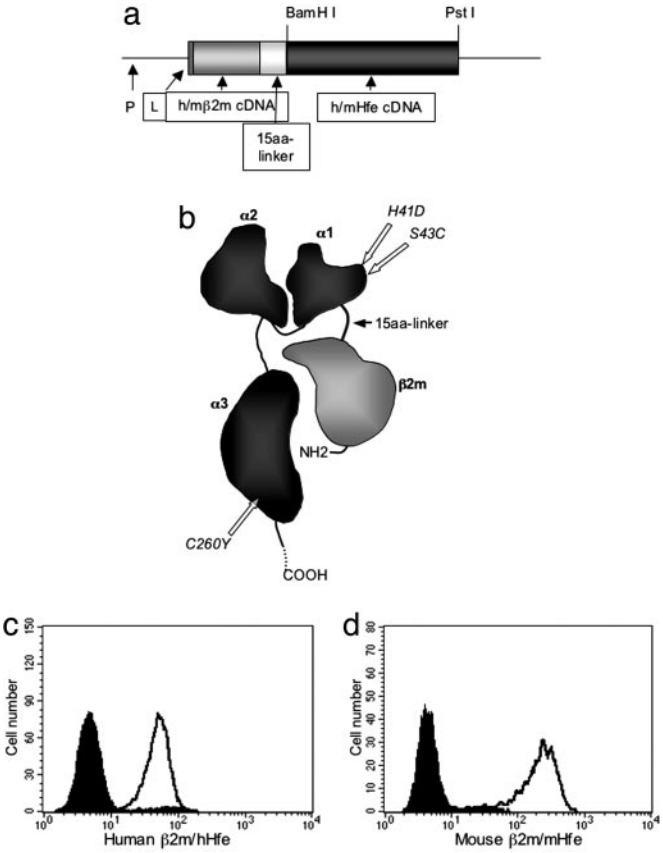

Fig. 1. Hfe monochain constructs and surface expression by transfected cells. (*a*) Schematic representation of the constructs. P and L indicate the *HLA-A*0201* promotor and leader sequence, respectively; h/m indicates human or mouse β2m and Hfe cDNA linked by synthetic oligonucleotides encoding a (GGGGS)×3 peptide. (*b*) Cartoon representation of the β2m/Hfe monochain with the three most frequent Hfe mutations in humans. (*c*) Surface expression of human β2m/hHfe monochain on EL4S3-Rob-transfected cells as detected with G2.3 anti-hHfe mAb (open curve) and negative control (BB7.2, anti-HLA-A2) mAb (filled curve). (*d*) Surface expression of mouse β2m/mHfe monochain on P815 HTR-transfected cells as detected with 25.4 anti-mHfe mAb (open curve) and negative control (BB7.2) mAb (filled curve).

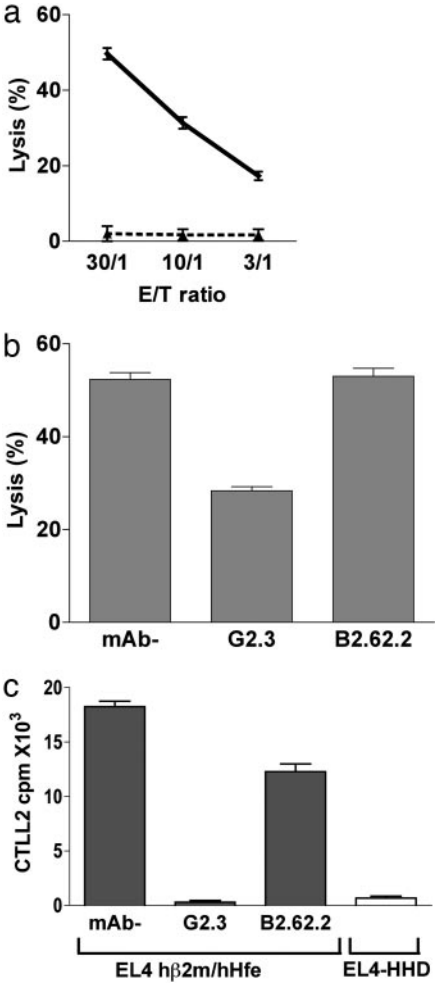

Fig. 2. CTL recognition of hHfe molecule. *HHD*-mice were immunized with human *β2m/hHfe*-transfected EL4S3-Rob cells and splenocytes tested for cytolytic activity after *in vitro* restimulation. (*a*) Cytolytic activity of bulk cell population of a representative mouse against human *β2m/hHfe*-transfected (solid line) and human *β2m/HLA-A0201* (dashed line) ^{51}Cr-labeled EL4S3-Rob target cells at different effector/target (E/T) ratios. (*b*) Inhibition of cytolysis at the 30:1 effector/target ratio by G2.3 anti-hHfe or B2.62.2 anti-human β2m mAb. (*c*) CTL clone 6 was fused with BW51.47 cells. TCR$^+$ T cell hybridomas were cocultivated for 1 day with human *β2m/hHfe*-transfected EL4S3-Rob cells (filled bars) in the absence (mAb−) or presence of anti-hHfe (G2.3) or anti-human β2m (B2.62.2) mAb. Culture supernatants were then assessed for the presence of IL-2 in a CTLL2-proliferation assay. As negative control, we used the T cell hybridoma cocultured with *HHD* (*HLA-A0201*)-transfected EL4S3-Rob cells (open bar).

Similar results were obtained against hHfe in a physiological configuration, i.e., when the hHfe heavy chain is noncovalently associated with the human β2m on the surface of MHC class I-negative VAD1279 cells cotransfected with human *β2m* and *hHfe* cDNAs (Fig. 3*b Top*). These results strongly suggest a direct recognition of hHfe by the CTL TCR.

TCR Recognition of hHfe. To prove that the CTL TCR was responsible for the recognition of hHfe, the AV14 and BV2 chains were cloned in lentiviral vectors, which were used to cotransduce a ζ$^+$ derivative of BW51.47 (Fig. 3). The TCR-positive transductant Tr6 specifically produced IL-2 in response to hHfe expressing VAD1279 cells, as illustrated (Tr6) in Fig. 3*b Middle*, whereas the T8.1 control hybridoma did not.

The ζ-associated protein tyrosine kinase ZAP-70 is implicated in controlling the early steps of the TCR signaling cascade (20). To confirm that the interaction with hHfe leads to TCR triggering, we analyzed the phosphorylation status of the ZAP-70 molecule after contact of hybridoma 6 with *hHfe*-expressing VAD1279 cells. As shown in Fig. 3*c*, ZAP-70 was strongly tyrosine-phosphorylated upon incubation of T cell hybridoma 6 with *hHfe*-expressing VAD1279 cells (lane 5) and upon stimulation with anti-CD3 mAb (lane 2). No phosphorylation was found in the presence of hHfe-negative VAD1279 cells. These results demonstrate that specific interaction between TCR and hHfe molecule leads to T cell activation.

T Cell Recognition of mHfe. To gain insight into the potential impact of Hfe on the CD8$^+$ T cell repertoire, we hyperimmunized *mHfe* KO and wild-type DBA/2 mice with mouse *β2m/mHfe*-transfected P815 cells (the only murine cell type in which we obtained stable mHfe surface expression; see Fig. 1*d*) or with a lentiviral vector encoding the mouse β2m/mHfe monochain. The splenocytes of immunized animals were restimulated *in vitro* with wild-type splenocytes. Although wild-type DBA/2 mice never developed cytolytic responses against mHfe, splenocytes from nine of 30 *mHfe* KO mice specifically lysed mHfe$^+$ target cells. This lysis, also documented at the CTL clonal level, was

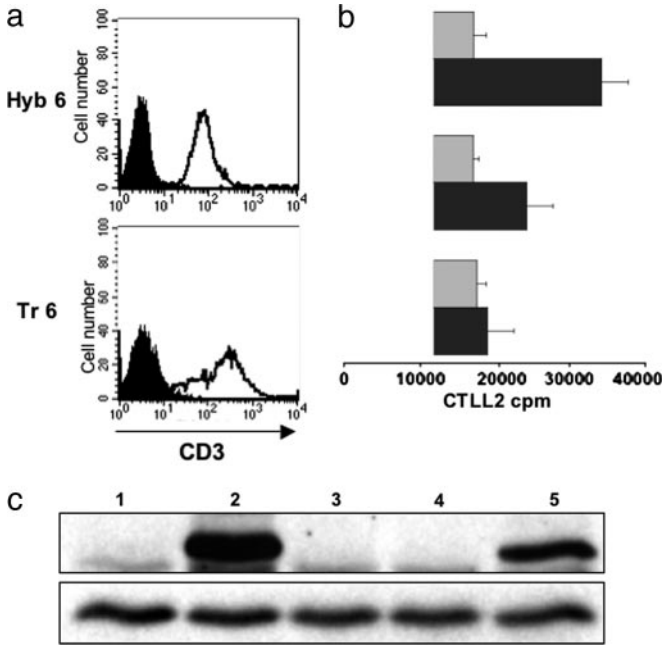

Fig. 3. TCR recognition of hHfe molecules. BW51.47×CTL clone 6 hybridoma (Hyb6), BW51.47 ζ^+ cells cotransduced with lentiviral vectors encoding the AV and BV chains of CTL clone 6 (Tr6) and a HLA-DR1-restricted, tetanus toxoid-specific hybridoma (T8.1, negative control) were used for these analyzes. (*a*) Detection of TCR surface expression on Hyb6 and Tr6 was performed with FITC anti-mouse CD3 (open curves), and irrelevant mAb (filled curves). (*b*) IL-2 secretion in response to hHfe molecules. Hyb6 (*Top*), Tr6 (*Middle*), and negative control hybridoma T8.1 (*Bottom*) were cocultured for 1 day with untransfected (light gray bars) or human *β2m*/*hHfe*-cotransfected (dark gray bars) VAD1279 cells. The IL-2 content of culture supernatants was evaluated in a CTLL2-proliferation assay. (*c*) The following cell lysates were prepared to analyze the tyrosine phosphorylation status of ZAP-70: lysates of Hyb6, unstimulated (lane 1), stimulated by CD3 cross-linking (lane 2), incubated with either untransfected (lane 4), or human *β2m*- and *hHfe*-cotransfected VAD1279 cells (lane 5). Lane 3 corresponds to mix lysates of unstimulated Hyb6 and *hHfe*-transfected VAD1279 cells. Zap-70 was precipitated with a rabbit anti-ZAP-70 serum (IB60), separated by SDS/PAGE, and transferred to nitrocellulose membrane. (*Upper*) Tyrosine phosphorylated ZAP-70 was probed with anti-pTyr mAb (4G10) and enhanced chemiluminescence analysis. (*Lower*) The total amount of ZAP-70 molecules in the precipitates was visualized by immunoblot with the IB60 serum after membrane stripping.

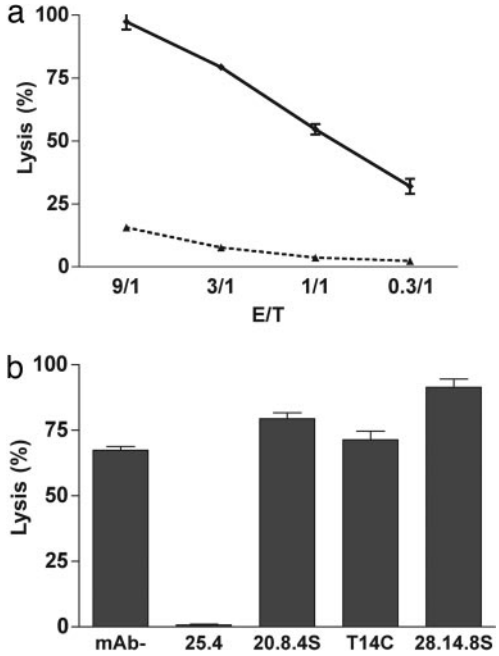

Fig. 4. CTL recognition of mHfe molecule. DBA/2 *mHfe* KO mice were injected i.p. with mouse *β2m*/*mHfe*-transfected P815 HTR cells or mouse β2m/mHfe-encoding lentiviral vector. Splenocytes were tested for cytolytic activity after *in vitro* restimulation with wild-type irradiated DBA/2 splenocytes. (*a*) Lysis at different effector/target (E/T) ratios against mouse *β2m*/*mHfe*-transfected (solid line) or untransfected (dashed line) ^{51}Cr-labeled P815 cells is illustrated for one representative clone. (*b*) The cytolytic activity of the same CTL clone was assayed at the 1/1 effector/target ratio in the absence (mAb−) or presence of anti-mHfe (25.4), anti-H-2K^d (20.8.4S), anti-H-2D^d (T14C), or anti-H-2L^d (28.14.8S) mAb, respectively.

inhibited by anti-mHfe mAb and not by anti-H-2^d mAb, implying a direct recognition of the mHfe molecule (Fig. 4). Although several Hfe-derived peptides possess major anchoring residues for binding to H-2^d class Ia molecules, no mHfe-specific, H-2^d-restricted responses were detected in any DBA/2 *mHfe* KO mice.

These results show that wild-type DBA/2 mice are tolerant to mHfe, whereas *mHfe* KO DBA/2 mice can develop unrestricted cytolytic responses with direct recognition of membrane-bound mHfe molecule, as demonstrated by specific inhibition of the cytolysis with the anti-mHfe mAb. Finally, lysis was not inhibited by anti-mouse CD8 mAb (data not shown).

mHfe-Specific TCR Repertoire. To characterize the diversity of the TCR repertoire emerging in response to mHfe, we isolated 47 specific CTL clones from six *mHfe* KO DBA/2 mice. Molecular analyses of the AV and BV chains of the 47 CTL clones showed that they derived from eight CTL precursors only (Table 1). Six of those precursors use an AV6 gene segment (two use AV6.1 and four use AV6.6), the two others use AV2 or AV14. These AV segments were linked to different AJ segments without significant structural similarities of their CDR3. Five distinct BV gene segments were mobilized, with usage of BV13.2 and BV4.1 in three and two cases, respectively, a likely consequence of their abundant peripheral representation in the naive T cell repertoire of normal mice. A recurrent expression of acidic residues was noticed in the C terminus of the CDR3*β*. These residues are germline-encoded by most BJ segments and potentially interact with positively charged conserved residues of the mHfe *α*2-helix, as they do with other MHC class I molecules (21). Thus, in DBA/2 *mHfe* KO mice, mHfe can be directly recognized by CTLs expressing different AV and BV TCR gene segments with preferential usage of two members of the AV6 segment family.

Experiments were devised to evaluate the impact of mHfe on the AV TCR repertoire in normal DBA/2 mice. Quantitative global RNA analysis of the whole AV6 segment family usage (12 members) in purified CD8$^+$ CD4$^-$ splenic naive T cells did not reveal significant differences between wild-type (13.28 ± 2.12%) and *mHfe* KO (10.66 ± 1.83%) DBA/2 mice. By contrast, as illustrated in Fig. 5, a 3- to 4-fold increase in CD8$^+$ T cells expressing AV6.1 or AV6.6 segments was found in wild-type DBA/2 compared with *Hfe* KO DBA/2 mice. Quantitative RNA analyses of the other AV families did not disclose appreciable differences between wild-type and *Hfe* KO DBA/2 mice. Thus, in normal DBA/2 mice, Hfe contributes to the selection of part of the CD8$^+$ T cell repertoire.

Discussion

In this report we have shown that human and mHfe molecules can be recognized directly by mouse *αβ* TCR. T cell responses against hHfe were obtained in HHD mice. Approximately one-third of these mice developed hHfe-specific TCR*αβ* CD8*αβ* CTL. Three sets of experiments established that these CTL recognize the hHfe molecule directly through their TCR: (*i*) All CTL clones killed specifically hHfe-transfected cells, and their

Table 1. Molecular characterization of mHfe-reactive TCR

Mice	TCR	CDR3 size	CDR3 sequence
Mouse 13			
Clones 6, 12, 18, 37, and 38	TRAV6.6*01-AJ5*01	7	CAL GGVGQLT FG
	TRBV1*01-BJ2.5*01	10	CTC SADWGDQDTQ YF
Clone 11	TRAV6.6*01-AJ40*01	11	CAL GDPVTGNYKYV FG
	TRBV4*01-BJ1.4*01	9	CAS SRQFSNERL FF
Mouse 8			
Clone 102	TRAV8.8*01-AJ20	10	CAL SAHNYAQGLT FG
	TRBV13.2*01-BJ2.1*01	11	CAS GGGLGGLYAEQ FF
Mouse 16			
Clones 1–4, 7–9, 11, 12, 16, 17, 40, and 42	TRAV14.1*02-AJ35*01	7	CAG GFASALT FG
	TRBV13.2*01-BJ2.1*01	8	CAS GDAFYAEQ FF
Clones 5 and 14	TRAV6.1*01-AJ27*01	10	CVL GDNTNTGKLT FG
	TRBV5*01-BJ1.1*01	11	CAS SQETLANTEVF FG
Mouse 23			
Clone 73	TRAV6.6*1-AJ20	10	CAL GPNNYAQGLT FG
	TRBV13.2*01-BJ2.1*01	11	CAS GDGLGGLYAEQ FF
Mouse 27			
Clones 47, 49, 51, 54, 56, 57, 60, 61, 92, and 93	TRAV6.1*01-AJ22*01	11	CVL GATSSGSWQLI FG
	TRABV1*01-BJ2.7*01	9	CTP GLGGEEQ YF
Mouse 22			
Clones 14, 15, 17–19, 22, 23, 28, 32, 36, 38, and 40–42	TRAV2*01-AJ11*01	9	IVT HDSGYNKLT FG
	TRBV4*01-BJ1.4	11	CAS SPGTGGNERLF FG

Retrotranscripts of TCR α- and β-chains from mHfe-specific CTL clones (identified in the left column by numbers) isolated from six DBA/2 *mHfe* KO mice were sequenced to determine their AV/BV segment usage and the structure of their CDR3 subregions. The nomenclature of International ImMunoGeneTics Information System (http://imgt.cines.fr) is used for AV and BV numbering (37). Spaces in amino acid sequences delimit CDR3 region boundaries.

cytolytic effect was blocked by anti-hHfe mAb.(*ii*) AV14$^+$/BV12$^+$ BW 51.47 derived T cell hybridomas and AV14/BV12 TCR transductants specifically recognized cells expressing the hHfe molecule (Figs. 2*c* and 3*b Top*). (*iii*) Phosphorylation of the tyrosine kinase ZAP-70 was observed upon contact of AV14$^+$/BV12$^+$ T cell hybridoma with hHfe-expressing cells. mHfe-specific CTL responses were similarly generated in a third of DBA/2 *mHfe* KO mice that had been hyperimmunized with *mHfe*-expressing cells. The lysis mediated by these CTL and by all CTL clones was inhibited by anti-mHfe mAb, not by anti-H-2^d mAb (as shown for one of them in Fig. 4), demonstrating that these CTL did not recognize mHfe-derived peptide presented by H-2^d molecules. Therefore, as can also be deduced from cytolytic and structural studies concerning TL (for thymus leukemia) antigen, another H-2 class 1b molecule (22, 23), $\alpha\beta$ TCR can recognize histocompatibility molecules with a nonfunctional peptide-binding groove.

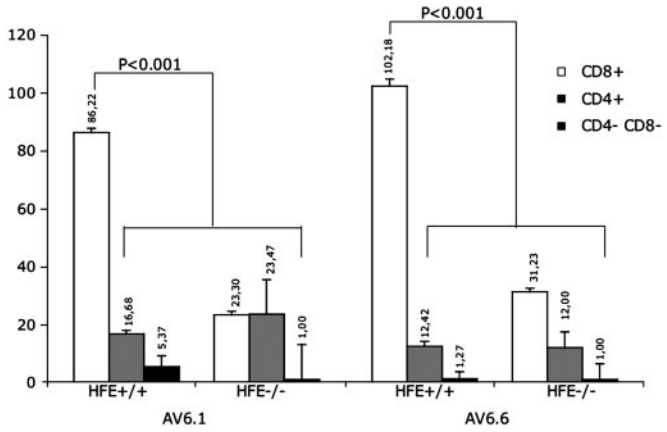

Fig. 5. Representation of AV6.1 and AV6.6 gene segments in naive peripheral T cells from wild-type and *Hfe* KO DBA/2 mice. CD4$^+$CD8$^-$, CD8$^+$CD4$^-$, and CD4$^-$ CD8$^-$ T lymphocytes were purified from splenocytes of two wild-type and two *mHfe* KO DBA/2 mice by using FACS cell sorting. Quantitative RT-PCR amplification of AV6.1 and AV6.6 α-chain transcripts of these T cell subsets was performed by using specific primers and β-actin retrotranscripts as internal standard. Results (means of two mice tested individually) of one experiment representative of three were normalized relative to the AV6.1 and AV6.6 retrotranscripts of the CD4$^-$ CD8$^-$ T cell subsets of *mHfe* KO DBA/2 mice.

Because TCRs in *HHD* and *mHfe* KO DBA/2 mice have most likely been educated by MHC molecules associated with self-peptides, one may wonder how some of these TCRs can recognize Hfe. The crystal structures of 11 TCR/peptide/MHC complexes indicate that the MHC-bound peptides are mainly contacted by the TCR α- and β-chain CDR3s (4, 24). The α1 and α2 helices of the MHC molecules are interacting with the TCR α- and (in most cases to a lesser extent) β-chain CDR1 and CDR2 regions. Such interaction suggests a functional dichotomy with initial docking of the TCR on MHC molecules mainly ensured by the α-chain and subsequent stabilization resulting from β-chain CDR3/ligand interaction (25). According to that scheme [although not uniformly accepted (24)], TCR recognition of Hfe molecules (which are deprived of bound ligand) would rely mainly on α-chains. In fact, analyzes of the T cell repertoire of 47 mHfe-specific CTL clones derived from six *mHfe* KO DBA/2 mice showed that these clones originated from eight CTL precursors only, and that, although the Vβ gene segment usage was rather diverse, six CTL clones expressed an AV6 segment. A Vα bias in TCR usage has been similarly suggested for two other MHC class Ib molecules, CD1 and MR1 (2, 3), and never documented formally for MHC class Ia molecules. This bias could suggest that class Ib molecules and CDR1/CDR2 "rudimentary" interactions, in particular those contributed by the Vα-chain, have predated the emergence of more "sophisticated" MHC class Ia molecule and CDR3 interactions. We would not, however, exclude that, to some extend, AV biased

usages could also apply to MHC class Ia molecules. In view of the polymorphism of MHC class Ia $\alpha1\alpha2$ helices, the positive and negative T cell selection processes would result in differential peripheral representation of AV segments after education by one MHC class Ia molecule versus another. In support of this hypothesis, Sim *et al.* (26) have demonstrated that CDR1 and CDR2 sequences from AV9D-4 and AV9–4 were involved in the preferential selection of $CD8^+$ or $CD4^+$ T cells, respectively, and they were able to reverse this CD8/CD4 bias by a single amino acid substitution of CDR1 or CDR2. Along the same line of thinking, we would like to suggest that the peptide-independent recognition of foreign MHC molecules by some alloreactive CTLs (4, 7, 8) results mainly from an AV CDR1/CDR2-mediated interaction. Peptide-independent recognition of allogeneic MHC molecules mediated by AV segments may explain that, in certain mismatched combinations, up to 10% of naive peripheral T cells are allo-activated (27).

Interestingly, although mHfe-specific CTLs obtained in *mHfe* KO mice preferentially express AV6.1 or AV6.6 segments, comparative quantitative RT-PCR analyses showed that, in wild-type DBA/2 mice, mHfe is responsible for the positive education of most $AV6.1^+$ and $AV6.6^+$ $CD8^+$ T cells. This finding is reminiscent of the positive selection of characteristic AV11 or AV5 rearrangements by the MHC class Ib molecules CD1 or MR1, respectively (2, 3). However, whereas in the two latter cases, expression of these AV11 or AV5 segments is restricted to the $CD4^+CD8^-$ and $CD4^-CD8^-$ T cell compartments, the expression of the $AV6.1/AV6.6^+$ T cells was observed essentially in the $CD8^+$ compartment of DBA/2 wild-type mice. However, Hfe-positively selected $CD8^+$ T cells may not differentiate into cytolytic effector cells but may acquire other regulatory functions, as was recently observed for $CD8^+$ T cells expressing self-reactive TCR of high avidity (28).

Indirect evidence [iron overload in *TNF receptor 2* KO mice or in *mHfe* KO mice deleted of the *RAG 1* recombinase gene (29, 30)] suggests that the immune system could participate in the control of iron metabolism. In humans with a C282Y Hfe homozygous mutation, a low $CD8^+$ peripheral T cell number increases the risk of severe hemochromatosis (31), but iron overload is not observed in CD8 KO mice (32). This last observation agrees with our results showing that none of the Hfe-specific CTL clones tested were inhibited by anti-CD8 mAb. We hypothesize that, although most Hfe-educated T cells are $CD8^+$ in wild-type mice (this study), the CD8 molecules might be dispensable for their education and effector function. Hfe, a sensor for the iron body level, lowers the affinity of the transferrin receptor for holotransferrin (33). In addition, Hfe could act as a signal molecule for TCR$\alpha\beta$ $CD8^+$ specialized T cells, like the macrophage inhibitory cytokine stress-inducible molecule for TCR$\gamma\delta$ T cells (34). $AV6.1/6.6^+$ T cell activation through Hfe could result in the production of cytokines like IL-6 and TNF-α, which are known to modulate the liver production of hepcidin, a small polypeptide that regulates iron metabolism (35, 36). The detailed functional analysis in DBA/2 wild-type mice of this identified T cell subset warrants further investigation.

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