TAP-independent, β_2-Microglobulin–dependent Surface Expression of Functional Mouse CD1.1

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Summary

CD1 molecules consist of β_2-microglobulin (β_2m) noncovalently complexed to a non-major histocompatibility complex (MHC)–encoded monomorphic integral membrane protein homologous to MHC class I α chains. Little is known about the requirements for cell surface expression and T cell recognition of CD1. We inserted the mouse CD1.1 gene into vaccinia virus to create a recombinant virus expressing CD1.1 under the control of a viral promoter. Using this recombinant virus to infect normal or mutant cell lines, we found that the expression of molecules reactive with the CD1.1-specific monoclonal antibody 3C11 requires the expression of β_2m but was not affected by the absence of the MHC-encoded peptide transporter (TAP). Consistent with these results, IL-2 production by the mCD1.1-specific T cell hybridoma DN32.D3 was induced by thymocytes from normal mice or mice with a homozygous deletion of the *TAP1* gene, but not by thymocytes from mice with a homozygous deletion of the β_2m gene. These results indicate that expression of functional mCD1.1 occurs in a β_2m-dependent, TAP-independent manner.

MHC class Ia molecules consist of a highly polymorphic integral membrane protein (α chain) noncovalently associated with β_2-microglobulin (β_2m)[1] to form a binding site that carries peptides to the cell surface for surveillance by $CD8^+$ T cells (T_{CD8+}). Most class I–associated peptides derive from a cytosolic pool of proteins through the action of cytosolic and/or nuclear proteases (1, 2). Peptides associate with newly synthesized class I molecules in an early secretory compartment, probably the endoplasmic reticulum (ER) (3). Cytosolic/nuclear peptides reach the ER through the action of a membrane transporter known as TAP, which is formed by association of the products of *TAP1* and *TAP2* genes located within the MHC. After their assembly, α-β_2m complexes associate with TAP via the TAP1 subunit until released upon peptide binding. The heterotrimeric complex is then transported through the Golgi complex to the cell surface. In the absence of TAP, class I molecules are exported from the ER, but slowly and in a form that is rapidly denatured once they reach the plasma membrane (4–9). Class Ia molecules are constitutively expressed in many body tissues and can be expressed in most others after exposure of cells to the appropriate cytokines. This is consistent with the primary function of class Ia molecules in the presentation of peptides from intracellular pathogens to the immune system.

A number of molecules highly similar to class Ia molecules are encoded by the mammalian MHC. These molecules, variously termed class Ib molecules, medial histocompatibility molecules, or nonclassical class I molecules, are structurally highly similar to class I molecules in consisting of an α chain noncovalently complexed to β_2m. Unlike class Ia molecules, class Ib molecules display little, if any, polymorphism and have a highly restricted tissue distribution. Some class Ib molecules have been directly demonstrated to bind short peptides (10–15) and present them to T cells (10–13, 16–18). Some class Ib molecules acquire peptides in a TAP-dependent manner (19–21); others appear to procure peptides independently of TAP (19, 22).

Mammals also express non–MHC-encoded class I–like molecules. These exhibit less homology to class Ia molecules than the MHC-encoded class Ib molecules, and, in the case of the neonatal Fc receptor, the known function is completely unrelated to T cells (23–25). The CD1 family of class Ib molecules, on the other hand, is involved in T cell function. CD1 molecules are expressed by virtually all mammals examined (26). Five human CD1 genes have been identified (hCD1a–e). hCD1a–c, and -e are more homologous to each other than CD1d (27). hCD1a–c, which

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; BFA, brefeldin A; ER, endoplasmic reticulum; MOI, multiplicity of infection; rVV, recombinant vaccinia virus; T_{CD8+}, $CD8^+$ T cells.

are expressed primarily on cortical thymocytes and on GM-CSF–treated monocytes, can be recognized by T cells expressing either the α/β or γ/δ T cell receptors (28–30). Most remarkably, it was recently demonstrated that one of these T cells apparently recognizes mycobacterial lipid presented by hCD1b (31).

Two CD1 genes have been identified in mice, mCD1.1 and mCD1.2. Both are predominantly expressed in the gut and on cortical thymocytes (32–34), and both are homologous to hCD1d, whose expression is largely limited to the gut. We recently demonstrated that mCD1.1 is recognized by a subset of $NK1.1^+$ T cells expressing an invariant $V\alpha$ T cell receptor chain with $V\beta$ chains of limited variability (35). This extended prior findings that a human jejunal intraepithelial T cell line could lyse hCD1d-transfected target cells (36). In the present study we examine the requirements for β_2m and TAP in the surface expression of mCD1.1 as detected by the CD1-specific mAb 3C11 (32) or the CD1-specific $NK1.1^+$ T cell–derived hybridoma DN32.D3.

Materials and Methods

Mice. C57BL/6 mice were purchased from Taconic Farms, Inc. (Germantown, NY). C57BL/6 mice with a homozygous disruption of the β_2m gene were obtained from The Jackson Laboratory (Bar Harbor, ME). (B6 × 129)F_1 mice with a homozygous disruption of the *TAP1* gene (37) were kindly provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN). Control (B6 × 129)F_1 mice were kindly provided by Dr. P. Love (National Institute of Child Health and Human Development, Bethesda, MD). Mice ranged in age from 7 wk to 7 mo.

Cell Lines. MC57G, a fibroblast cell line derived from C57BL ($H\text{-}2^b$) mice and TK^-p54, a human osteosarcoma cell line, were grown in DME (GIBCO BRL, Gaithersburg, MD), supplemented with 7.5% heat-inactivated fetal bovine serum (Biofluids, Inc., Rockville, MD), Na_2CO_3, and L-glutamine. The human lymphoblastoid cell line T2, which contains a deletion encompassing the MHC class II region, including the genes encoding the TAP subunits (38, 39), was provided by Dr. P. Creswell (Yale University, New Haven, CT) and was propagated in Iscove's medium (GIBCO BRL) with the same supplements as above. The human β_2m–deficient SS melanoma cell line (provided by Dr. N. Restifo, National Cancer Institute, Bethesda, MD) was grown in RPMI 1640 medium (Biofluids, Inc.) with the same additives as with DMEM above. FO-1 cells, a human β_2m–deficient melanoma (40) (provided by Dr. S. Ferrone, New York Medical College, Valhalla, NY) were maintained in DME as above. The murine IL-2–dependent cell line CTLL-2 was provided by Dr. E. W. Shores (Food and Drug Administration, Bethesda, MD).

Recombinant Vaccinia Virus (rVV) Generation. cDNA encoding mCD1.1 in Bluescript (Stratagene Inc., La Jolla, CA) (provided by Dr. S. Balk, Beth Israel Hospital, Boston, MA) was excised using XhoI and BamHI. The fragment was then inserted into the SalI/BglII sites of pSC11 modified by addition of a multiple cloning site downstream of the vaccinia virus p7.5 promoter. The generation of an rVV encoding mCD1.1 (VV-mCD1.1) was performed using standard techniques as previously reported (41). rVV coexpressing *TAP1* and *TAP2* genes (VV-Tap[1+2]), or expressing H-2 K^b (VV-K^b), K^d (VV-K^d), or human β_2m (VV-hβ_2) have been described (42, 43). In all experiments, cells were infected with the rVV at a multiplicity of infection (MOI) of 10 for the indicated lengths of time.

Thermostability Analysis. T2 cells (5×10^6) infected overnight with rVV at 26°C were washed and plated in 96-well U-bottom plates (Costar Corp., Cambridge, MA) at 10^5 cells per well. To one-half of the plates, brefeldin A (BFA; Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 5 μg/ml. Plates were then incubated at 26°C or 37°C for 3 h, and indirect immunofluorescence was performed as described below.

Antibodies and Cytofluorography. The following mAbs were purchased from American Type Culture Collection (ATCC, Rockville, MD): W6/32 (44) (ATCC No. HB-95), panreactive anti-HLA; BBM.1 (45) (ATCC No. HB-28), anti-human β_2m; SF1.1.1 (ATCC No. HB-159), anti-$H\text{-}2K^d$; and Y3 (46) (ATCC No. HB-176), anti-$H\text{-}2K^b$. 3C11 (32) (anti-mCD1.1, rat IgM) was provided by Drs. C. Terhorst (Beth Israel Hospital) and R. Blumberg (Brigham and Women's Hospital, Boston, MA). Fluorescein-labeled rabbit anti–mouse Ig antiserum was purchased from Dako Corp. (Carpenteria, CA). PE-conjugated goat anti–rat μ chain antiserum was obtained from Southern Biotechnology Associates (Birmingham, AL). Staining of uninfected and rVV-infected cells was performed as previously described (47, 48) and was quantitated using a FACScan® cytofluorograph (Becton Dickinson & Co., Mountain View, CA). Ethidium homodimer (Molecular Probes, Inc., Eugene, OR) was added to the cells at a final concentration of 10 μg/ml immediately before analysis; ethidium-stained, presumably nonviable cells were excluded from analysis.

Hybridoma Assay. Thymocytes (5×10^5 cells per well) were cultured with or without the mCD1.1-specific hybridoma DN32.D3 (49) (3×10^4 cells per well) in flat-bottom microtiter plates (Costar Corp.) as described previously (35). 20 h later the plates were centrifuged, and the supernatants were assayed for IL-2 using the murine IL-2–dependent cell line CTLL-2 as previously described (35).

Results and Discussion

To conveniently study the expression and function of mCD1.1 in a variety of cells lines (and for use in future in vivo studies), we inserted a cDNA encoding mCD1.1 into VV under the control of the p7.5 VV promoter (42). The expression of mCD1.1 on the surface of viable cells was monitored using the 3C11 mAb (32). This mAb is likely to react with native mCD1.1, since it blocks recognition by mCD1.1-specific T cells (35). After infection of MC57G mouse fibroblast cells with VV-mCD1.1, CD1.1 expression on the surface of viable cells was detected by the CD1-specific 3C11 mAb via indirect immunofluorescence and cytofluorography as early as 3 h after infection, reaching near-maximal levels 3 h later (Fig. 1). By contrast, cells infected with a control rVV were not stained above background levels observed using uninfected cells, thus demonstrating the specificity of staining for CD1.1. CD1.1 expression at the cell surface reflects its biosynthesis by infected cells and not simply adsorption onto the cell surface from the virus inoculum (which consists of a crude lysate from infected cells) since infection of cells in the presence of the protein synthesis inhibitor cycloheximide completely blocked CD1.1 cell surface expression (data not shown).

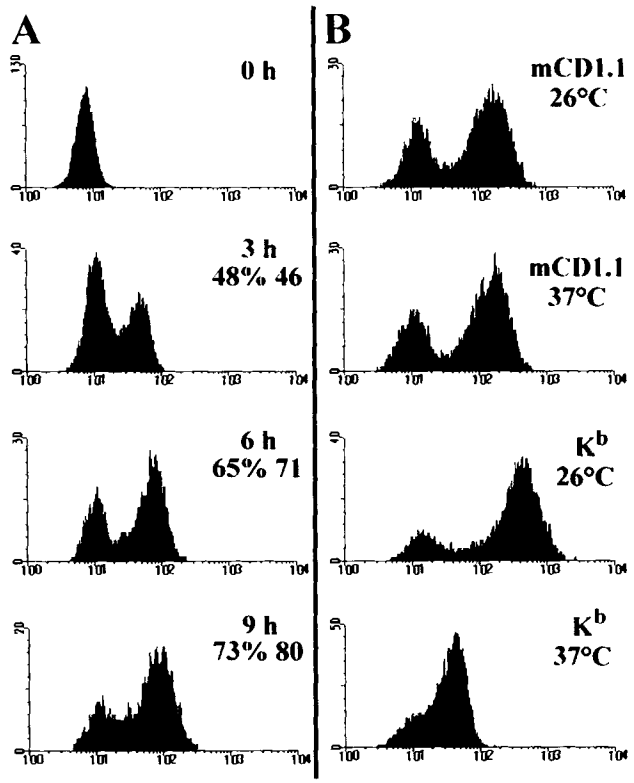

Figure 1. Characterization of mCD1.1 cell surface expression. (*A*) Murine MC57G fibroblasts (H-2^b) were either mock infected or infected with VV-mCD1.1 at an MOI of 10 for the indicated number of hours at 37°C. The cells were then tested for the expression of mCD1.1 by staining with the anti-mCD1.1 mAb 3C11, followed by an FITC-conjugated rabbit anti-mouse antiserum. Analysis of viable cells was by FACScan®. The percentage of positive cells and median channel fluorescence of positive cells are indicated. (*B*) T2 cells were infected with VV-mCD1.1 or VV-K^b (control) at an MOI of 10 overnight at 26°C. The cells either remained at 26°C or were incubated at 37°C for 3 h more in the presence of 5 μg/ml BFA. The cells were then stained with 3C11 (mCD1.1) or Y3 (H-$2K^b$), followed by an FITC-conjugated rabbit anti-mouse Ig antiserum. Analysis of viable cells was by FACScan®. In both *A* and *B*, the x- and y-axes correspond, respectively, to the fluorescence intensity and the number of cells.

Both human and murine CD1 molecules associate with β_2m (32, 50, 51). It has been reported, however, that cobiosynthesis of β_2m is not required for the expression of mouse or human CD1 on the surface of β_2m-deficient FO-1 human melanoma cells transfected with the respective genes (52). To explore this issue, we infected a β_2m-deficient human melanoma cell line (SS) with VV-mCD1.1 with or without an rVV expressing human β_2m (VV-hβ_2). SS cells express virtually no cell surface class I molecules reactive with the W6/32 mAb, which binds only native class I molecules. Infection with VV-hβ_2 greatly increases expression of W6/32-reactive cell surface HLA molecules (Table 1, experiment 1). Infection of cells with VV-mCD1.1 or an rVV expressing the mouse H-$2K^d$ class Ia α chain resulted in marginally detectable levels of mCD1.1 or K^d. Coinfection with VV-hβ_2 greatly increased the surface expression of both mCD1.1 and K^d (Table 1, experiment 1).

These data demonstrate that the expression of 3C11-reactive mCD1.1 on the surface of VV-mCD1.1-infected cells is greatly enhanced by coexpression of β_2m, although we cannot rule out the possibility that free mCD1.1 heavy chains are present on the surface of β_2m-deficient cells and are undetectable by 3C11. This finding appears to conflict with the conclusion of Balk et al. that mCD1.1 expression on FO-1 cells transfected with mCD1.1 is independent of β_2m (52). To explore this discrepancy, we obtained FO-1 cells and performed a similar experiment. As seen in Table 1 (experiment 2), FO-1 cells expressed greater amounts of CD1.1 than SS cells, and again only very small amounts of 3C11-reactive material were expressed in the absence of β_2m, whose expression enhanced 3C11 staining ~50-fold (after subtracting background staining). This is likely to be an underestimation of the effect of β_2m, since mCD1.1 biosynthesis will be compromised to some extent because of competition from VV-hβ_2 for gene expression. Since

Table 1. *Cell Surface Expression of mCD1.1 Requires Endogenous β_2m*

	Mean channel fluorescence			
Cells infected with	mCD1.1	human β_2m	HLA	H-$2K^d$
Experiment 1				
SS				
Mock	0	1	2	1
VV-wβ_2m	0	145	153	0
VV-mCD1.1	1	7	6	0
VV-mCD1.1 + VV-wβ_2m	56	150	128	2
VV-K^d	0	3	3	2
VV-K^d + VV-wβ_2m	0	184	197	102
Experiment 2				
SS				
Mock	0	2	2	
VV-wβ_2m	1	82	78	
VV-mCD1.1	2	6	5	
VV-mCD1.1 + VV-wβ_2m	82	94	60	
FO-1				
Mock	2	1	1	
VV-wβ_2m	3	121	140	
VV-mCD1.1	9	13	9	
VV-mCD1.1 + VV-wβ_2m	245	94	55	

SS and FO-1 cells were infected with the indicated rVV at an MOI of 10 for either 5.5 (experiment 1) or 12.5 h (experiment 2) at 37°C. The cells were then stained for mCD1.1, human β_2m, human HLA, or H-$2K^d$ expression, using 3C11, BBM.1, W6/32, and SF1-1.1.1 mAb, respectively, followed by an FITC-conjugated rabbit anti-mouse Ig antiserum. In experiment 2, 3C11 was followed by a PE-labeled goat anti-rat μ chain antiserum. Analysis was by FACScan®.

Balk et al. did not examine the effect of coexpression of mCD1.1 with β_2m in FO-1 cells, it is possible that the amount of mCD1.1 they detected on cells transfected with the mCD1.1 gene corresponds to the very low levels of mCD1.1 we detect on the surface of cells not coinfected with VV-hβ_2. An additional complexity is that Balk et al. used a different mAb to detect mCD1. Since this mAb may be better able to detect unfolded CD1.1 than 3C11, our findings do not eliminate the possibility that mCD1 is transported to the cell surface in β_2m-deficient cells. As demonstrated below, however, β_2m is clearly required for expression of at least one form of mCD1.1 recognized by T cells. Whether or not β_2m would have a similar effect on the cell surface expression of hCD1d in FO-1 cells remains a question.

We next examined the requirement for TAP in the surface expression of 3C11-reactive CD1.1 molecules. Class Ia molecules expressed on the surface of TAP-deficient cells at 26°C lose reactivity with mAbs reactive with native molecules when cells are shifted to 37°C, because of the absence of high-affinity peptides needed to stabilize class I molecules. T2 cells, which lack a megabase region of the MHC encoding TAP, were infected for 18 h at 26°C with VV-K^b or VV-mCD1.1 and incubated for an additional 3 h in the presence of BFA at 37°C or 26°C before surface expression of class I molecules was determined by indirect immunofluorescence using either 3C11 or the Y3 mAb specific for native K^b. BFA was included to minimize the exocytosis of additional intracellular class I molecules (53, 54). As expected, K^b expression was greatly diminished by the shift in temperature to 37°C (Fig. 1 *B*). By contrast, CD1.1 expression was unaffected by this shift. This demonstrates, first, the thermostability of CD1.1 protein at the cell surface, and second, that CD1.1 is not detectably internalized or shed over the 3-h incubation period.

We further examined the effect of TAP expression of CD1.1 cell surface expression by coinfecting T2 cells for 18 h at 37°C with VV-mCD1.1 and an rVV expressing human TAP1 and TAP2 (VV-TAP[1+2]). As shown in Table 2 (and reported previously in reference 54a), VV-TAP[1+2] infection increased the expression of endogenous HLA class molecules reactive with the W6/32 mAb. Similarly, cells coinfected with VV-CD1.1 and VV-TAP[1+2] demonstrated an increase in HLA expression. Despite the coexpression of TAP, CD1.1 expression actually decreased relative to cells infected only with VV-CD1.1 (additional experiments show that the decrease is attributable to interrecombinant competition for gene expression).

We previously reported that murine $NK1.1^+$ T cells, which express an invariant T cell receptor α chain, specifically recognize mCD1.1, as demonstrated using cells infected with VV-CD1.1 (35). To examine the effects of β_2m and TAP expression on T cell recognition of mCD1.1, we examined the ability of the $NK1.1^+$ T cell–derived mCD1.1-specific T cell hybridoma DN32.D3 (35, 49) to release IL-2 in response to a 20-h coincubation with thymocytes derived from normal mice or mice with homozygous deletions in genes encoding β_2m or TAP1. As shown in Table 3, IL-2 production by the DN32.D3 mCD1.1–specific T cell hybridoma was induced by normal C57BL/6 thymocytes, as we have previously reported (35). Thymocytes from wild-type (B6 × 129)F_1 mice were also able to induce the production of IL-2 by DN32.D3 cells (these mice were used as a control for the TAP1-deficient mice, which were only available on this background). As we have reported previously (35), thymocytes from β_2m-deficient mice were unable to stimulate IL-2 production by DN32.D3 cells. By contrast, thymocytes from mice lacking TAP1 stimulated IL-2 release to an extent similar to thymocytes derived from C57BL/6 or (B6 × 129)F_1 thymocytes (Table 3). Consistent with this finding, thymocytes derived from the TAP1-deficient mice bound similar amounts of the 3C11 mAb as thymocytes derived from normal mice (not shown).

Taken together, these findings demonstrate that both the surface expression of mCD1.1 and recognition by T cells occur mostly in a β_2m-dependent, TAP-independent manner. The TAP independence of mCD1.1 is similar to findings made with hCD1b, which was shown to present a mycobacterial lipid in a TAP-independent manner (31). The nature of the ligands presented by mCD1.1, or, indeed, whether mCD1.1 presents a ligand at all, remains to

Table 2. *Surface Expression of mCD1.1 Is TAP Independent*

	Mean Channel Fluorescence			
	Experiment 1		Experiment 2	
T2 Cells infected with*	Anti-mCD1.1	Anti-HLA	Anti-mCD1.1	Anti-HLA
Mock	0	899	1	772
VV-mCD1.1	56	833	84	692
VV-mCD1.1 + VV-TAP[1 + 2]	52	1222	65	1275
VV-TAP[1 + 2]	2	1326	0	1674

*T2 cells were either mock infected or infected with VV-mCD1.1, VV-TAP[1 + 2], or both, overnight at 37°C. The cells were then stained with antibodies against mCD1.1 (3C11) or HLA (W6/32), followed by an FITC–conjugated rabbit anti–mouse Ig antiserum. Analysis was by FACScan®.

Table 3. *Recognition of mCD1.1 by the mCD1.1-specific T Cell Hybridoma, DN32.D3, is TAP Independent*

	Average dpm*	
	Experiment 1	Experiment 2
Medium only	584	539
C57BL/6 thymocytes only	1,133	522
β_2m (−/−) thymocytes only	371	431
(B6 X 129) F_1 thymocytes only	534	597
TAP1 (−/−) thymocytes only	554	472
DN32.D3 only	1,015	1,448
DN32.D3 + B6 thymocytes	47,887	38,479
DN32.D3 + β_2m (−/−) thymocytes	1,671	1,455
DN32.D3 + (B6 X 129) F_1 thymocytes	47,407	31,355
DN32.D3 + TAP1 (−/−) thymocytes	48,087	37,549

*Thymocytes (5×10^5) from the indicated mouse strains were cocultured with the mCD1.1-specific T cell hybridoma DN32.D3 (3×10^4 cells) for 20 h at 37°C. Supernatants were then tested for IL-2 by their ability to support the growth of the murine IL-2–dependent cell line CTLL-2 for 24 h at 37°C. 6 h before harvest, 1 μCi of [^{3}H]TdR was added to each well, and the incorporation (average dpm of duplicate samples) was determined. The values of DN32.D3 + thymocytes lie on the linear part of the IL-2 standard curve.

be determined. Assuming that the NK1.1$^+$ cells are the major immune cell population that interacts with mCD1.1, the invariant nature of their TCR suggests that they recognize mCD1.1 bound either to a highly conserved ligand or to no ligand at all. In the latter case, mCD1.1 would be acting strictly as a regulator of immune responses, which is consistent with the prodigious ability of NK1.1$^+$ T cells to secrete IL-4 (55–57). In the former case, the conserved ligand should be a cellular product, which, as demonstrated by findings with hCD1b (31), need not be peptidic in nature. If mCD1.1 is presenting such a ligand, then the TAP-independent nature of this presentation makes it imperative in future studies to understand its intracellular trafficking, as this could offer an important clue to the cellular locale of ligand binding.

We thank Judy Stephens and Beth Buschling for expert technical assistance. We gratefully acknowledge Drs. S. Balk, C. Terhorst, R. Blumberg, P. Cresswell, S. Ferrone, N. Restifo, E. W. Shores, L. Van Kaer, and P. Love for generously providing reagents, cell lines, and mice.

R. R. Brutkiewicz is the recipient of a National Research Council–National Institutes of Health Research Associateship.

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Received for publication 11 May 1995 and in revised form 17 July 1995.

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