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Human CD1d Functions as a Transplantation Antigen and a Restriction Element in Mice

Bin Wang,* Taehoon Chun,* Ingrid C. Rulifson,† Mark Exley,‡ Steven P. Balk,‡ and Chyung-Ru Wang**

To study the potential functions of human CD1d (hCD1d), we developed transgenic (Tg) mice that ectopically express hCD1d under the control of H-2Kb promoter. High levels of hCD1d expression were detected in all Tg tissues tested. Skin grafts from the KbhCD1d Tg mice were rapidly rejected by MHC-matched non-Tg recipient mice, suggesting that hCD1d can act as transplantation Ags. Furthermore, we were able to elicit hCD1d-restricted CD8+ CTLs from mice immunized with KbhCD1d Tg splenocytes. These CTLs express TCR rearrangements that are distinct from invariant TCR of NK T cells, and secrete significant amounts of IFN-γ upon Ag stimulation. Analysis with various hCD1d-expressing targets and use of Ag presentation inhibitors indicated the recognition of hCD1d by CTLs did not involve species or tissue-specific ligands nor require the processing pathways of endosomes or proteasomes. Additionally, the reactivity of hCD1d-specific CTLs was not affected by acid stripping followed by brefeldin A treatment, suggesting that CTLs may recognize a ligand/hCD1d complex that is resistant to acid denaturation, or empty hCD1d molecules. Our results show that hCD1d can function as an alloantigen for CD8+ CTLs. The hCD1d Tg mice provide a versatile model for the study of hCD1d-restricted cytolytic responses to microbial Ags. The Journal of Immunology, 2001, 166: 3829–3836.

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3 Abbreviations used in this paper: hCD1d, human CD1d; αGalCer, α-galactosylceramide; βm, β2-microglobulin; IEC, intestinal epithelial cell(s); m, mouse; Tg, transgenic.

CD1 molecules are MHC-unlinked class Ib molecules that have been conserved throughout mammalian evolution (1). Although the overall structure of CD1 resembles that of MHC class Ia molecules, the homology between CD1 and MHC class Ia is only between 25% and 30% (2). Unlike class Ia molecules, CD1 is relatively nonpolymorphic, and its expression is TAP independent (3, 4). CD1 proteins can be divided into two distinct groups: group 1 CD1, including human CD1 (hCD1)3 a, b, and c, and group 2 CD1, consisting of hCD1d and two mouse CD1 molecules, mCD1.1 and mCD1.2 (1, 5). They differ in their sequence, tissue distribution, and possibly function. Group 1 CD1 are expressed by cortical thymocytes (6), a subset of B cells (7), and APCs, such as dendritic cells, and activated monocytes (8). The tissue distribution of group 2 CD1 molecules is more widespread. hCD1d is expressed on most thymocytes, activated T cells, peripheral B cells, monocytes (9), and intestinal epithelial cells (IEC) (10, 11). The expression of hCD1d on IEC is predominantly localized to the apical and lateral regions of small and large intestinal epithelia (12), placing it in a critical location for interaction with intraepithelial lymphocytes. mCD1 is expressed on cells of multiple hemopoietic lineages, including B and T cells, macrophages, and dendritic cells (13–15). However, the expression of CD1 on mouse IEC (mIEC) remains controversial, as anti-CD1 mAbs differ in detection of CD1 expression on mIEC (16–18).

Human group 1 CD1 can present lipid and glycolipid Ags derived from mycobacterial cell wall to different subsets of T cells, including CD4+ T cells (17), CD8+ T cells (18), and CD4−CD8− T cells (19, 20). Group 2 CD1 can also bind lipid Ags, such as glycosylceramide and phospholipids (21, 22). Additionally, mCD1.1 has been shown to bind hydrophobic peptides as well (23). Both hCD1d and mCD1.1 can be recognized by a unique subset of T cells, the NK T cells, which express a restricted range of TCRs bearing a single invariant Vα-chain (Var14Jα28l in mice and Var24αQ in humans) paired with limited sets of Vβ-chains (24–26). NK T cells can recognize CD1d in the absence of exogenous Ags, but their reactivity can be enhanced by the addition of synthetic lipid Ags, such as α-galactosylceramide (α-GalCer) (3) (21, 27). Upon activation, NK T cells promptly produce large amounts of cytokines, in particular IL-4. Several studies suggest that NK T cells may have important functions in regulating immune responses (28–31). In addition to NK T cells, T cells expressing diverse TCR α- and β-chains have also been found to recognize mCD1. These include some CD4+ T cells from class II-deficient mice (32, 33), some CD8+ CTLs from mice immunized with plasmid DNA containing chicken OVA (34), and from mice immunized with a mCD1 transfected coated with CD1-binding peptide (23, 35). However, no hCD1d-restricted CD4+ or CD8+ CTLs have been isolated to date.

Expression of two biochemically distinct forms of hCD1d, β2-microglobulin (β2m)-associated and non-β2m-associated hCD1d, has been demonstrated (9, 12, 36). The β2m-associated hCD1d is a mature 48-kDa glycoprotein, which is expressed on the surface of thymocytes and B cells. The non-β2m-associated hCD1d is a 37-kDa nonglycosylated isofrom, which is predominately expressed on the IEC. The functional role of hCD1d on IEC has been
implicated by studies showing that anti-hCD1d mAb inhibit IEC-induced proliferation of CD8+ T cells (37). Recently, it was also shown that cross-linking hCD1d on the surface of human IEC with the anti-hCD1d Ab specifically induced epithelial IL-10 expression, which may serve to dampen the epithelial proinflammatory signals (38).

Little is known about the Ag presentation by hCD1d other than its ability to present α-GalCer to CD4+ CD8− or CD4+ NK T cells. To further the study of hCD1d functional properties, we have derived transgenic (Tg) mice expressing hCD1d molecules. We report in this work that hCD1d behaves as a transplantation Ag in mice, as observed in rapid rejection of skin grafts from hCD1d Tg mice. Furthermore, hCD1d Tg spleen cells are effective in inducing CD8+ hCD1d-specific CTLs in normal mice. The hCD1d-specific T cells are capable of killing both hCD1d-positive mouse and human cells, suggesting CTLs recognize hCD1d as an intact molecule. Our data demonstrate that, similar to hCD1a, b, and c, hCD1d could function as a restriction element for CTLs.

Materials and Methods

Generation of K0/hCD1d Tg mice

Genomic clone containing full-length hCD1d was isolated from human genomic library (Stratagene, La Jolla, CA). The chimeric gene in which hCD1d gene was driven by the H2-Kb promoter was constructed as shown in Fig. 1. This fragment was injected into the pronuclei of fertilized eggs of (C57BL/6 (B6) × CBA)F1 mice to produce Tg founder mice. Tg-positive mice were identified by PCR using primers specific for hCD1d exon 2 (5′-CGAGGGCCCCAAGCGGCGCATA-3′) and exon 3 (5′-CAGAGAGCGAGGGTGC-3′). Two lines of Tg mice, line 1 and line 3, were established by crossing Tg founder mice with B6 mice. Line 1 was chosen for further backcrossing with B6 mice because it showed higher surface expression of hCD1d than line 3 (data not shown). The Tg mice used in this study have been backcrossed four to seven generations onto B6 background.

Expression of hCD1d mRNA in Tg mice

RNA was extracted from various tissues of Tg mice with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was prepared using random hexamer primers, and amplified by PCR using primers specific for hCD1d exon 2 and exon 3. The amount of template cDNA used in each reaction was normalized to the amount of hypoxanthine phosphoribosyltransferase mRNA amplified with primers 5′-GGTTGATACAGGCCAGACTTT GTTG-3′ and 5′-GGAGTTAGCGTGGCTATAGGC-3′.

Cell lines and CD1 transfectants

RMA-S and L929 cells were transfected with K0/hCD1d chimeric gene by electroporation, followed by G418 selection and FACS analysis to generate lines stably expressing hCD1d. The derivation of RMA-S and L929 transfectants expressing mCD1, or mCD1d, and CIR transfectants expressing CD1a, b, c, or CD1d/a chimeric protein have been described previously (26, 39). Human cell lines, U937, THP-1, K-562, Jurkat, MOLT-3, MOLT-4, Raji, and JY, were kindly provided by Gijs van Severent (University of Chicago, Chicago, IL). All transfectants and human cell lines were maintained and cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FCS (Sigma, St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 50 μM β-mercaptoethanol (RPMI 10). Human intestine epithelial cell lines (HT29, T84, and CACO2) were kindly provided by Eugene Chang (University of Chicago). Con A- and LPS-induced blasts were prepared by incubating spleen cell suspension (5 × 106) with Con A (2.5 μg/ml; Sigma) or LPS (5 μg/ml; Sigma) for 72 h in RPMI 10. Bone marrow-derived macrophages were obtained by culturing bone marrow cells (2 × 106 cells/ml) for 6 days in RPMI 10 supplemented with 30% L929 cell supernatant.

Abs, cell preparations, and FACS analysis

The following Abs were purchased from PharMingen (San Diego, CA): anti-CD11a (2D7), anti-CD102 (3C4), anti-CD54 (3E2), FITC anti-H2-Kb (AF6-88.5), FITC anti-CD8α (53-6.7), PE anti-CD4 (RM4-5), PE anti-CD11c (M175), FITC anti-H2-IAa (M5114), biotinylated goat anti-mouse IgG2b, and biotinylated goat anti-mouse IgG1. The hCD1d-specific Abs, 51.1 (mlg2b) and 42.1 (mlgG1), have been described previously (9). PK136, anti-mouse NK1.1; YTS.169.69, anti-mouse CD8; GK1.5, anti-mouse CD4; 16-1-11N, anti-H2-Kb; Y3, anti-H2-Kk; B22, anti-H2-D3; BB7.2, anti-HLA-A2 (mlgG2b); and 4D12, anti-HLA-B5 (mlgG1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Single cell suspensions from thymus, spleen, lymph nodes, and Peyer’s patches were prepared using standard procedure and stained in immunofluorescence buffer (HBSS containing 2% FBS and 0.1% NaN3) using combinations of fluorescent-conjugated Abs for 30 min at 4°C. The IEC were prepared and purified through the discontinuous 25/40/70% Percoll gradient centrifugation, as described by Yamamoto et al. (40). Cells that layered between the 40 and 25% interface were collected as IEC. For isolating hepatic epithelial cells, mice livers were perfused with 10 min with perfusion I medium (in mmol/L: NaCl, 120; KCl, 5; KH2PO4, 0.4; Na2HPO4, 0.2; NaHCO3, 25; EGTA, 0.5; d-glucose, 5.5; pH 7.4), then for 10 min with perfusion II medium (in mmol/L: NaCl, 120; KCl, 5; KH2PO4, 0.2; NaHCO3, 25; MgSO4, 0.4; MgCl2, 0.5; CaCl2, 3; d-glucose, 5.5; pH 7.4) containing 0.05% collagenase, 0.5 ml insulin (5 mg/ml), and 0.8 U trypsin inhibitor per unit tryptic activity in the collagenase (41). Then heptocytes were minced, and separated on a Percoll density gradient described as above. Thymic stromal cell suspensions were prepared by digesting fetal thymus in 0.1% trypsin, 0.5 mM EDTA for 40 min at 37°C. Digestion was stopped by addition of immunofluorescence (IF) buffer. After mechanical disruption of the lobe, cells were washed by IF buffer before cell surface staining experiments. Cells (106) were stained with anti-hCD1d, followed by biotin-conjugated goat anti-mouse IgG2b and a second incubation with streptavidin-conjugated PE and FITC anti-I-Ak. The stained cells were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Mountain View, CA) with the CellQuest software.

Skin grafting

Female hCD1d Tg or Tg−/− mice (6–8 wk old) were used as donors. Full-thickness sections of skin (∼1 × 1 cm in size) were harvested from the tail of donors and grafted onto the dorsal side of female C57BL/6. Bandages were removed on day 7 post transplant, and grafts were monitored for 55 days for evidence of rejection. Rejection was defined as complete necrosis of the skin grafts.

Generation of anti-hCD1d CTLs

B6 × CBAF1 mice were primed with 106 irradiated hCD1d+ splenocytes (in B6 background) through i.p. injection and footpad injection. After 10 days, lymphocyte suspensions were prepared from draining lymph nodes and spleen, and then cultured with irradiated hCD1d− splenocytes (2–5 × 106 cells/ml) in RPMI 10 medium. One week later, cultures were restimulated with hCD1d+ splenocytes and maintained in supplemented Michelli Dutton medium (SMDM) with IL-2 supplement (20 U/ml). IL-2 for restimulations was partially purified from the supernatant of EL4.II.2 cells (ATCC). After that, the CTLs were restimulated weekly with the irradiated L929/hCD1d1 transfectants. CTL clones were established by limiting dilution method in the presence of IL-2 (10 U/ml; PharMingen) and 2.5 × 106 irradiated L929/hCD1d1 transfectants. The established clones were maintained as weekly stimulation with irradiated L929/hCD1d1 transfectants. The CTL activity was tested by 31Cr release assay, as described below.

CTL assay

One million target cells were labeled with 50 μCi [31Cr]sodium chromate (Amersham, Arlington Heights, IL) for 45 min at 37°C. Target cells (1 × 106) were incubated with effector T cells in round-bottom microtiter wells. After 4 h at 37°C, 100 μl of supernatant from each well was collected and assayed for [31Cr] release. The percentage of specific [31Cr] release was calculated by the following equation: (experimental release − spontaneous release)/spontaneous release) × 100. For Ab-blocking studies, the CTL activity of clones against various targets was tested in the presence of 25% of supernatant or 30 μg/ml of purified mAbs.

Inhibitor treatment of target cells

Target cells were incubated with lacticystin (40 μM; Calbiochem, San Diego, CA), chloroquine (20 μM; Sigma), or brefeldin A (1 μg/ml; Sigma) for 18 h before CTL assay (42, 43). For the acid-stripping experiment, target cells (RMA-S/hCD1d, RMA, and P388) were washed with HBSS, and incubated for 90 s with acid-stripping medium (0.3 M glycine-HCL and 1% BSA in water, pH 2.4) at cell densities of 2 × 106 cells/ml (43). Culture medium (100 vol) was added to neutralize pH. Cells were washed three times and suspended in RPMI 10 at 106/ml. Completeness of acid stripping was assessed by flow cytometric analysis on RMA and P388 cells using anti-Kb and anti-Kk Ab, respectively.
CTCs (10⁶ cells/well) were cultured with the same number of irradiated stimulators in round-bottom 96-well plate in a total volume of 200 µl/well. After 48 h, the supernatants were harvested for cytokine assay. A sandwich ELISA was used to determine the concentration of IFN-γ, IL-4, and IL-2. Abs specific for cytokines and recombinant mouse cytokines were obtained from PharMingen and used according to the manufacturer’s directions.

Cloning and sequencing of TCR genes

Total RNA was isolated from CTL clones using TRizol reagent (Life Technologies). First strand cDNA synthesis and PCR of DC-tailed cDNA were conducted with 5’ rapid amplification of cDNA end (RACE) system (Life Technologies, Rockville, MD), according to the manufacturer’s protocol. In brief, first strand cDNA synthesis was done using Superscript II reverse transcription and Ca-specific primer (5’-CAGGAGTTCGAGGTC CCA-3’) or CB-specific primer (5’-CCAGAAGTACAGAGACC-3’). The synthesized cDNA was then isolated with GlassMax DNA Isolated Spin Cartridge, tagged with TdT, and amplified by PCR. Oligonucleotide primers used were as follows: abridged anchor primer (5’-ACTTAG TACGGGIIGGGIIGGGIIGG-3’) and Ca-inner primer (5’-CTGTCCT GAGACCGGAGGATC-3’) for TCR α gene amplification; and abridged anchor primer and CB-inner primer (5’-CTGGGTT GGAGTCACATT TTCTC-3’) for TCR β gene amplification.

The PCR products were cloned into pGEM-T vector (Promega, Madison, WI). Positive clones were screened by nested PCR with abridged anchor primer pairing with the primer specific for either Ca (5’-ACTGG TACACAGCAGGGTCTG-3’) or CB (5’-CTGGGT TGGAGTCACATT TTCTC-3’). Nucleotide sequences were determined by PCR sequencing method using Taq DNA polymerase, BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA).

Results

Expression of hCD1d in Kb/hCD1D Tg mice

The expression of hCD1D in the different tissues of Tg mice was examined by RT-PCR. hCD1D message was detected from all tissues tested from Tg animals, including thymus, spleen, lymph nodes, liver, kidney, intestine, and skin (Figs. 1 and 2A). Cell surface expression of hCD1d in the Tg mice was determined by immunofluorescence staining with mAbs specific to hCD1d (mAb51.1) (Fig. 2B). High levels of surface expression can be detected on the majority of lymphocytes isolated from spleen and lymph nodes of the Tg animals, suggesting the hCD1d epitope recognized by mAb51.1 was not affected by the species origin of the associated β₂m. A bimodal staining pattern was observed from Tg thymocytes with high levels of hCD1d expression on mature thymocytes (CD3high) and low levels of hCD1d expression on immature thymocytes (CD3low) (data not shown). Surface expression of hCD1d can also be detected on epithelial cells from the thymus, intestine, and liver (Fig. 2B). The expression pattern of hCD1d in the Tg mice is similar to that of H2-Kb molecules.

hCD1d is a strong transplantation Ag in mice

To determine whether hCD1d can serve as a transplantation Ag, B6 mice were grafted with tail skin from hCD1d Tg1 and Tg2 littermate control mice. The donor mice used in skin graft experiments were backcrossed seven generations onto B6 background. All skin grafts (n = 10) from hCD1d+ mice were rapidly rejected with a mean survival time of 14 days. Five of six skin grafts from hCD1d− mice showed no sign of rejection and survived for at least 55 days. One of the hCD1d− skin grafts was rejected at day 28, which might be due to the remaining minor histocompatibility Ag disparities. These results reveal that hCD1d molecules are recognized as functional transplantation Ags in mice (Fig. 3).

Generation of CTLs against hCD1d

To investigate whether hCD1d is capable of stimulating hCD1d-restricted CTL response, we immunized B6 × CBA/F₁ (H-2k/b) mice with hCD1d Tg² splenocytes. The lymphocytes isolated from the primed mice were stimulated with irradiated Tg² splenocytes in vitro. After 2 wk of culture with Tg² splenocytes (H-2b background), the CTLs were restimulated with H-2-mismatched hCD1d-transfected L929 cells (H-2k background), to eliminate H-2b-restricted CTLs. RMA-S/hCD1d (H-2b) and L929/hCD1d (H-2k) transfectants were used to screen hCD1d-specific CTLs. Two CTL lines, BN1 and BN4, which lysed both transfectants but not untransfected parental cells, were established from two individual mice. Lysis of H-2-mismatched hCD1d-positive cells indicated that these CTL lines recognized hCD1d as an intact molecule and not as an hCD1d-derived peptide presented by a mouse MHC.
FIGURE 3. Rejection of skin grafts from K\(^b\)/hCD1d Tg mice by B6 recipients. Skins from tails of transgene-positive mice or transgene-negative littermates were grafted onto the flank of a recipient mouse. The grafts were covered with bandages. Seven days later, grafts were observed and scored daily for 55 days. Rejection was defined as complete necrosis of the skin graft. All hCD1d Tg\(^{+}\) grafts (n = 10) were rapidly rejected with a mean survival time of 14 days, while Tg\(^{−}\) skin grafts (n = 6) had prolonged survival time (p < 0.002).

molecule (Fig. 4). Inhibition of target cell lysis by H2-K\(^b\), D\(^b\), or hCD1d-specific mAb showed that only the Ab to hCD1d exerted a complete inhibitory effect, which further supports the notion that BN1 and BN4 CTLs recognized hCD1d as a restriction molecule (Fig. 4).

Characteristics of anti-hCD1d CTLs

FACS analysis of BN1 and BN4 showed that they are CD8\(^{+}\)/CD4\(^{−}\) and negative for NK cell surface markers (data not shown), suggesting that they are distinct from the NK T cell subset. Consistent with this finding, both lines did not express invariant V\(\alpha 14\) or V\(\alpha 281\) transcript (data not shown), the canonical TCR rearrangement found in most of the mCD1-restricted NK T cells. Thus, we used 5'-rapid amplification of cDNA end (RACE) protocol followed by DNA sequencing to determine the TCR usage of CTL clones derived from line BN1 and BN4. All four clones from line BN1 expressed V\(\alpha 5\)j\(\alpha 4\) and V\(\beta 3\)D\(\beta 2\)B\(\beta 2\), and all eight clones derived from line BN4 expressed V\(\alpha 17\)j\(\alpha 25\) and V\(\beta 2\)D\(\beta 1\)B\(\beta 6\). These CTL clones secreted significant amounts of IFN-\(\gamma\) upon Ag stimulation, but not IL-2 or IL-4 (Table I). CTL clones derived from these two CTL lines were used for further study.

Specificity of anti-hCD1d CTLs

Unlike some mouse and human NK T cells that can recognize CD1 molecules from both species (44), BN1 and BN4 CTLs did not recognize mCD1A-, B-, C- molecules, human C1R cells transfected with CD1a, b, c transfectants, even at the high E:T ratio (data not shown).

Reactivity of hCD1d-restricted CTLs to various CD1-transfected CIR cells

To determine whether anti-hCD1d CTLs recognize other hCD1 molecules, human CD1A-, B-, C-, or D-transfected CIR cells were used as targets. Both clones showed preferential reactivity toward hCD1d transfectant (Fig. 7). Clone BN1 showed some cross-reactivity to CD1c and CD1a transfectants, while BN4 did not react with CD1a, b, c transfectants, even at the high E:T ratio (data not shown).

Table I. Characteristics of anti-hCD1d CTLs

<table>
<thead>
<tr>
<th>Phenotype(^{d})</th>
<th>TCR Usage(^{b})</th>
<th>Cytokine Production(^{c})</th>
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<td>CD4</td>
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<td>BN1</td>
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<tr>
<td>BN4</td>
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\(^{a}\) Anti-hCD1d CTL clones were stained with anti-CD4, anti-CD8, B, or anti-NK1.1.1 and analyzed by flow cytometry.

\(^{b}\) The TCR usage of hCD1d-reactive T cell clones were determined by RT-PCR with 5'-RACE system as described in Materials and Methods. The gene segments used to encode the TCRs were defined by comparing the sequences to the NCBI Genbank database using the BLAST algorithm. Nomenclature for V\(\alpha\) and V\(\beta\) family numbers is based on the recommendation (57). These sequence data are available from GeneBank under accession number AF296659-62.

\(^{c}\) Cytokine levels were determined by ELISA. Results are shown as U/ml of cytokine from 10\(^{5}\) of CTLs. CTLs cultured with B6 splenocytes did not secrete detectable amounts of IFN-\(\gamma\) (<1 U/ml). –, Cytokine levels were below detection limit (<0.05 and <1 U/ml for IL-2 and IL-4, respectively).

FIGURE 4. Recognition of hCD1d by CTL lines is not H2 restricted. Untransfected and hCD1d-transfected RMA-S or L929 cells were labeled with \(^{51}\)Cr, and used as targets in a standard cytotoxicity assay. The CTLs were incubated with target cells in the presence of an anti-hCD1d mAb (51.1) or anti-H2-K\(^b\)/D\(^b\) mAbs (Y3 and B22) or medium alone. The E:T ratio was 3:1. The results are representative of two experiments.
Similar to CD1b and CD1c, the cytoplasmic tails of hCD1d contain a putative tyrosine base-sorting motif, Y-X-X-Z (in which X is any amino acid, and Z is a hydrophobic amino acid) (45). Several studies demonstrated that this motif is required for CD1b, c, and d internalization and access to endosomal compartments (46, 47). Therefore, we examined the reactivity of CD1d-specific CTLs to C1R line expressing the chimeric hCD1d molecules (hCD1d/a), which lacks the endosomal targeting sequence. Fig. 7 shows that the hCD1d/a transfectants could be killed as efficiently as the hCD1d transfectants, suggesting that the recognition of these CTLs did not involve endosomally derived ligands.

To gain insights into the cellular processes required to generate epitopes recognized by these anti-hCD1d CTLs, inhibitors known to interfere with discrete stages of Ag processing were used, specifically for proteasomal degradation (lactacystin) and endosomal acidification (chloroquine) requirements. As shown in Fig. 8A, neither lactacystin nor chloroquine affected the recognition of hCD1d-specific CTLs to C1R line expressing the chimeric hCD1d molecule (hCD1d/a), which lacks the endosomal targeting sequence. Fig. 7 shows that the hCD1d/a transfectants could be killed as efficiently as the hCD1d transfectants, suggesting that the recognition of these CTLs did not involve endosomally derived ligands.

Effect of various Abs on CTL response

Accessory molecules, such as ICAM/LFA-1, are known to play important roles in hCD1d-restricted NK T cell-mediated cytotoxicity (49), and therefore we examined the role of these molecules in cytotoxicity mediated by hCD1d-restricted CTLs. The require-
CTLs. The differential effect of anti-LFA-1 and anti-ICAMs Abs on hCD1d-transfected RMA-S (LFA-1+, ICAMs+) and L929 cells (LFA-1−, ICAMs−) correlated with the expression levels of LFA-1 and ICAMs on these two target cell lines (data not shown). This result indicates that an LFA-1/ICAM interaction is critical for the cytotoxicity of BN1 and BN4 for hCD1d-expressing T cell line, but not fibroblast cell lines, and the requirement for accessory molecules by BN1 and BN4 to exert CTL activity is similar. Although these CTL clones express CD8 on their cell surface, anti-CD8 Ab did not block the CTL response, suggesting the affinity of the TCRs and hCD1d is sufficient enough to trigger the CTL response.

Discussion

Unlike hCD1a, b, and c, the surface expression of hCD1d on peripheral blood monocytes is not up-regulated upon activation with inflammatory cytokines (9). This property may explain why no hCD1d-restricted CTLs have been derived to date using in vitro activated human monocytes as APCs. To overcome this limitation and to examine the functional potential of hCD1d, we have generated hCD1d Tg mice that expressed high levels of hCD1d on many cell types. We have shown that, similar to other HLA class I molecules that have been expressed in Tg mice (50), hCD1d mediates a transplantation rejection response and elicits CTLs that recognize hCD1d as alloantigen. The hCD1d-specific CTLs recognized their target Ag regardless of whether they were expressed on H-2-mismatched mouse cells or on human cells; the results suggested these CTLs recognized hCD1d molecules as restriction elements, which was confirmed by anti-hCD1d mAb blocking.

Most of the allo-specific CTLs to MHC class I molecules recognize epitopes that are dependent on both MHC molecules and specifically bound peptides (51–54). In contrast, our data showed that the recognition of hCD1d by anti-hCD1d CTLs may not involve a specific ligand, as hCD1d-specific CTLs can react with various hCD1d-expressing cells of either mouse or human origin. Thus, the hCD1d-restricted CTLs may recognize empty hCD1d molecules or, alternatively, the target could be a complex of hCD1d and a broadly distributed, conserved cellular Ag. If both BN1 and BN4 CTLs recognized empty hCD1d, one would expect that the reactivity of CTLs to different targets would entirely depend on the surface expression levels of hCD1d on the target cells; furthermore, the reactivity patterns of both CTLs against varied hCD1d-expressing cells should be the same. Although this was the case to a certain extent, differential reactivity between BN1 and BN4 to some hCD1d target cells was detected. For example, BN4 had lower reactivity to hCD1d+ bone marrow-derived macrophages and MOLT-4 cell line than BN1 at all ranges of E/T ratio tested (Figs. 5 and 6). Our Ab-blocking experiment indicated the requirement for accessory molecules is similar between BN1 and BN4 (Fig. 9). Therefore, it is most likely that differential activity between BN1 and BN4 may depend on the relative abundance of the conserved cellular ligand(s) for hCD1d on different cells. Because the recognition of hCD1d-restricted CTLs is resistant to acid denaturation and independent of TAP and proteasomal activity, it is possible that anti-hCD1d-specific CTLs may recognize nonpeptide Ags, probably cellular lipid Ags, in the context of hCD1d.

The response of hCD1d-specific CTLs to hCD1d-expressing target cells was not enhanced by addition of α-GalCer or glycosylation variants of ceramides (data not shown). These results indicated that hCD1d-specific CTLs have different ligand specificity from CD1d-restricted NK T cells. This is consistent with the finding that the hCD1d-specific CTLs did not express characteristic invariant TCR found in CD1-restricted NK T cells. Our analysis of TCR sequences of hCD1d-specific CTLs showed that all CTLs

FIGURE 8. Effect of metabolic inhibitors and acid stripping on the recognition of CD1d-restricted CTLs and H-2-M3-restricted CTLs. A, RMA-S/CD1d transfectants were treated with lactacystin, chloroquine, brefeldin A, or acid, as described in Materials and Methods, and used as targets for a CTL assay with clone BN4. B, P388 cells were treated with the indicated inhibitors and used as targets for alloreactive anti-M3 CTLs. The E:T ratios are shown in the figure. Results were comparable in two experiments.

FIGURE 9. Effect of various Abs on CTL response. CTL clones were incubated with 51Cr-labeled RMA-S/hCD1d to L929/hCD1d transfectants for 4 h in the presence of mAbs against CD8α, CD11a (LFA-1), CD54 (ICAM-1), and CD102 (ICAM-2), or medium alone.
clones derived from each individual mouse have identical DNA sequences. The homogeneity of CTL population from each individual mouse may be due to the limited TCR repertoire against hCD1d molecules and/or preferential clonal expansion during in vitro stimulation. Although the expression of hCD1d on human IEC lines, HT29, T84, and Caco2, has been demonstrated (56), we found that hCD1d-specific CTLs fail to recognize the target Ag expressed on these IEC lines (data not shown). It has been shown that the majority of hCD1d on IEC is nonglycosylated and non-β2m associated (36). Our data suggested that hCD1d-specific CTLs could distinguish the different conformational state of hCD1d molecules.

Tg mice expressing HLA class I and class II molecules have been used to provide a suitable animal model for the study of the functions of HLA molecules. The ability of hCD1d to serve as a restriction element in allogeneic recognition for CD8+ CTLs suggested that similar to group 1 CD1, hCD1d might present unique microbial Ags to CTLs. We are attempting to challenge the hCD1d Tg mice with various bacterial pathogens and examine whether the bacterial Ags to CTLs. We are attempting to challenge the hCD1d Tg mice with various bacterial pathogens and examine whether the hCD1d might play a role as a restriction element for the microbial Ags in vivo. Crossing hCD1d Tg mice onto CD1-deficient background will further provide an animal model to study the functional role of hCD1d in T cell development and immune response against infectious disease.

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