Regulation of multiple myeloma survival and progression by CD1d

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Introduction

In multiple myeloma (MM), a mostly incurable malignancy of plasma cells, survival, and growth during disease progression, in addition to accumulating intrinsic oncogenic events, also involves active cross-talk between neoplastic plasma cells with the tumor microenvironment.1,2

Human leukocyte antigen (HLA) class I and II molecules, required for the development of antmyeloma T-cell immune responses responsible for halting disease progression at early stages, are important mediators in this cross-talk.3-5 Loss of HLA molecule expression by tumor cells is a common mechanism for tumor immune evasion, survival, and eventually disease progression.6,7 CD1d, a nonconventional, glycolipid-presenting HLA class I-like molecule instructing the function of the immunoregulatory invariant NKT cells can affect tumor cell survival is not known. Here we show that CD1d is highly expressed in premalignant and early myeloma, but with disease progression its expression is reduced and eventually in advanced stages and myeloma cell lines is lost altogether, suggesting that CD1d impacts negatively on myeloma cell survival. Consistent with this, engagement of CD1d by anti-CD1d monoclonal antibodies (mAbs) induces cell death of myeloma cell lines with restored CD1d expression and primary myeloma cells. Cell death induced by monoclonal antibody engagement of CD1d is associated with overexpression of proapoptotic Bax and mitochondrial membrane potential loss but it is caspase-activation independent; in addition, it requires the cytoplasmic tail but not the Tyr residue critical for lysosomal sorting of CD1d. Finally, anti-CD1d cooperates with antimyeloma agents in the killing of myeloma cells. Thus, this work provides evidence linking a novel function of CD1d in the regulation of cell death with tumor survival and progression in humans. (Blood. 2009; 113:2498-2507)

Down-regulation of conventional human leukocyte antigen (HLA) class I and II molecules from the surface of tumor cells is an important mechanism for tumor immune evasion, survival, and progression. Whether CD1d, a nonconventional, glycolipid-presenting HLA class I-like molecule instructing the function of the immunoregulatory invariant NKT cells can affect tumor cell survival is not known. Here we show that CD1d is highly expressed in premalignant and early myeloma, but with disease progression its expression is reduced and eventually in advanced stages and myeloma cell lines is lost altogether, suggesting that CD1d impacts negatively on myeloma cell survival. Consistent with this, engagement of CD1d by anti-CD1d monoclonal antibodies (mAbs) induces cell death of myeloma cell lines with restored CD1d expression and primary myeloma cells. Cell death induced by monoclonal antibody engagement of CD1d is associated with overexpression of proapoptotic Bax and mitochondrial membrane potential loss but it is caspase-activation independent; in addition, it requires the cytoplasmic tail but not the Tyr residue critical for lysosomal sorting of CD1d. Finally, anti-CD1d cooperates with antimyeloma agents in the killing of myeloma cells. Thus, this work provides evidence linking a novel function of CD1d in the regulation of cell death with tumor survival and progression in humans. (Blood. 2009; 113:2498-2507)

CD1d possesses a cytoplasmic tail YXXZ (X: any amino acid, Z: hydrophobic amino acid) motif required for targeting surface CD1d to the endosomal compartment where loading of the definitive glycolipid ligands to the hydrophobic presentation groove takes place.20,21 In addition to endosomal sorting, the cytoplasmic tail of CD1d has been shown to be important in other processes. For example, on monoclonal antibody (mAb) ligation on the surface of myeloid or epithelial cells, CD1d is capable of initiating downstream signaling and cytokine secretion, a process that may involve Y phosphorylation of the YXXZ motif22 or activation of nuclear factor-κB (NF-κB) transcription.23 Recent work has also demonstrated that herpes viruses and HIV might be able to subvert antiviral iNKT cell–dependent immune responses by down-regulating expression of surface CD1d,24,25 either through interaction of the cytoplasmic tail with viral proteins such as Nef or through its covalent modification such as ubiquitination, representing thus another example of viral immune escape.24

Viruses often share the same mechanisms used by tumors for immune evasion and in particular down-regulation of HLA class I molecules.27 Whether down-regulation of CD1d expression as a means of tumor and especially myeloma escape also takes place is not known.

In addition to the potential for CTL-mediated induction of tumor death, HLA molecules, when they are engaged by specific mAbs, have been shown to mediate tumor programmed cell death, often in a caspase-independent manner.28,29 Although the in vivo
significance of this phenomenon is not known, it has led to the development and validation of anti–HLA class I and II mAb with promising therapeutic potential.30,31

In this study, we investigate the effect of CD1d ligation on the survival of myeloma cells and expression of CD1d during disease progression. Highlighting the role of CD1d as a myeloma antisurvival factor, we demonstrate that surface CD1d expression is significantly down-regulated during disease progression in vivo. We also find that mAb-mediated ligation of CD1d leads to death of myeloma cells, a process that is caspase-independent and requires the cytoplasmic tail of CD1d but not the Y residue of the cytoplasmic sorting motif. In addition, CD1d ligation can cooperate with other antmyeloma agents in inducing more effective killing of myeloma cells.

Methods

Patient samples and myeloma cell selection

Collection of bone marrow samples from patients with MM was approved by the Hammersmith Hospitals NHS Trust Research Ethics Committee, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. After density gradient centrifugation on Ficoll, primary myeloma cells were positively selected from the lymphomononuclear cell fraction, using CD138-conjugated magnetic beads (MiniMACS; Miltenyi Biotec, Auburn, CA) and LS and MS columns from the same provider.

Cell culture and retroviral transduction

All myeloma cell lines were a kind gift of Professor Junia Melo, Imperial College (London, United Kingdom). Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (all from Invitrogen). The same forward primer 5′-GGAATTCGCCAAAATGCTAC-3′ (Invitrogen) was used for polymerase chain reaction (PCR) amplification of all 3 CD1d constructs. The reverse primers were as follows: for full-length CD1d cDNA 5′ (PCR) amplification of all 3 CD1d constructs. The reverse primers were as follows: for full-length CD1d cDNA (and reverse 5′-GGAATTCGCCAAAATGCTAC-3′ (Invitrogen)). The same forward primer 5′-GGAATTCGCCAAAATGCTAC-3′ (Invitrogen) was used for polymerase chain reaction (PCR) amplification of all 3 CD1d constructs. The reverse primers were as follows: for full-length CD1d cDNA (and reverse 5′-GGAATTCGCCAAAATGCTAC-3′ (Invitrogen)).

To generate retroviral plasmids containing various human CD1d cDNA constructs, the human CD1d-coding region was amplified from a pCΔN3 plasmid carrying the full-length CD1d using high-fidelity Taq polymerase (Invitrogen). The same forward primer 5′-GGCCCAAGATCTGAGTCGATCT-GGATATGGGGTGCCTGC-3′ was used for polymerase chain reaction (PCR) amplification of all 3 CD1d constructs. The reverse primers were as follows: for full-length CD1d cDNA 5′-GTTCCTGTCAGCAGTCACAG-GACGCCCTGTAGG-3′; for full-length CD1d cDNA in which the cytoplasmic tail sorting motif Y was changed to A was 5′-GGCCCAAGATCTGAGTCGATCT-GGATATGGGGTGCCTGC-3′; and for CD1d with truncated cytoplasmic tail was 5′-GTTCCTGTCAGCAGTCACAG-GACGCCCTGTAGG-3′. PCR products were subcloned into the pBABE-hygro retroviral vector using BamH1 and Sal1 as restriction-enzyme cloning sites. All constructs were confirmed by direct sequencing. To establish myeloma cell lines expressing CD1d variants, CD1d retroviral plasmids were transfected into the Phoenix packaging cell line using Lipofectamine 2000 (Invitrogen). The supernatants containing retroviruses were collected 48 hours after transfection and after filtration through a 0.45-μm filter were applied to KMS11 cells in the presence of 8 μg/mL polybrene. After transduction and hygromycin selection (750 ng/mL) for 2 weeks, CD1d expression was confirmed by flow cytometry using the mouse anti–human CD1d 42.1 antibody.

Antibodies, flow cytometry, and reagents

Mouse anti–human CD1d mAb clone 42.1, CD138–fluorescein isothiocyanate (FITC), CD38–allophycocyanin (APC), CD45–peridinin chlorophyll protein (PerCP), and IgG isotypic controls were from BD Pharmingen (San Diego, CA). Mouse anti–human CD1d mAb clone 51.1 supernatant was a kind gift of Dr S Porcelli, Albert Einstein Medical School (New York, NY). CD1d expression on primary myeloma cells was performed by staining total bone marrow mononuclear cells in a 2-stage staining procedure, using unconjugated CD1d 42.1 and phycoerythrin (PE)–conjugated goat anti–mouse Fab (Dako North America, Carpenteria, CA) secondary antibody. Dexamethasone 21-acetate was from Sigma-Aldrich (St Louis, MO); caspase inhibitors were from Biovision (Mountain View, CA).

RT-PCR

Total RNA was extracted using the RNeasy RNA Extraction Kit (Qiagen, Valencia, CA). First-strand cDNAs were reversely transcribed from 0.5 μg RNA using superscript II first-strand cDNA synthesis kit (Invitrogen) following the manufacturer’s instructions. For PCR amplification of CD1d, the forward 5′-GGAATTCGCCAAAATGCTAC-3′ and reverse 5′-TTGGCGTTCACCTGGCTCTT-3′ primers were used under the following conditions: 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, for a total of 35 cycles.

Cell death assays

Annexin-propidium iodine staining. KMS-11 cells expressing various CD1d constructs and C1R-CD1d were plated in 48-well plates at 10⁵ cells per well. Cells were cultured with different concentrations of unconjugated anti-CD1d 42.1, IgG1 isotypic control, or medium at the indicated concentrations. At different time points, cells were stained with annexin V fluorescein isothiocyanate together with propidium iodine (Pharmingen, San Jose, CA) and analyzed in a FACSCalibur flow cytometer (BD Biosciences). C1R-CD1d cells were subjected to the same assays using a second anti–human CD1d clone 51.1 mAb as well as the anti-CD1d 42.1. Where applied, caspase inhibitors were added at 20 μM into the cells 1 hour before CD1d antibody. Because in some samples of primary myeloma cells spontaneous cell death at 24 hours was more than 50%, data presented are for assays in which spontaneous cell death was less than 50%.

Detection of mitochondrial potential Δψm depolarization by DiOC3 staining. After treatment with anti-CD1d or isotypic controls, cells were stained with DiOC3 (MitoProbe; Invitrogen) according to the manufacturer’s instructions followed by flow cytometric analysis.

Western blot analysis

Myeloma cell lines and primary myeloma cells were lysed in RPMI lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with protease inhibitors: 40 μg of lysates after sonication was mixed with reducing sample buffer (Laemmli buffer; Bio-Rad, Hercules, CA; plus β-mercaptoethanol), boiled at 100°C for 5 minutes, loaded in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted in polyvinylidene difluoride membranes. The following antibodies were used: GAPDH, anti–Bcl-xL (clone 54H6), anti-Bax, anti-Bad, anti-Bid, anti-Puma, anti–cleaved caspase-8 (clone 18C8), anti–caspase-9 (clone C9), and appropriate secondary horseradish peroxidase (HRP)–conjugated antibody (from Cell Signaling Technology, Danvers, MA); anti-CD1d (clone NOR3; Serotec, Oxford, United Kingdom). Films were developed by chemoluminescence using ECL Developing Kit (GE Healthcare, Little Chalfont, United Kingdom).

Metaphase fluorescent in situ hybridization

Myeloma cell lines and primary myeloma cells were lysed in RPMI lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with protease inhibitors: 40 μg of lysates after sonication was mixed with reducing sample buffer (Laemmli buffer; Bio-Rad, Hercules, CA; plus β-mercaptoethanol), boiled at 100°C for 5 minutes, loaded in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted in polyvinylidene difluoride membranes. The following antibodies were used: GAPDH, anti–Bcl-xL (clone 54H6), anti-Bax, anti-Bad, anti-Bid, anti-Puma, anti–cleaved caspase-8 (clone 18C8), anti–caspase-9 (clone C9), and appropriate secondary horseradish peroxidase (HRP)–conjugated antibody (from Cell Signaling Technology, Danvers, MA); anti-CD1d (clone NOR3; Serotec, Oxford, United Kingdom). Films were developed by chemoluminescence using ECL Developing Kit (GE Healthcare, Little Chalfont, United Kingdom).
Expression of CD1d in myeloma cell lines

It was previously reported that, unlike primary myeloma cells, which are CD1d+, expression of CD1d in 3 myeloma cell lines (MCLs) was not detectable by flow cytometry. To investigate this further, we studied expression of CD1d in a set of another 4 MCLs by reverse-transcribed polymerase chain reaction (RT-PCR) as well as flow cytometry (Figure 1).

Of 4 MCLs studied, only line U266 expressed low-level surface CD1d by flow cytometry compared with the CD1d-transduced CIR-CD1d cell line, whereas the other 3 were almost completely negative (Figure 1A) and by RT-PCR, CD1d expression could only be demonstrated in U266 cells (Figure 1B). Taken together, these findings suggested that loss of CD1d expression in the other 3 MCL is at a pretranscriptional level, possibly at the level of genomic DNA.

To address this, we used FISH analysis on all MCLs using a CD1d BAC as a probe. Because CD1d is known to map within the region of chromosome 1q, which is commonly amplified in MM, it is possible that the rearrangement events leading to 1q amplification may also give rise to simultaneous submicroscopic deletions. Using the sequenced BAC clone RP11-404O13, which spans the CD1d gene locus, we screened the cell lines for loss of this genomic region. We found that the chromosome number of all cell lines was approximately 70; however, at least 4, and up to 10, hybridization signals from the 404O13 BAC were observed. Signals were usually seen within marker chromosomes containing large regions of chromosome 1, resulting from structural abnormalities, such as complex unbalanced translocations (Figure 1C). These data exclude large deletions as possible causes for lack of expression of CD1d in these MCLs. They would, however, be consistent with smaller deletions or point mutations not identifiable by FISH or epigenetic silencing of CD1d preceding its genomic amplification, hence the absent CD1d expression by RT-PCR in MCLs.

Expression of CD1d in primary myeloma cells ex vivo and in situ

Because MCLs are usually derived from patients with end-stage or extramedullary disease, loss of CD1d expression may represent an event associated with disease progression. To address this, we studied expression levels of CD1d on bone marrow plasma cells (identified as CD138+CD38+CD45+low/n−) from patients with monoclonal gammopathy of undetermined significance (MGUS), a premalignant form of MM (n = 8), patients with newly diagnosed MM (n = 18), and patients with relapsed/advanced (n = 17) disease by flow cytometry (Figure 2A). Using the Geo MFI CD1d/isotypic IgG1 ratio (to account for the day-to-day variability of the isotype control) as a measure of surface expression, we found that, in MGUS and newly diagnosed patients, CD1d expression varied but was always detectable and overall similar between the 2 groups (MGUS: Geo MFI ratio mean ± SD, 8.61 ± 4.3 vs new MM 7.1 ± 4.72, P > .05). By contrast, in patients with relapsed/advanced disease, expression of CD1d was considerably lower (Geo MFI ratio, 1.95 ± 0.9) than in MGUS (P = .003) and newly diagnosed (P = .001) patients and displayed much less variation per patient (Figure 2B). Analysis of CD1d expression by immunoblotting in some representative cases showed considerable reduction of total CD1d in CD138-purified myeloma cells from relapsed compared with newly diagnosed disease and MGUS patients (Figure 2C).

We investigated further the dynamics of surface CD1d expression at different phases of the disease using immunohistochemistry. Expression of CD1d in available bone marrow trephine biopsies or biopsy from extramedullary disease from 13 patients was assessed in a blinded fashion by intensity of staining (0-3) and localization (ie, whether predominantly membranous or cytoplasmic; Table 1; Figure 2D).

Statistical analysis

Data are shown as mean plus or minus SEM, analyzed using an unpaired Student t test. Wilcoxon rank test was used for comparing cell death in patient samples treated or not with anti-CD1d. The level of significance was set at P less than .05.
Advanced disease compared with MGUS (plasmacytoma). Hematopoietic stem cell transplantation) disease were stained with anti-CD1d; in P6, therapy. For patients P5, P6, P8, and P11, paired bone marrow trephine biopsies from control. (D) Assessment of CD1d expression in myeloma cells by immunohistochemistry. Figure 2. CD1d expression in primary myeloma cells ex vivo and in situ. (A) Surface expression of CD1d in bone marrow myeloma cells from patients in different stages of the disease was assessed by multicolor flow cytometry. Histograms are gated on plasma cells identified as CD138 CD38 CD45 (dotted line represents isotype; continuous line, CD1d). (B) CD1d expression as assessed by the Geo MFI CD1d/isotypic IgG1 ratio (to account for the day-to-day variability of the isotype control) in MGUS (n = 8), newly diagnosed (n = 18), and relapsed patients (n = 17). Geo MFI CD1d IgG1 ratio was similar between MGUS and newly diagnosed patients (P > .05), but considerably lower in patients with relapsed/advanced disease compared with MGUS (P < .01) and newly diagnosed patients (P < .01). (C) Western blot analysis of CD1d in plasma cells selected with CD138 immunomagnetic beads from a MGUS, a newly diagnosed and a patient with terminal disease. Thus, in 69% of 9 of 13) of patients, loss of membranous expression of CD1d was observed in advanced disease.

Three patterns were observed in terms of relative intensity of cytoplasmic (C) and membranous (M) staining, that is, C > M, C = M, or M > C; in some samples, both C and M staining were absent altogether.

Using this semiquantitative approach, we found that, in 4 patients (P1-P4), an initial C > M pattern did not change from diagnosis/early disease to disease progression samples; whereas in 7 of 13 patients (P6-P8, P10-P13), a C > M, M > C, or C = M pattern changed to almost complete lack of both C and M staining. In P5 and P9, there was reduction or loss of M but not of C staining. Interestingly, in P6 where 3 trephine biopsy samples from different stages of the disease were available, decreased M but preservation of C was followed by complete lack of M as well as C staining in terminal disease. Thus, in 69% (9 of 13) of patients, loss of membranous expression of CD1d was observed in advanced disease.

Taken together, these findings show that myeloma progression is associated with decreased surface CD1d expression. In advanced disease, such as extramedullary relapse or relapse postautologous and allogeneic hematopoietic stem cell transplantation (HSCT), in a manner similar to the MCL, expression of CD1d may be lost altogether suggesting that surface CD1d might be linked to myeloma cell survival.

Ligation of CD1d induces cell death in B and plasma cells

To test its role in regulating cell survival, we first studied the effect of engaging CD1d on the surface of the C1R-CD1d B lymphoblastoid cell line using 2 different anti-CD1d mAbs (clones 42.1 and 51.112). We found that both mAbs induced significant surface dissipase/tolerase exposure (ie, annexin+ compared with IgG or medium control as assessed by PI/annexin staining (Figure 3A,B). This effect was not observed with the CD1d–C1R cell line (ie, the parental line of C1R-CD1d), nor did it require ligation by a secondary mAb (data not shown), and was associated with higher rate of mitochondrial membrane potential (MMP) loss as assessed by DiOC3 staining (ie, DiOC3– cells) and flow cytometry (Figure 3C). In addition, anti-CD1d mAb adsorbed on protein A agarose beads triggered considerably higher cell death than adsorbed isotypic IgG, suggesting that anti–CD1d–induced cell death does not involve Fc receptors (data not shown). To study the effect of CD1d ligation on myeloma cell survival, we restored CD1d expression in the CD1d-negative MCL KMS11 by retroviral transduction of the full-length human CD1d cDNA (see Figure 6A bottom). Using anti-CD1d 42.1 to ligate surface CD1d and without secondary mAb, we found a dose- as well as time-dependent induction of cell death of KMS11-CD1d, that is, annexin+PI cells (Figure 3D,E), an effect associated with loss of MMP (Figure 3F). We also found that anti-CD1d induces apoptosis of the Ramos lymphoma B-cell line, which spontaneously expresses CD1d as well as on CD1d-expressing primary human monocytes (data not shown).

Next, we assessed induction of cell death in primary myeloma plasma cells PC from 5 patients with newly diagnosed and 5 patients with relapsed disease in whom CD1d expression was detectable on myeloma cells as assessed by flow cytometry (Figure 3G). In all cases, treatment of purified myeloma cells with 5 µg/mL anti-CD1d resulted in higher rate of cell death than treatment with medium alone, although the magnitude of the effect varied. The mean plus or minus SEM increase of cell death induced by anti-CD1d compared with control was 63% plus or minus 11.7%. No correlation between extent of cell death and level of CD1d expression was found (r² = 0.081). The reason for this is not clear, but lack of correlation between HLA level of expression and cell death has been also observed with the anti–HLA class I diabody remain 2D7,29,34 and may reflect the increased affinity of the corresponding mAb. Therefore, consistent with a role of CD1d in myeloma cell survival, its ligation on the surface of myeloma cell lines and primary myeloma cells induces cell death.

CD1d-induced apoptosis is associated with cell aggregation and is caspase-independent

In many cases, apoptosis induced by monoclonal antibodies used to ligate surface molecules is characterized by cell aggregation. We found that ligation of CD1d in the surface of the KMS11-CD1d cell line also induces considerable cell aggregation compared with KMS11-vector control (Figure 4A).
As discussed above, CD1d-induced apoptosis of KMS11-CD1d as well as C1R-CD1d was associated with loss of MMP. Maintenance or loss of MMP is a process directly regulated by Bax and Bak, 2 proapoptotic members of the Bcl-2 family, and these in turn are under the control of the prosurvival Bcl-2 family proteins (Bcl-2, BclxL, Mcl-1) and proapoptotic BH3-only proteins (Bim, Bid, Bad, Puma, and Noxa).35 Using immunoblotting, we tested the involvement of some of these proteins in CD1d-induced cell death. Of the prosurvival Bcl-2 family proteins, after CD1d ligation and over a period of 24 hours, the levels of Bcl-xL and Bcl-2 did not change from baseline, ie, cells not treated with anti-CD1d (Figure 5B; and data not shown), nor was there a significant change in the BH3-only proteins Bim, Bid, Bad, and Puma. However, CD1d ligation induced a significant increase in the expression of Bax (Figure 4B), implying that loss of MMP might be the result of increased expression of Bax.

Caspases are cysteine proteases required for induction of apoptosis, a form of programmed cell death that proceeds through loss of MMP.36 For example, after activation of the extrinsic pathway of apoptosis, cleavage of caspase-8 leads to MMP loss, whereas caspase-9, cleaved as a result of MMP loss, participates in the formation of the apoptosome and eventually cleavage of caspase-3, the final caspase effector of apoptosis (Figure 4C). We tested whether CD1d-dependent cell death after ligation with anti-CD1d results in caspase activation, but we found no evidence of either caspase 8 or 9 cleavage, suggesting that CD1d-induced cell death is caspase-independent. Confirming this, specific (ie, anticaspase 1, 2, 3, 5, 6, 8, and 9) and general (Z-V AD) caspase inhibitors failed to reduce anti-CD1d-induced cell death (Figure 4D).

Overall, these findings suggest that cell death after CD1d ligation is associated with increased expression of Bax and loss of MMP but does not require caspase activation.

**CD1d structural requirements for induction of apoptosis**

CD1d similar to conventional HLA class I molecules possesses a cytoplasmic tail containing a YXXZ motif required for AP2/3

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**Table 1. CD1d expression in myeloma cells as assessed by immunohistochemistry**

<table>
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<th>Patient No./year</th>
<th>Disease status/tissue</th>
<th>Score</th>
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<td>1</td>
<td>C &gt; M</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>1998 Post-chemo/BM</td>
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</tr>
<tr>
<td>5</td>
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<td>C &gt; M</td>
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<td>6</td>
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<td>18</td>
<td>2008 Relapse post-chemo and --auto-HSCT</td>
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HSCT indicates hematopoietic stem cell transplantation; BM, bone marrow; and None; staining not higher than background.

*C indicates cytoplasmic staining; and M, membranous staining.
adaptor binding and trafficking to the cell surface to the endosomal compartment for ligand exchange and loading.\textsuperscript{37,38} The Y residue of this motif has been shown to be phosphorylated after CD1d ligation leading to secretion of IL-12 by a human monocytic cell line, an effect that is abrogated when the CD1d tail is exchanged for that of CD1a.\textsuperscript{22} We hypothesized that the Y residue of the YXXZ motif in mediating CD1d-induced cell death could be the result of somatic mutations in the cytoplasmic tail affecting the endoplasmic reticulum–cell surface–endosome-cell surface trafficking of CD1d or posttranslational covalent modification of the cytoplasmic tail. Consistent with the latter, it has been shown that the Kaposi sarcoma herpes virus MHR2 protein through its ubiquitin ligase activity could ubiquitinate a tail K residue of human CD1d, resulting in its accelerated endocytosis and reduced cell surface expression.\textsuperscript{24} As suggested by our combined fluorescence-activated cell sorter, RT-PCR, and FISH analysis, the drastic loss of CD1d expression is more drastic and at pretranscriptional level. The mechanism of loss of surface CD1d expression and its primarily cytoplasmic localization in relapsed disease could be the result of somatic mutations in the cytoplasmic tail affecting the endoplasmic reticulum–cell surface–endosome-cell surface trafficking of CD1d or posttranslational covalent modification of the cytoplasmic tail.

**Discussion**

This is the first work to link CD1d with tumor survival and progression in humans. Our ex vivo and in situ findings clearly showed a correlation of loss of surface CD1d expression with myeloma progression. The combined studies of CD1d expression at genomic, transcriptional, and protein level suggest that predominantly cytoplasmic rather membranous localization could be one possible mechanism for reduced surface CD1d expression (eg, P5 in Figure 2D); whereas in more advanced and end-stage disease as represented by extramedullary relapse, for example, skin plasmacytoma and the MCLs, loss of expression is more drastic and at pretranscriptional level. The mechanism of loss of surface CD1d expression and its primarily cytoplasmic localization in relapsed disease could be the result of somatic mutations in the cytoplasmic tail affecting the endoplasmic reticulum–cell surface–endosome-cell surface trafficking of CD1d or posttranslational covalent modification of the cytoplasmic tail. Consistent with the latter, it has been shown that the Kaposi sarcoma herpes virus MHR2 protein through its ubiquitin ligase activity could ubiquitinate a tail K residue of human CD1d, resulting in its accelerated endocytosis and reduced surface expression.\textsuperscript{24} As suggested by our combined fluorescence-activated cell sorter, RT-PCR, and FISH analysis, the drastic loss of CD1d expression in MCLs probably resulted from the presence of microdeletions in the CD1d gene or its epigenetic silencing, which took place before genomic amplification of CD1d. An interesting feature disclosed by studying CD1d expression in primary tumor cells in situ in serial trephine biopsy samples was the identification of focal areas of surface CD1d expression loss next to areas of predominantly surface expression (P6, Figure 2D middle panel), followed by complete loss of all CD1d expression in terminal disease (P6, Figure 2D middle panel).
bottom panel), a finding consistent with surface CD1d loss being associated with clonal progression.

These findings strongly suggested that loss of CD1d expression favors tumor survival and progression. Two functional properties of CD1d might be important in this process. The first is its ability to activate iNKT cells through glycolipid ligand presentation and interaction with the invariant Vα24Jo18 T-cell receptor (TCR). Activated iNKT cells are able to enhance experimental antitumor T-cell responses, a property that is critically dependent on their ability to secrete rapidly large quantities of cytokines.

Figure 4. CD1d ligation induces cell aggregation, up-regulation of Bax, and caspase-independent cell death. (A) Treatment with anti-CD1d of KMS11-CD1d but not KMS11-vector cells induces cell aggregation. (B) Immunoblotting for various Bcl-2-related proteins at the indicated time points after treatment of the KMS11-CD1d cell line with anti-CD1d 42.1 mAb. (C) Immunoblotting for cleaved caspase 8 and 9. No difference in cleaved caspase 8 products compared with baseline was observed after treatment of KMS11-CD1d with anti-CD1d for the indicated time points; similarly, no cleaved caspase 9 higher than background was observed after treatment with anti-CD1d. (D) Cell death induced by CD1d ligation of KMS11-CD1d is not inhibited by various caspase inhibitors. Data shown in all panels are representative of 3 independent experiments with similar results.

Figure 5. CD1d cytoplasmic tail structural requirements for CD1d-induced cell death. (A) The CD1d-negative KMS11 myeloma cell line was transduced with empty retroviral vector only, full-length of human CD1d cDNA, full-length CD1d cDNA in which the Y residue of the YXXZ motif was mutated to A (CD1dY→A) or cDNA encoding CD1d with truncated cytoplasmic tail (CD1dTr). CD1d expression by flow cytometry (top) and immunoblotting (bottom) is shown. (B) Role of cytoplasmic tail in myeloma cell aggregation. Aggregation of KMS11-CD1d, KMS11-CD1dY→A, and KMS11-CD1dTr cells at 12 hours after treatment with anti-CD1d or IgG control. (C) Cell death of KMS11-vector only, KMS11-CD1d, KMS11-CD1dY→A, and KMS11-CD1dTr cells as detected by annexin V at 24 hours after treatment with anti-CD1d: P < .05 for KMS11-CD1d vs KMS11-CD1dY→A at 24 and 36 hours; P < .01 for KMS11-CD1d vs KMS11-CD1dTr at 24 and 36 hours; n = 5. (D) Mitochondrial membrane potential loss shown as DiOC3- cells in KMS11-vector only, KMS11-CD1d, KMS11-CD1dY→A, and KMS11-CD1dTr cells after treatment with anti-CD1d. Data shown represent mean plus or minus SEM of 3 independent experiments.
amounts of IFN-γ. Consistent with this, tumor bed and peripheral blood iNKT cells from myeloma patients have been shown to lose their ability to secrete IFN-γ with disease progression. Further highlighting its potential role in antitumor immune responses, CD1d expressed in human myeloid as well as lymphoid tumors has been also shown to activate directly tumoricidal iNKT cells in the presence of the potent glycolipid ligand alpha-galactosylceramide in vitro. Yet another potential mechanism that may compromise the CD1d-iNKT cell axis of immunosurveillance and antitumor immune responses was suggested in a mouse model of lymphoma whereby tumor-derived glycolipids were shown to interfere with productive CD1d-iNKT cell interaction. Our findings suggest that loss of CD1d expression may constitute another means for compromising activation and thus the direct or indirect antimyeloma effect of iNKT cells, contributing further to tumor immune escape.

A second pathway through which loss of CD1d could favor tumor survival might relate to its ability for downstream signaling after ligation by mAb, as it was demonstrated in previous work. Using the same strategy and in keeping with a role of CD1d in myeloma cell survival, we demonstrated that mAb ligation of CD1d on myeloma cells induces cell death. Investigation of CD1d-triggered cell death revealed several important features: cell aggregation, increased Bax expression, MMP loss, lack of caspase activation, and specific CD1d structural requirements.

Downstream signaling after CD1d ligation has been shown to require the cytoplasmic tail and the Tyr residue of the YXXZ motif. We found that CD1d-induced cell death was dependent on the whole tail but not on the Tyr residue. The same structural requirements were shown for the interaction of the HIV protein Nef with CD1d, resulting in the surface down-regulation of the latter. Although the potential partners and the specific signal transduction pathways involved downstream of CD1d leading to cell death remain to be identified, we found that MMP loss is linked to increased expression of Bax, a proapoptotic member of the Bcl-2 family of proteins, which along with Bak is crucial for regulating MMP and thus different forms of cell death. Biochemical and pharmacologic analyses showed this process to be caspase-independent.

Caspase-independent, but MMP loss- and cell aggregation-dependent, cell death triggered by mAb targeting surface and specifically HLA molecules has been previously observed. For example, a diabody specific for the α2 domain of HLA class I was shown to induce caspase-independent, but cell aggregation-dependent, cell death. The latter required activation of Rho GTPase family of proteins and actin polymerization. Similarly, mAb against the α2 domain of murine major histocompatibility complex class I (MHC I) was also shown to induce caspase-independent, actin polymerization- and cell aggregation-dependent cell death of activated T cells. Earlier work showed that mAb against the α3 domain of HLA class I can also induce caspase-independent cell death, whereas Pettersen et al found that anti-HLA A2-induced, caspase-independent death of lymphoid cells was cytoplasmic tail-independent. By contrast to anti-HLA class I heavy chain-specific mAb, myeloma cell death induced by a series of mAb specific for β2m were found to be caspase-dependent.

Therefore, similar to the effect of anti–HLA class I mAb, anti-CD1d–induced cell death is caspase-independent and is associated with cell aggregation, although we have not addressed whether the latter is strictly required for cell death induction. Unique to CD1d, cell death and aggregation were largely dependent on the presence of the CD1d cytoplasmic tail but did not require Tyr residue of the YXXZ sorting motif. As the caspase-independent cell death induced by anti-CD1d is associated with MMP loss, downstream effectors could include mitochondrial enzymes, such as apoptosis-inducing factor (AIF) and Endoglin G. On MMP loss, AIF and Endoglin G have been shown to translocate to the nucleus where they can directly induce chromatin condensation and DNA fragmentation. Indeed, AIF was shown to mediate the caspase-independent cell death effect of Hu1D10 (apolizumab), an anti-HLA DR mAb currently tested in clinical trials.

Finally, while this work was under revision, it was reported by Ozeki and Shively that engagement of CD1d by anti-CD1d (clone 42.1) on the promyelocytic cell line HL60 induces considerable apoptosis, thus independently corroborating our findings.

What might be the relevance of CD1d-depentent tumor cell death in vivo? It is possible that it reflects a CD1d-iTCR interaction that is not only unidirectional toward activating iNKT cells and consequently their ability to directly exert a cytotoxic effect on tumor cells through effectors such as perforin and granzymes or Fas-FasL, but it could also involve generation of death-inducing signals downstream of CD1d, dependent on its tail. Alternatively,

Table 2. Human CD1d retroviral constructs used in this study

<table>
<thead>
<tr>
<th>Retroviral constructs</th>
<th>Full-length</th>
<th>Y &gt; A</th>
<th>Truncated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic tail amino acids are shown in italics.</td>
<td>FLLLGVFTSRFKRTSAGQVL</td>
<td>FLLLGVFTSRFKRTSAGQVL</td>
<td>FLLLGVFT</td>
</tr>
</tbody>
</table>
CD1d, like other nonconventional major histocompatibility complex–like molecules, such as HFE and neonatal FcR, might have acquired additional functions, which do not require it interacting with TCR but with other yet to be defined ligands. In line with this, there is evidence of a low-affinity NK-cell receptor interacting with CD1d.

We also showed that CD1d can cooperate with other antmyeloma agents in inducing more effective cell death raising the prospect of targeting myeloma or other CD1d-expressing tumors therapeutically using anti-CD1d mAb alone or in combination with other antitumor agents. However, such an approach should probably be intended for early myeloma because, in advanced stages, loss of CD1d expression might lead to selection of therapy-refractory disease.

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References


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Authorship

Contribution: E.S., M.H., E.T., V.M., A. Reid, L.K., and K.N. performed research; A.K., A. Rahemtulla, A. Reid, S.A., and K.N. designed and supervised research and analyzed data; and A.K. wrote the manuscript with contributions from all coauthors.

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Regulation of multiple myeloma survival and progression by CD1d

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