

# The Requirement for DNAM-1, NKG2D, and NKp46 in the Natural Killer Cell-Mediated Killing of Myeloma Cells

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## Abstract

Recent evidence suggests a role for natural killer (NK) cells in the control of multiple myeloma. We show that expression of the NK cell receptor DNAM-1 (CD226) is reduced on CD56<sup>dim</sup> NK cells from myeloma patients with active disease compared with patients in remission and healthy controls. This suggested that this receptor might play a role in NK-myeloma interactions. The DNAM-1 ligands Nectin-2 (CD112) and the poliovirus receptor (PVR; CD155) were expressed by most patient myeloma samples analyzed. NK killing of patient-derived myelomas expressing PVR and/or Nectin-2 was DNAM-1 dependent, revealing a functional role for DNAM-1 in myeloma cell killing. In myeloma cell lines, cell surface expression of PVR was associated with low levels of NKG2D ligands, whereas cells expressing high levels of NKG2D ligands did not express PVR protein or mRNA. Furthermore, NK cell-mediated killing of myeloma cell lines was dependent on either DNAM-1 or NKG2D but not both molecules. In contrast, the natural cytotoxicity receptor NKp46 was required for the killing of all myeloma cell lines analyzed. Thus, DNAM-1 is important in the NK cell-mediated killing of myeloma cells expressing the cognate ligands. The importance of NKp46, NKG2D, and DNAM-1 in myeloma killing mirrors the differential expression of NK cell ligands by myeloma cells, reflecting immune selection during myeloma disease progression. [Cancer Res 2007;67(18):8444–9]

## Introduction

Multiple myeloma, a plasma cell malignancy, is the second most common hematologic malignancy in the Western world. Thalidomide shows promise in myeloma therapy and this treatment is associated with increased numbers of natural killer (NK) cells with greater cytotoxic activity (1). This suggests that NK cells might play a role in the control of myeloma, and strategies that harness NK cells might provide new avenues of therapy. Defining the molecular interactions between NK cells and myeloma cells is a key step toward achieving this goal.

NK cells recognize tumor cells via changes in the target cell surface phenotype. This can occur either through the reduced expression of inhibitory ligands, such as MHC class I, or the

presence of activating ligands, such as those that bind to NKG2D or the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46 (2). Activation signals delivered by NKG2D and the NCRs have been implicated in tumor recognition and killing (2, 3). Expression of NKG2D ligands is largely confined to infected cells and tumors. However, cellular ligands of the NCRs are poorly defined. Functional studies showed that MHC class I, NKG2D, and NCR molecules play a role in myeloma cell killing and that myeloma expression of MHC class I and NKG2D ligands is shaped by immune selection (4).

The cell surface receptor DNAM-1 (CD226) has also been implicated in the recognition of target cells by cytotoxic cells (5). DNAM-1 has two known ligands: Nectin-2 (CD112) and the poliovirus receptor (PVR), also known as Nectin-like molecule 5 or CD155 (6, 7). Unlike NKG2D ligands, DNAM-1 ligands are expressed by healthy cells (7, 8). Nevertheless, DNAM-1 regulates the NK cell killing of several tumor types, including those of hematologic origin (5–7, 9–11). However, the role of DNAM-1 in NK cell-myeloma interactions is currently unknown.

Studies of other malignancies have shown that changes in NK cell receptor expression reflect their functional role in NK-tumor interaction (12, 13). We searched for NK cell receptors whose expression was altered by myeloma disease activity. Our results indicate a role for the DNAM-1 molecule in NK-myeloma interactions.

## Materials and Methods

**Patients.** Fresh peripheral blood lymphocytes were collected from patients with active myeloma ( $n = 31$ ; ages 41–88 years), myeloma patients with complete response to therapy ( $n = 15$ ; ages 43–84 years), and healthy individuals ( $n = 11$ ; ages 37–88) and used immediately. Blood and bone marrow samples were collected following ethical approval and informed consent.

**Antibodies and proteins.** The following antibodies were used for flow cytometry: CD56 (allophycocyanin-labeled clone B159; BD Biosciences), CD3 [phycoerythrin (PE)/Cy5-labeled OKT3; conjugated in house], DNAM-1 (PE-labeled DX11; PharMingen), CD138 (PE-labeled Mi15; PharMingen), CD38 (FITC-labeled HIT2; PharMingen), PVR [TX21 (MBL International) or PV404.19 (Beckman Coulter)], Nectin-2 (R2.525; PharMingen), CD96/TACTILE (NK92.39; HyCult Biotechnology), NKG2D (PE-labeled 1D11; PharMingen), and NKp46 (9E2/nkp46; PharMingen). NKG2D ligand expression was detected using an NKG2D-Fc fusion protein (R&D Systems) with a PE-conjugated human Fc-specific secondary antibody (The Jackson Laboratory). For receptor blocking, we used unconjugated antibodies from the clones listed above against DNAM-1, NKG2D, CD96, NKp46, and an isotype control.

**DNAM-1 expression on NK cells and T cells.** Flow cytometry was used to analyze expression of a panel of markers on CD56<sup>dim</sup>CD3<sup>neg</sup> and CD56<sup>bright</sup>CD3<sup>neg</sup> NK cell subsets (14) and CD56<sup>neg</sup>CD3<sup>+</sup> T cells from the

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peripheral blood of myeloma patients and controls. This panel included CD94, KIR molecules, CD16, CD161, HLA-DR, CD57 (data not shown), and DNAM-1 (Fig. 1). Cells were stained with antibodies against CD56, CD3, and DNAM-1 and expression was determined on NK subsets and T cells using the strategy shown in Fig. 1. The position of the gates for the CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets was confirmed using CD16 staining. The percentage of cells expressing a particular marker and the geometric mean of fluorescence (as a measure of expression density) were analyzed using Statistical Package for the Social Sciences (SPSS) v.14.0.2 (SPSS, Inc.). The Mann-Whitney (nonparametric) test was used to compare the groups. Myeloma cells express CD56 but were not present in the peripheral blood of the patients analyzed. In addition, DNAM-1 expression was absent from patient-derived myelomas and cell lines (data not shown).

**Purification and flow cytometric analysis of patient myeloma samples.** All plasma cells in myeloma bone marrow had a neoplastic phenotype as judged by flow cytometry, in keeping with diagnostic criteria (data not shown). RBCs from bone marrow aspirates (1–3 mL) were lysed using ammonium chloride (0.86%, w/v) and myeloma cells were selected using anti-CD138 magnetic beads (Miltenyi Biotech) and CD138, CD38, PVR, and Nectin-2 expression was determined using flow cytometry. More than 95% of the purified cells expressed CD138 and CD38.

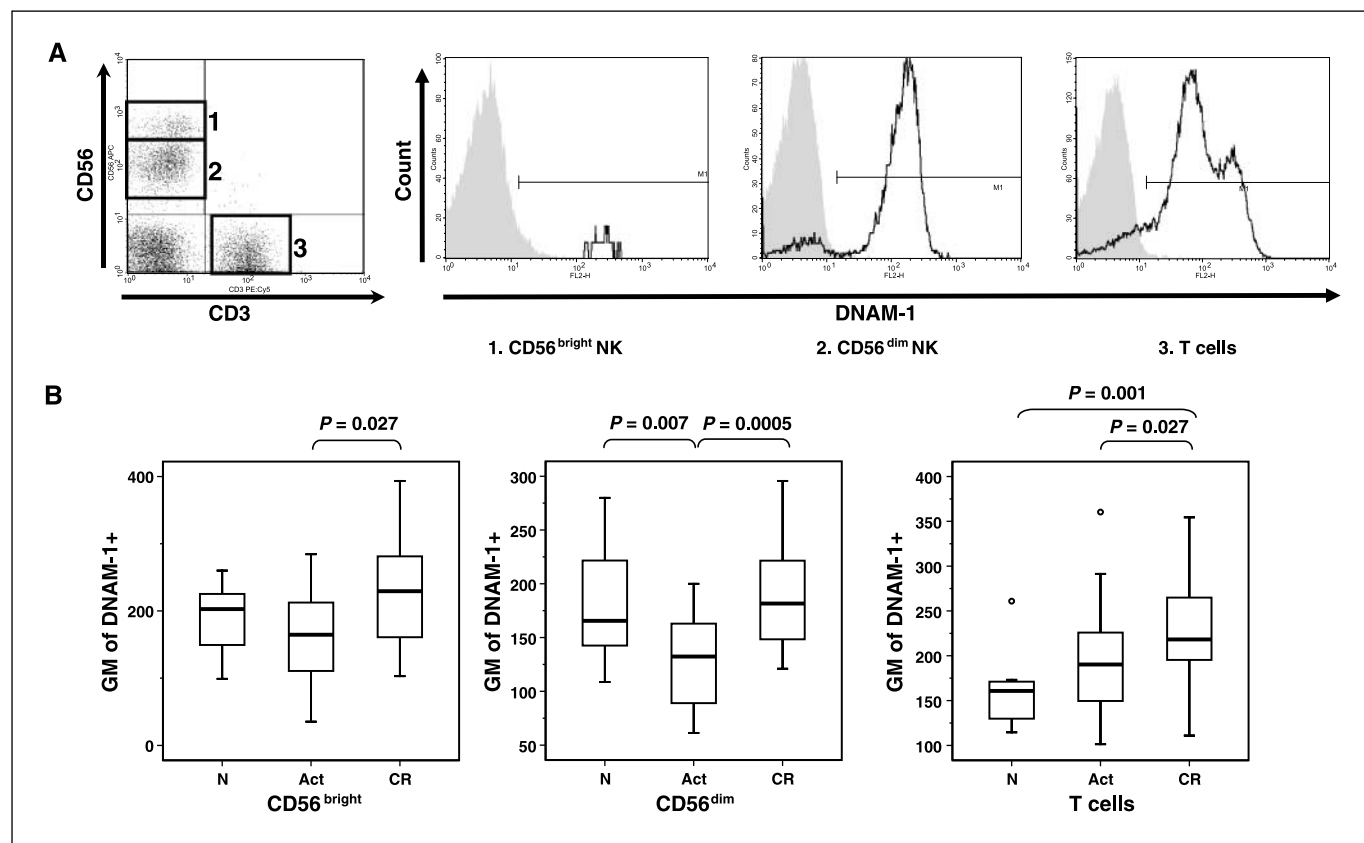
**NK cell killing assays.** Peripheral blood NK cells from healthy donors were isolated using indirect magnetic selection (Miltenyi Biotech), activated with 100 units/mL interleukin-2 (IL-2) for 5 to 7 days, and used in killing

assays as described (15) using an E:T ratio of 5:1. For blocking experiments, activated NK cells were preincubated with or without 30 µg/mL blocking antibody or isotype control for 30 min at room temperature before coculture with targets. Inhibition of killing was calculated as a percentage of the inhibition observed with the isotype control antibody. Isotype controls inhibited killing by <5%.

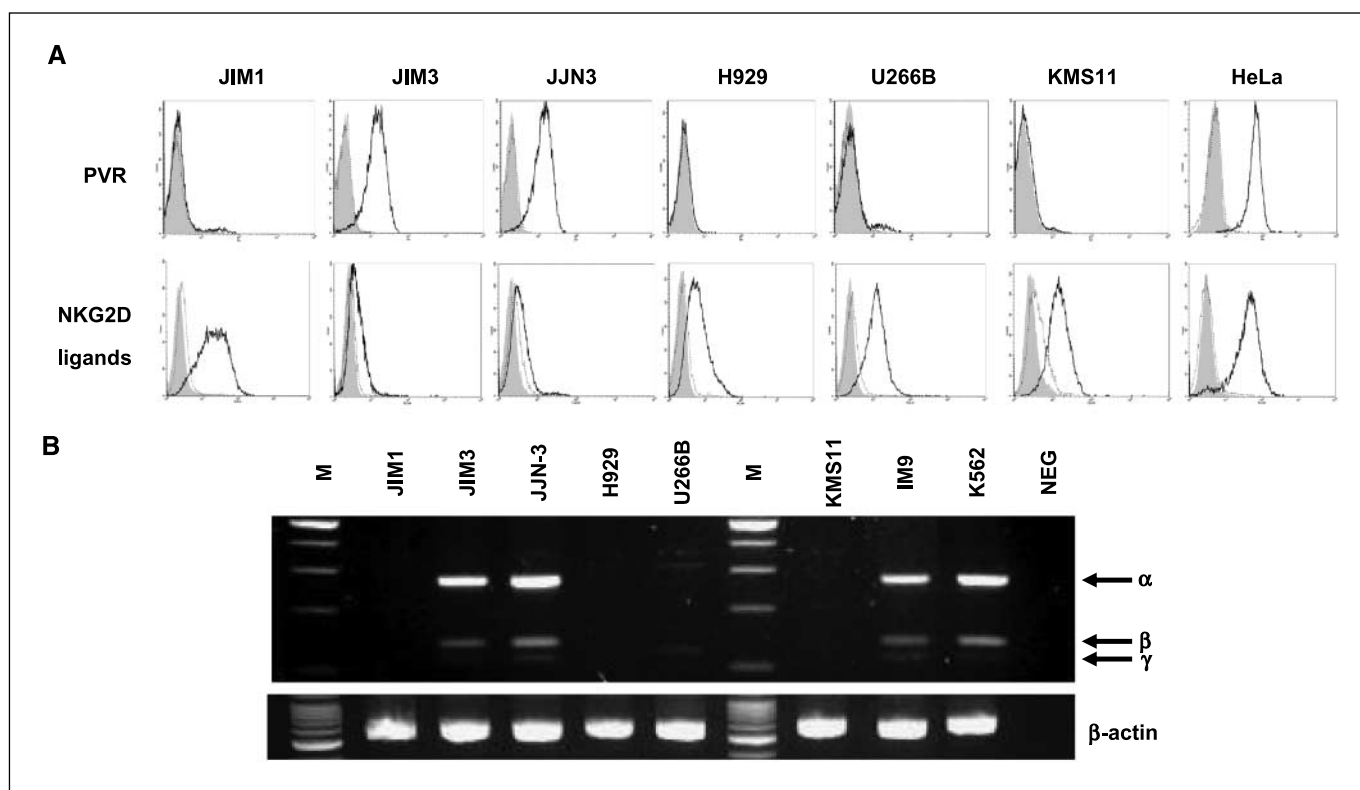
**Cell lines and tumor cell phenotyping.** Cell lines KMS11, U266B, JIM1, JIM3, and NCI-H929 originate from myeloma patients, whereas JIN3 is from a plasma cell leukemia (16). IM9, a lymphoblastoid cell line (16), HeLa, and K562 were used as controls. Cell lines were analyzed for expression of Nectin-2, PVR, and NKG2D ligands by flow cytometry. PVR transcripts were detected as described previously (8).

## Results

**NK cell expression of DNAM-1 in myeloma patients.** We analyzed expression of NK cell receptors in myeloma patients with active disease, myeloma patients with complete response to therapy, and healthy donors. Among a panel of receptors (described in Materials and Methods), DNAM-1 showed significant changes in expression (Fig. 1). More than 85% of CD56<sup>dim</sup>CD3<sup>neg</sup> and CD56<sup>bright</sup>CD3<sup>neg</sup> NK cells and CD3<sup>+</sup> T cells expressed DNAM-1 in all three sample groups (an example is shown in Fig. 1A).



**Figure 1.** DNAM-1 expression in patients with active myeloma compared with patients in remission. *A*, strategy for analysis of DNAM-1 expression on NK cell subsets and T cells in patients and controls. Fresh peripheral blood lymphocytes were stained with antibodies against CD56, CD3, and DNAM-1 (as described in Materials and Methods). *Left*, expression of DNAM-1 was analyzed on CD56<sup>bright</sup>CD3<sup>neg</sup> and CD56<sup>dim</sup>CD3<sup>neg</sup> NK cells and CD56<sup>neg</sup>CD3<sup>+</sup> T cells using gates 1, 2, and 3, respectively. *Solid line*, expression of DNAM-1 on these individual subsets; *gray-filled areas*, isotype control antibody staining on whole lymphocytes. More than 85% of cells in gates 1, 2, and 3 express DNAM-1; the differences in the size of DNAM-1-expressing populations in the two NK cell subsets reflects the bright to dim NK cell ratio in peripheral blood (~1:10). This analysis was done on the patient and control samples. Demarcation between CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets was confirmed by analysis of CD16 expression (14), the CD56<sup>dim</sup> cells being CD16<sup>high</sup> whereas CD56<sup>bright</sup> cells have a CD16<sup>low</sup> phenotype (data not shown). *B*, comparison of DNAM-1 expression in the patient and control groups. Plots (generated using SPSS v.14.0.2) show the range of data values obtained. Thirty-one patients with active myeloma (*Act*; ages 41–88 y) were compared with 15 patients with complete response to therapy (*CR*; ages 43–84 y) and 11 healthy donors (*N*; ages 37–88 y). *Top and bottom whiskers*, values of the top and bottom 25% of the cases, respectively; *boxed area*, interquartile range and the significant *P* values between groups; *horizontal black line*, median value; *circles*, outlying values (as defined by SPSS).



**Figure 2.** Expression of PVR and NKG2D ligands by myeloma cell lines. *A*, myeloma cell lines and controls were analyzed for cell surface expression of PVR and NKG2D ligands by flow cytometry. HeLa cells (a cervical carcinoma cell line) were used as a positive control for PVR and NKG2D ligand expression. *Solid line*, PVR and NKG2D ligand staining; *gray areas*, unstained cells; *dotted lines*, isotype controls. *B*, expression of PVR transcripts. The position of products encoding the transmembrane ( $\alpha$ ) and secreted forms ( $\beta$  and  $\gamma$ ; ref. 8) is indicated. The EBV-transformed lymphoblastoid cell line IM9 and the erythroleukemic cell line K562 were used as control cell lines. Analysis of actin transcripts was used as a control for RNA quality and cDNA synthesis. *NEG*, no added template.

Interestingly, DNAM-1 expression density was reduced on the CD56<sup>dim</sup>CD3<sup>neg</sup> NK subset of patients with active disease compared with patients with complete response to therapy and controls (Fig. 1). This NK cell subset has the most potent cytotoxic activity (14). Changes in DNAM-1 expression were also observed in CD56<sup>bright</sup>CD3<sup>neg</sup> NK cells (believed to have a cytokine-producing role) and T cells. The apparent reduction of DNAM-1 expression levels in the CD56<sup>dim</sup>CD3<sup>neg</sup> NK subset in active myeloma suggested that DNAM-1 might functionally interact with myeloma cells. We did functional studies to test this hypothesis.

**Expression of NK cell receptor ligands and killing of myeloma cell lines.** We first used a panel of myeloma cell lines to investigate the role of DNAM-1 in myeloma cell killing. Two of the six myeloma cell lines expressed cell surface PVR (JIM3 and JIN3; Fig. 2A), whereas Nectin-2 was undetectable (data not shown). The expression of cell surface PVR was mirrored at the mRNA level and transcripts encoding cell surface and secreted forms of PVR (8) were detected (Fig. 2B). Interestingly, the PVR-expressing cell lines (JIM3 and JIN3) expressed low levels of cell surface NKG2D ligands, whereas the cell lines lacking PVR expression showed much higher levels of NKG2D ligand expression (Fig. 2A).

Killing assays were then done using IL-2-activated NK cells (to maximize cytotoxicity) in conjunction with receptor blocking antibodies (Fig. 3A). The PVR-expressing myeloma lines were killed in a DNAM-1-dependent, NKG2D-independent manner, whereas myeloma lines expressing NKG2D ligands were killed in an NKG2D-dependent but DNAM-1-independent manner. In contrast, killing of the lymphoblastoid cell line IM9, which

expresses PVR and NKG2D ligands, was dependent on both receptors (Fig. 3A). Recently, Carlsten et al. (11) showed that resting NK cells could kill PVR-expressing ovarian carcinoma cells. In agreement with these data, JIM3 (PVR<sup>+</sup>) was killed (albeit with reduced efficiency) in a DNAM-1-dependent manner by resting NK cells, whereas U266B (PVR<sup>neg</sup>) was resistant to resting NK cells (data not shown).

The PVR molecule also binds to CD96, a receptor expressed by IL-2-activated NK cells and T cells (7, 17). NK cell surface expression of CD96 was induced by IL-2, whereas DNAM-1 was constitutively expressed (Fig. 3B). Expression of NKG2D and Nkp46 was also increased by IL-2 activation (Fig. 3B). However, an antibody that blocks CD96-PVR interactions (17) did not inhibit the killing of JIM3 and JIN3 (Fig. 3C). Furthermore, no additional inhibition was observed when blocking antibodies against DNAM-1 and CD96 were combined. In contrast, all of the myeloma cell lines tested were killed in an Nkp46-dependent manner (Fig. 3D).

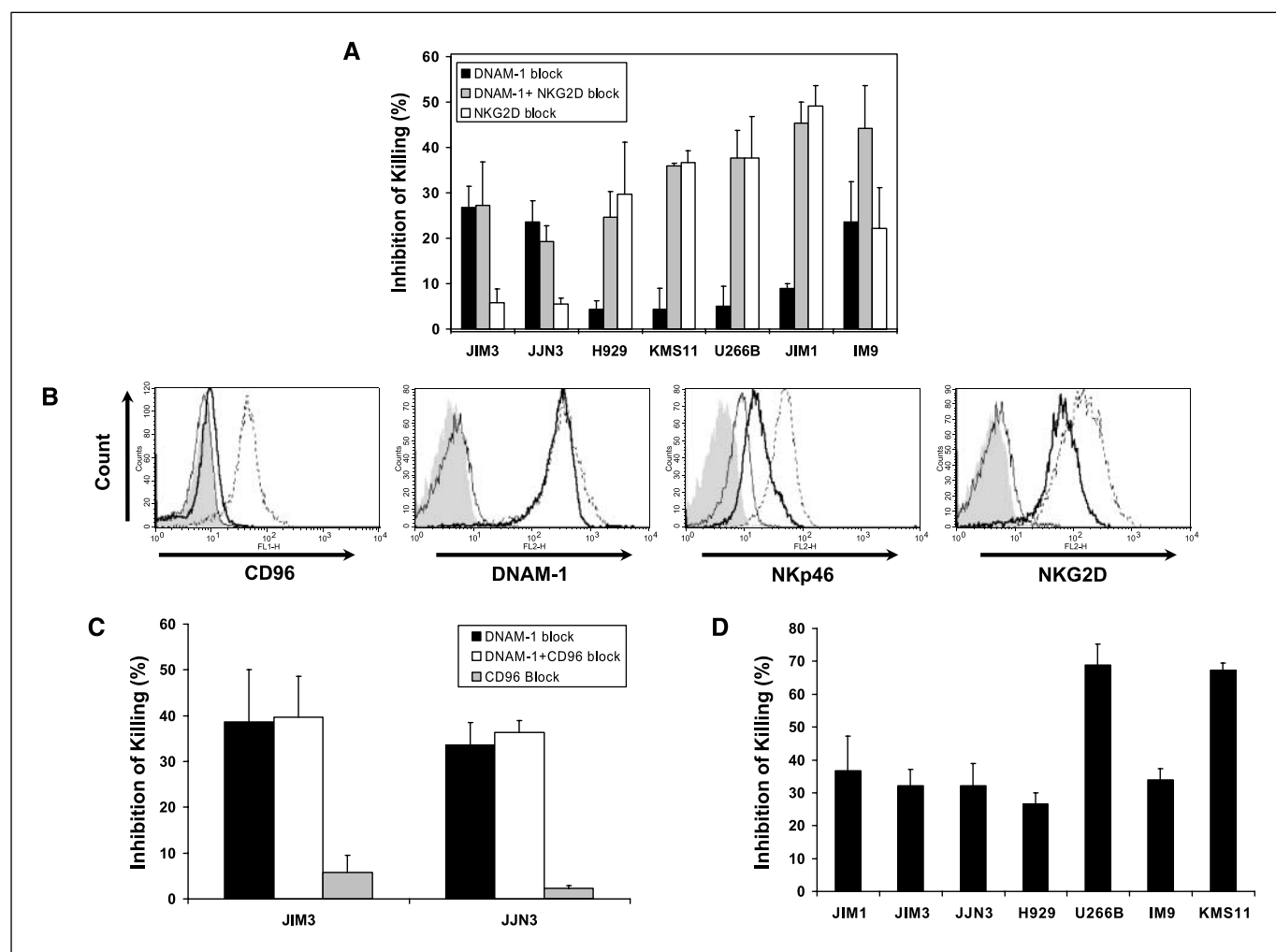
**DNAM-1 is required for the NK cell-mediated killing of patient-derived myeloma cells.** Functional studies were then extended to patient-derived myeloma cells from bone marrow samples. Analysis of 12 patient myeloma samples revealed PVR and/or Nectin-2 expression on all but two samples. Data from four patients (P1, P2, P3, and P4) are shown in Fig. 4A. Nonneoplastic plasma cells from two normal bone marrow samples showed expression of Nectin-2 (data not shown) in agreement with the widespread expression of this molecule (7). These results indicated that DNAM-1 ligands were expressed by patient-derived myeloma cells at varying levels.

The bone marrow–derived myeloma samples P1 to P4 (Fig. 4) were used in killing assays. Killing of myeloma P1, which lacked DNAM-1 ligands, was DNAM-1 independent (Fig. 4B). However, this sample yielded sufficient myeloma cells to do additional assays and was killed in an NKG2D- and NKp46-dependent manner (data not shown), similar to the myeloma cell lines that lacked DNAM-1 ligand expression (Fig. 3). DNAM-1 ligands were expressed by myeloma samples P2, P3, and P4 and killing of these three myelomas was DNAM-1 dependent (Fig. 4B). Thus, patient-derived myeloma samples confirm observations made with the myeloma cell lines and reveal that DNAM-1 is important in the NK cell-mediated killing of myelomas expressing cognate ligands.

## Discussion

These results establish a functional role for the DNAM-1 molecule in the NK cell-mediated killing of myeloma cells. We

focused our attention on the DNAM-1 molecule because this receptor showed reduced expression density on the CD56<sup>dim</sup> NK cell subset of myeloma patients with active disease compared with patients with complete response to therapy and healthy donors (Fig. 1). This subset of NK cells possesses potent killing activity (14), reflecting the role of DNAM-1 in cytotoxic function (5–7, 9–11). There was no significant difference in DNAM-1 expression between healthy controls and patients with complete response to therapy in this subset. To the best of our knowledge, this is the first time that DNAM-1 expression has been analyzed in the context of malignant disease. In other malignancies, changes in NK receptor expression are mediated by the shedding of soluble NK receptor ligands (12) and receptor down-regulation following target contact (13). Furthermore, tumor-derived cytokines can down-regulate NK cell receptor expression (18). Tumor burden impedes NK cell development (19) and the presence of both myeloma cells and developing NK cells in the bone marrow suggests that myeloma



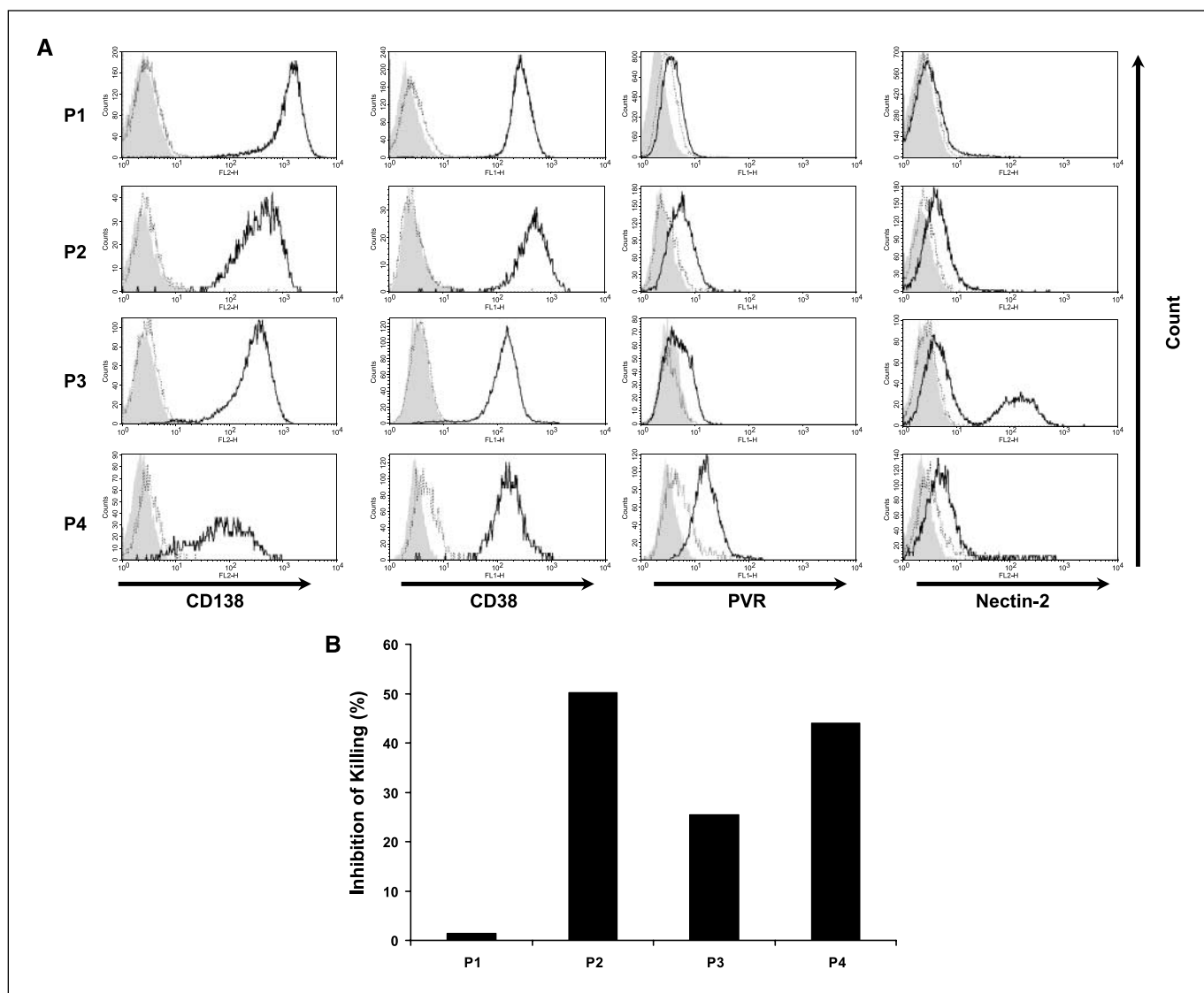
**Figure 3.** NK cell killing of myeloma cell lines. *A*, role of DNAM-1 and NKG2D in killing of myeloma cell lines. The graph shows inhibition of killing expressed as a percentage of the killing observed with the isotype control antibody (which gave <5% inhibition compared with no added antibody). The blocking antibodies used are depicted in the key. *B*, NK cell receptor expression by unstimulated and IL-2-activated NK cells. Expression of CD96, DNAM-1, NKp46, and NKG2D was analyzed on unstimulated NK cells (thick solid line) and NK cells were activated with 100 units/mL IL-2 for 7 d (dotted line). Gray-filled area, unstimulated cells; thin solid line, isotype control stain. *C*, comparison of DNAM-1 and CD96 blocking antibodies in killing of PVR-expressing myeloma cell lines. The assay shows the inhibition of killing of JIM3 and JLN3 cells expressed as a percentage of killing observed with the isotype control antibody. *D*, role of NKp46 in killing of myeloma cell lines. The graph shows the inhibition of killing expressed as a percentage of killing observed with the isotype control antibody for six myeloma cell lines and the lymphoblastoid cell IM9. All of the killing assay data shown in Fig. 3 are from single donors, with experiments done using NK cells purified on at least three separate occasions. Columns, mean inhibition of killing; bars, SD. Results were confirmed using other donors.

cells may influence NK cell differentiation. We are currently investigating the mechanism of changes in DNAM-1 expression and whether altered expression has functional consequences. Soluble PVR molecules may play a role in this process (Fig. 2B; ref. 8).

We speculated that the differences in DNAM-1 expression in the different patient and control groups (Fig. 1) reflected a functional role for DNAM-1 in myeloma. Most patient-derived myeloma cell samples and some myeloma cell lines expressed DNAM-1 ligands (Figs. 2 and 4). Not surprisingly, only those myeloma cells that express PVR and/or Nectin-2 were killed in a DNAM-1-dependent manner. Interestingly, one patient sample (P1) and four myeloma cell lines lacked DNAM-1 ligands and were killed in a DNAM-1-independent but NKG2D-dependent manner. Previous studies have revealed that differential expression of MHC class I and NKG2D

ligands by myelomas reflects immune selection (4). The differential requirement for DNAM-1 and NKG2D shown here is also suggestive of immune selection events that have shaped the myeloma cell surface phenotype.

In contrast to the differential requirements for DNAM-1 and NKG2D, all of the myeloma cell lines were killed in an NKp46-dependent manner (Fig. 3D). In the previous study, simultaneous blocking of all three NCR molecules was shown to inhibit myeloma cell killing (4). Our results identify NKp46 as playing an important role in this process. However, we were unable to show a role for CD96 in the killing of PVR-expressing myeloma cells despite strong induction of CD96 by IL-2 (Fig. 3B and C). Similarly, DNAM-1, but not CD96, was shown to be important in the killing of ovarian carcinoma cells (9).



**Figure 4.** The role of DNAM-1 in killing of patient-derived myeloma cells. *A*, cell surface phenotype of patient-derived myeloma cells. Myeloma cells were magnetically selected from bone marrow aspirates using anti-CD138 beads (as described in Materials and Methods). Selected cells were >95% pure based on expression of CD138 and CD38. Purified cells were then analyzed for expression of PVR and Nectin-2. Plots show unstained cells (gray-filled area), isotype controls (dotted line), and the test antibody (solid line). Four patient samples (P1, P2, P3, and P4) are shown; samples P2 to P4 expressed PVR and Nectin-2, whereas sample P1 did not express either molecule. *B*, NK cell-mediated killing of patient-derived myeloma cells. Myeloma samples P1 to P4 were used as targets in the presence of a blocking antibody against DNAM-1 (or an isotype control antibody). Data are expressed as the percentage inhibition of killing compared with the isotype control. Insufficient numbers of myeloma cells were recovered from P2 to P4 to do additional assays, whereas sample P1 yielded sufficient myeloma cells to do additional killing assays and was killed in an NKG2D- and NKp46-dependent manner (data not shown).

The importance of DNAM-1 in tumor immune surveillance has been shown *in vivo* using mouse models (20). The data presented here support a role for this NK receptor in the killing of myeloma cells. Defining the molecular interactions between NK cells and tumor cells, and understanding how this is altered by malignant disease progression, will contribute to the design of immunotherapeutic strategies, which tip the balance in favor of antitumor immune responses rather than immune evasion.

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