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DNAM-1/CD155 Interactions Promote Cytokine and NK Cell-Mediated Suppression of Poorly Immunogenic Melanoma Metastases

Christopher J. Chan,*†,1 Daniel M. Andrews,*†,1 Nicole M. McLaughlin,* Hideo Yagita,‡ Marco Colonna,§ and Mark J. Smyth*†

A role for NK cells in therapeutic intervention for hematologic malignancies, such as acute myeloid leukemia and multiple myeloma, and nonhematologic malignancies, such as melanoma, is becoming more apparent. DNAM-1 is an NK cell receptor whose importance in facilitating activation signals received by NK cells in natural and cytokine-driven responses to tumor metastases in vivo is poorly explored. In this study, we used matched tumor lines expressing a variety of relevant ligands, neutralizing monoclonal Abs, and DNAM-1 gene-targeted mice to determine the relative importance of DNAM-1-ligand interactions in controlling tumor metastases. Our results demonstrate that NK cells require DNAM-1 for natural or cytokine (IL-2, IL-12, or IL-21) suppression of tumor metastases or their variants expressing CD70 or CD80. In contrast, DNAM-1 was dispensable when tumor cells were targets of Ab-dependent cellular cytotoxicity or presented ligands for NKG2D. CD155 appeared to be a key ligand recognized by DNAM-1 in NK cell-mediated suppression of metastases, and DNAM-1-mediated suppression coincided with perforin activity. Overall, these data implied a general role for DNAM-1-CD155 interactions in NK cell-mediated killing of tumors, even in the presence of tumor CD70 or CD80 expression, and further defined the optimal efficacy requirements of cytokines that directly activate NK cells. The Journal of Immunology, 2010, 184: 902–911.

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artual killer cells have a predisposition to spontaneously kill cancer cells and their metastases (1, 2). When NK cells are adoptively transferred or activated in situ by cytokines, their clinical utility in selected malignancies, such as acute myeloid leukemia and malignant melanoma, is becoming apparent (3–5). Haploidentical NK cells can persist and expand in vivo and may have a role in the treatment of selected malignancies used alone or as an adjunct to hematopoietic stem cell transplantation (6). NK cell allogrecognition has been translated into the clinical practice of allogeneic hematopoietic transplantation, and it has opened innovative perspectives in the cure of leukemia. Amplifying the antitumor effect of NK cells by selecting donor/recipient mismatches in specific HLA or using Abs to reduce the inhibitory effect of killer Ig-like receptors (7) is not the only possible consideration. An alternative approach is to augment the antitumor effect of NK cells, or cytokines that mobilize NK cells, by selecting patients whose tumors express key ligands that provoke or costimulate NK cell activation.

NK cells require adhesion molecules for their migration and to interact with their targets (8). It has been shown that adhesion molecules can act independently, initiating intracellular pathways that trigger activation and adhesiveness of NK cells, or they may act in conjunction with activating receptors to optimize cellular contacts and NK cell effector function. NK cells express a range of adhesion molecules, such as integrins and Ig superfamily molecules. DNAX accessory molecule (DNAM-1; CD226) is one such Ig superfamily adhesion molecule. In humans, DNAM-1 is normally expressed on NK cells and CD8 T cells, as well as CD4 T cells and monocytes (9). In mice, there is variable expression of DNAM-1 on NK cells ranging from 25–50%; however, all CD8 T cells constitutively express DNAM-1. In addition, NKT cells, γδ T cells, and CD4 T cells express DNAM-1 when activated (10, 11). DNAM-1 binds to CD155 (Nect-5 or PVR) and CD112 (nectin-2), both of which have been found to be upregulated on tumors. In vitro studies have shown that DNAM-1 triggers NK cell-mediated killing of a range of tumor cells expressing CD155 and CD112 (12–16). DNAM-1 also facilitates NK cell interactions with dendritic cells that express CD155 (11, 17).

Because CD155 is expressed on many tumor cells, DNAM-1 has been implicated as a major trigger for NK cell antitumor activity (12–16). However, NK cells express additional receptors that mediate antitumor activity. NK cells can trigger Ab-dependent cellular cytotoxicity (ADCC) via CD16 (FcγIII) (18). NG2D triggers NK cell surveillance against tumor cells expressing stress-induced MHC I-related ligands, such as MIC-A, MIC-B, and ULBP in humans and Rael1, H60, and Multi1 in mice (19–21). Natural cytotoxicity receptors (NCRs), such as NKP46, NKP44, and NKP30, have also been shown to mediate an important role in antitumor activity (22). The interaction of NK cell CD27 and CD28 with CD70 and CD80, respectively, on tumor cells has also been shown...
to enhance their antitumor activity (23, 24). The relative contribution of DNAM-1 in the context of cytokine activation and these other NK cell receptors has not been addressed in vivo.

Our recently published study using DNAM-1-deficient mice suggested that NK cells require DNAM-1 for the elimination of tumor cells that lack NKG2D ligands (25). We have significantly broadened this study to evaluate the relative importance of DNAM-1 in the natural and cytokine-induced antitumor activity of NK cells against parental and engineered melanoma to express a range of NK cell-relevant ligands both in vitro and in vivo.

Materials and Methods

**Mice**

C57BL/6 wild-type (WT) mice, pfp−/−, IFN-γ−/−, pfp−/− x IFN-γ−/− (26) and DNAM-1-deficient (DNAM-1−/−) mice (25) were bred and maintained at the Peter MacCallum Cancer Centre. All gene-targeted mice were backcrossed to C57BL/6 at least 10 generations. All experiments were performed on mice 6–16 wk old and were approved by the Peter MacCallum Cancer Centre Animal Care Committee.

**Cell lines**

B16F10, B16, B16-Rae-1, B16-CD70, and B16-CD70 tumor cells were maintained in RPMI 1640 supplemented with 10% FCS. Stable B16 infected to express ligands were generated by retroviral infection using a murine stem cell virus plasmid as previously described (23).

**Abs and reagents**

The mAb 480.1 anti-mouse DNAM-1 (mDNAM-1) and mAb 4.24 anti-mouse CD155 were generated as previously described (25). The anti-mouse NKG2D mAb (CH7) hybridoma was a kind gift from Dr. Wayne Yokoyama (Washington University School of Medicine, St. Louis, MO) (27). The anti-NKG2D or anti–DNAM-1 mAbs do not deplete NK cells in vivo (data not shown).

**Results**

Cytokine suppression of B16F10 lung metastases requires DNAM-1

The cytokines IL-2, IL-12, and IL-21 all have been shown to mediate antitumor activities against either B16F10 or other experimental tumors in an NK cell-dependent fashion (30–32). Using doses of each that we had established as effective, we first evaluated the antitumor activity of IL-2, IL-12, and IL-21 against B16F10 lung metastases in WT and DNAM-1−/− mice (Fig. 1A). Whereas each cytokine significantly suppressed metastases in WT mice (from 125.5 ± 4.4 [PBS] versus 34.2 ± 6.4 [IL-2], 43.8 ± 7.5 [IL-12], 32.4 ± 3.9 [IL-21]), these cytokines were less effective in mice deficient in DNAM-1 (138.6 ± 3.8 [PBS] versus 75.0 ± 6.4 [IL-2], 86.6 ± 5.3 [IL-12], 83.7 ± 4.3 [IL-21]). The results in the DNAM-1−/− mice were also supported by the comparative effect of a neutralizing anti–DNAM-1 mAb in WT mice compared with an irrelevant control mAb (Fig. 1B). Overall these data indicated that cytokine-mediated suppression of B16F10 lung metastases were in part DNAM-1-dependent.

IL-2 mediated suppression of B16F10 lung metastases requires early NK cell and DNAM-1/CD1D55 activity

Given the similar role of DNAM-1 in cytokine-mediated suppression of B16F10 metastases, we focused our remaining experiments on the effects of IL-2. In support of our previous studies that had shown that the anti-B16F10 activities of IL-12 and IL-21 were NK cell-dependent (31, 32), we next illustrated that IL-2 suppression of B16F10 lung metastasis was NK cell-dependent in both WT and DNAM-1−/− mice (Fig. 2A). The high dose of B16F10 generated high numbers of lung colonies (mean >115) in all groups of mice treated with PBS regardless of genotype (WT, DNAM-1−/−) or cell depletion (control Ig [cIg], anti-CD8, anti-CD11c; Fig. 2A). By contrast, IL-2 was only optimally effective in WT and DNAM-1−/− mice where NK cells were intact (cIg- or anti-asGM1; Fig. 2A). In the absence of a mouse with DNAM-1 specifically deleted from NK cells, we next determined whether NK cells and DNAM-1 were acting at the same time (Fig. 2B). Clearly when the neutralization of DNAM-1 or depletion of NK cells was delayed until day 3, there was no significant change in B16F10 lung metastases, indicating that both IL-2–driven host NK cells and DNAM-1 acted early (in the first 48 h after tumor inoculation).

DNAM-1 is reportedly to interact with two ligands, CD155 (Necl-5 or PVR) and CD112 (nectin-2) (12, 33). B16F10 tumor cells express both CD155 and CD112 (25). To determine whether DNAM-1–CD155 interactions were critical in IL-2–mediated suppression of B16F10 lung metastases, we used a neutralizing anti-
mice (each symbol) and mean tumor colonies counted under a dissecting microscope. Data are depicted as individual (250 mice/group of B6 WT mice were treated with either cIg or anti-mouse DNAM-1 i.p. on days 1, 0, 1, and 2, or 500 ng IL-12 i.p. on days 1 and 0. B. Some groups of B6 WT mice were treated with either clg or anti-mouse DNAM-1 (250 μg i.p. on days −1, 0, and 7 after tumor inoculation). Lungs were harvested on day 14 and fixed in Bouin’s solution. Tumor colonies were counted under a dissecting microscope. Data are depicted as individual mice (each symbol) and mean tumor colonies ± SEM (cross bar) for each group. Statistical differences between treated WT and DNAM-1−/− mice or clg-treated and anti-DNAM-1−/− treated WT mice are depicted by asterisks. Mann-Whitney U test, p < 0.05.

mouse CD155 mAb in WT and DNAM-1−/− mice (Fig. 2C). Anti-CD155 mAb reduced the efficacy of IL-2 in WT mice and indeed increased B16F10 metastases to a level similar to that observed in IL-2–treated DNAM-1−/− mice. In contrast, anti-CD155 mAb had no effect in DNAM-1−/− mice (Fig. 2C). WT and DNAM-1−/− mice treated with PBS demonstrated equivalent levels of B16F10 lung metastases regardless of whether they received clg or anti-CD155. We did not have a neutralizing anti-mouse CD112 mAb available, but from these data it would appear that CD155 is a critical ligand for DNAM-1-dependent suppression.

**DNA-M-1-mediated suppression correlates with perforin function**

IL-2 suppresses many experimental tumors by an NK cell-dependent mechanism that requires perforin and IFN-γ (30, 34). The NK cell effector mechanisms used by IL-2 to control B16F10 have not yet been reported. Choosing a dose of B16F10 that metastasizes equivalently in WT, pfp−/−, IFN-γ−/−, and pfp−/− × IFN-γ−/− mice (Fig. 3A), we show that IL-2–mediated suppression of B16F10 lung metastases requires both pfp and IFN-γ (Fig. 3B). Surprisingly, anti-DNAM-1 treatment further enhanced metastases in IFN-γ−/− mice, but not pfp−/− mice, suggesting that DNAM-1 was required for IL-2 activated pfp-mediated cytotoxicity, but not critical for IL-2 driven IFN-γ antitumor activity.

**NKG2D-mediated suppression of B16-Rae-1 does not require DNAM-1**

NKG2D is a primary activation receptor used by NK cells to detect and eliminate tumor cells (35). We have previously shown it to be important in NK cell and perforin suppression of metastases mediated by IL-2 (34). B16-Rae-1 expresses the Rae-1 ligand for NKG2D, whereas B16 and B16F10 do not and 36). We next used parental B16 and B16-Rae-1 tumor cells to determine whether natural and IL-2 driven NK cell suppression of metastases required host DNAM-1. Both B16 and B16-Rae-1 expressed DNAM-1 ligands CD112 and CD155 in a similar manner to B16F10 tumor cells (Fig. 4). To examine natural host suppression, WT mice were inoculated with doses of B16-Rae-1−/− (Fig. 5A) and B16 (Fig. 5B) that resulted in low number of metastases—a 2-fold higher dose of B16-Rae-1 was required to achieve that purpose. When WT mice were concurrently treated with depleting anti-asGM1 Ab, significantly enhanced numbers of B16-Rae-1 and B16 metastases were detected (Fig. 5A, 5B), indicating that host NK cells naturally controlled the metastasis of both tumors. Not surprisingly, when WT mice were concurrently treated with neutralizing anti-NKG2D mAb, significantly enhanced numbers of B16-Rae-1- and B16 metastases were detected (Fig. 5A, 5B), indicating that in the presence of a functional NKG2D pathway DNAM-1 function was redundant. Genetic loss of host DNAM-1 similarly increased B16 metastases, with anti-NKG2D having no additional effect (Fig. 5B). A combination of anti-NKG2D and anti-DNAM-1 totally accounted for NK cell suppression of B16-Rae-1 metastases, suggesting that in the absence of NKG2D signaling, an effect of DNAM-1 was revealed (Fig. 5C).

**CD27- and CD28-mediated suppression of tumors requires DNAM-1**

CD70 and CD80 are important costimulatory receptors used by NK cells to help interact with target cells (37, 38). We have previously shown that NK cell CD27 and CD28 can interact with CD70 and CD80, respectively, on tumor cells to enhance their antitumor activity (23, 24). Parental B16 does not express the ligands for CD27 and CD28, so we next used parental B16, B16-CD70, and B16-CD80 tumor cells to determine whether IL-2–driven NK cell suppression of metastases required host DNAM-1. B16, B16-CD70, and B16-CD80 expressed DNAM-1 ligands CD112 and CD155 in a manner similar to B16F10 tumor cells (Fig. 4). To examine IL-2–mediated suppression of these tumor cells, WT mice were inoculated with a high dose of B16-CD70 (Fig. 6A) and B16-CD80

![Figure 1](http://www.jimmunol.org/) Cytokine suppression of B16F10 lung metastases requires DNAM-1. A. Groups of 5–20 B6 WT and DNAM-1−/− mice were challenged i.v. with 2 × 10⁵ B16F10 tumor cells as described in Materials and Methods. Groups of mice received PBS or cytokine treatment as follows: 100,000 IU IL-2 i.p. on day −1 relative to tumor inoculation, 50 μg IL-21 i.p. on days −1, 0, 1, and 2, or 500 ng IL-12 i.p. on days −1 and 0. B. Some groups of B6 WT mice were treated with either clg or anti-mouse DNAM-1 (250 μg i.p. on days −1, 0, and 7 after tumor inoculation). Lungs were harvested on day 14 and fixed in Bouin’s solution. Tumor colonies were counted under a dissecting microscope. Data are depicted as individual mice (each symbol) and mean tumor colonies ± SEM (cross bar) for each group. Statistical differences between treated WT and DNAM-1−/− mice or clg-treated and anti-DNAM-1−/− treated WT mice are depicted by asterisks. Mann-Whitney U test, p < 0.05.
When WT mice were concurrently treated with depleting anti-asGM1 Ab, significantly enhanced numbers of B16-CD70 (Fig. 6A) and B16-CD80 (Fig. 6B) metastases were detected, indicating that host NK cells controlled IL-2–mediated suppression of metastases of both tumors. Not surprisingly, when WT mice were treated with neutralizing anti-CD70 or anti-CD80 mAb, significantly enhanced numbers of B16-CD70 (Fig. 6A) or B16-CD80 (Fig. 6B) metastases, respectively, were detected. Interestingly, treatment

**FIGURE 2.** IL-2 mediated suppression of B16F10 lung metastases requires early NK cell and DNAM-1/CD155 function. A, Groups of 5 B6 WT and DNAM-1−/− mice were challenged i.v. with $2 \times 10^5$ B16F10 tumor cells. Groups of mice received PBS or 100,000 IU IL-2 i.p. on day −1 relative to tumor inoculation. Some groups of B6 WT or DNAM-1−/− mice received cIg or were depleted of CD8+ T lymphocytes or NK cells in vivo, by treatment with anti-CD8 mAb or anti-asGM1, respectively, on days −1, 0, and 7. B, Groups of B6 WT mice were treated with either cIg, anti-mouse DNAM-1 (250 μg i.p.), or anti-asGM1 (100 μg) early (on days −1, 0, and 7; d−1) or late (on days 3, 4, and 10; d3). C, Groups of 10 B6 WT and DNAM-1−/− mice were challenged i.v. with $2 \times 10^5$ B16F10 tumor cells. Groups of mice received PBS or 100,000 IU IL-2 i.p. on day −1. Some groups of B6 WT or DNAM-1−/− mice were treated with either cIg or anti-mouse CD155 (250 μg i.p.) on days −1, 0, and 7. Lungs were harvested on day 14 and fixed in Bouin’s solution. Tumor colonies were counted under a dissecting microscope. Data are depicted as individual mice (each symbol) and mean tumor colonies ± SEM (cross bar) for each group. Statistical differences between cIg-treated and anti–DNAM-1–treated or anti-CD155–treated WT mice are depicted by asterisks. Mann-Whitney U test, $p < 0.05$.

**FIGURE 3.** DNAM-1-mediated suppression correlates with perforin function. Groups of 5 B6 WT, B6 pfp−/−, B6 IFN-γ−/−, and B6 pfp−/− IFN-γ−/− mice were challenged i.v. with $2 \times 10^5$ B16F10 tumor cells as described in Materials and Methods. Groups of mice received (A) PBS or (B) 100,000 IU IL-2 i.p. on day −1 relative to tumor inoculation. Some groups of B6 WT mice were treated with either cIg or anti-mouse DNAM-1 (250 μg i.p. on days −1, 0, and 7). Lungs were harvested on day 14 and fixed in Bouin’s solution. Tumor colonies were counted under a dissecting microscope. Data are depicted as individual mice (each symbol) and mean tumor colonies ± SEM (cross bar) for each group. Statistical differences between cIg-treated and anti–DNAM-1–treated mice are depicted by asterisks. Mann-Whitney U test, $p < 0.05$.
with neutralizing anti–DNAM-1 mAb alone significantly enhanced the numbers of B16-CD70 (Fig. 8A) and B16-CD80 (Fig. 8B) metastases, suggesting that DNAM-1 was essential for the optimal suppression afforded by CD27-CD70 or CD28-CD80. Ab treatments in WT mice receiving PBS were without effect at such a high tumor inoculum (Fig. 6). A similar pattern of results comparing tumor metastases in WT and DNAM-1−/− mice supported a critical role for DNAM-1 in CD70- and CD80-mediated tumor suppression (Fig. 7A–C).

**DNAM-1 is not required for NKG2D-mediated and ADCC**

Given the correlation between the requirement for host DNAM-1 and perforin, we finally assessed the importance of DNAM-1 to the cytotoxicity of IL-2-activated NK cells against B16 tumor cells and matched B16 targets expressing each relevant ligand. Clearly, in concert with our previous studies using B16F10 (25), the in vitro cytotoxicity assays supported the in vivo studies in demonstrating that DNAM-1 was essential for IL-2/NK cell-mediated cytotoxicity of B16. Both anti-DNAM-1 and anti-CD155 were capable of inhibiting the cytotoxicity of B16 by IL-2-activated WT NK cells (Fig. 8A). Furthermore, the same NK cell populations killed B16-Rae-1e largely independently of DNAM-1 (Fig. 8B), whereas cytotoxicity of both B16-CD70 (Fig. 8C) and B16-CD80 (Fig. 8D) targets was DNAM-1-dependent. NK cells also mediate important cytotoxic effects by ADCC via their CD16 (FcγRIII) (18). By NP-labeling B16 targets cells, we showed at several E:T ratios that both naive (Fig. 8E) and IL-2–activated NK cells (Fig. 8F) could kill target cells in the presence of anti-NP Ab in a DNAM-1–independent manner. Thus, ADCC by NK cells appeared to be largely independent of NK cell DNAM-1.

**Discussion**

We have recently shown that DNAM-1 is an important adhesion receptor that serves to extend the range of target cells that lymphocytes, including NK cells, recognize and affect (25). In this study, we have used matched tumor lines expressing a variety of relevant target ligands, neutralizing mAbs, and DNAM-1–deficient mice to illustrate the relative importance of DNAM-1–ligand interactions in controlling tumor recognition and metastasis. We have shown that NK cells require DNAM-1 for natural or cytokine (IL-2, IL-12, or IL-21)-mediated suppression of nonimmunogenic tumor metastases. Tumor suppression dependent on the ligands CD70 or CD80 required an intact DNAM-1 pathway. By contrast, DNAM-1 was dispensable when tumor cells were targets of ADCC or presented ligands for NKG2D. CD155 appeared to be a key target ligand recognized by DNAM-1 in NK cell-mediated suppression of metastases and DNAM-1–mediated suppression coincided with host perforin activity. In this regard, for any particular tumor target, a strong correlation was observed between the importance of DNAM-1 to NK cell cytotoxicity and suppression of metastases in vivo. Thus it appears that there is a generally important role for DNAM-1–CD155 interactions in NK cell-mediated killing of tumors, unless the tumor triggers a strong activating receptor, such as NKG2D or CD16. With cytokines, such as IL-2 and IL-21, in clinical use for the treatment of cancer (39, 40), this work illustrates a potentially important requirement for their efficacy against aggressive and generally nonimmunogenic tumors.

Previous results using resting human NK cells and Drosophila target cells expressing various ligands have suggested that NKG2D, DNAM-1, 2B4, and CD2 provide early signals for interactions with target cells, with no particular order, but do not individually induce degranulation (41). Human coactivation receptors NKG2D, DNAM-1, 2B4, and CD2 facilitated contact and signals for LFA-1-dependent adhesion. Comparative studies using resting and activated mouse NK cells remain to be undertaken. A distinguishing feature of our study is that we have systematically demonstrated the importance of DNAM-1 in a number of different possible NK cell: tumor interactions in vivo, and we present the first evidence that the efficacy of cytokine therapies might be DNAM-1–dependent in some cases. In the context of a number of different tumor types, the majority of previous studies have only evaluated the role of this particular mouse and human adhesion molecule in vitro.

Although recognized for their ability to kill melanomas, NK cells appear clinically to play a crucial role in eradication of acute myeloid leukemia (4). Analysis of the ligands for triggering NK receptors revealed consistent CD112 and CD155 expression in
myeloid leukemias (42). In contrast, MHC class I-related chain molecules A/B (MICA/B) ULBPs were either absent or weakly expressed. Accordingly, NK cell-mediated killing of these leukemias was dependent on DNAM-1 and NCRs NKp46, but not NKG2D (42). We were unable to assess the role of NCRs in our mouse model of melanoma, because these molecules are restricted to NKp46 and the ligands poorly defined in the mouse (43). Notably, IL-2–activated NK cells killed B16 melanoma at very low levels in the absence of DNAM-1, so if the NCR pathway is operational in this context, then its effects are DNAM-1 dependent.

DNAM-1 participates in cancer cell recognition together with NCRs and NKGD2 in neuroblastoma, ovarian carcinoma, and multiple myeloma (13, 16, 44). Human ovarian carcinomas ubiquitously expressed CD155, but displayed sparse or heterogeneous expression of the NKGD2 ligands MICA/MICB and ULBP1, ULBP2, and ULBP3 (44). Again in vitro, Ab-mediated blockade of activating receptor pathways revealed a dominant role for DNAM-1; however, a complementary contribution of NKGD2 in tumor cell recognition was recognized (44). This result is somewhat distinct from ours, in which we observed a lack of importance of DNAM-1 in the context of functional NKGD2-ligand interactions. A recent study suggests that NK cell-mediated rejection of human ovarian carcinoma may be limited by reduced DNAM-1 expression on tumor-associated NK cells induced by chronic CD155 exposure (45). Ewing sarcoma (EWS) cell lines and primary cells express ligands for NKGD2 and DNAM-1. NK cell cytotoxicity toward EWS cells critically depends on the combination of NKGD2 and DNAM-1 signaling, because blocking either of these receptors inhibits killing by resting NK cells (46). Cytokine-activated NK cells more efficiently recognize EWS cells because only combined, but not single, blockade of NKGD2 and DNAM-1 by mAbs inhibit killing of EWS cells. In contrast, whereas we demonstrated that NKGD2-ligand expressing B16 tumors metastasized most effectively in IL-2 treated mice neutralized for both DNAM-1 and NKGD2, blocking DNAM-1 alone was ineffective. Thus with IL-2–activated mouse NK cells, DNAM-1 may be critical for some receptor–ligand interactions (i.e., in the killing of B16, B16-CD70, or B16-CD80), but it is not critical for NKGD2-mediated cytotoxicity or suppression of metastases. DNAM-1 is also expressed on other effector lymphocytes proposed for adoptive transfer in cancer therapy, such as Vg9Vd2 T cells (47). There is strong evidence that activating NK cell receptors play a role in γδT cell cytotoxicity, and mAb-mediated masking experiments revealed that cytotoxicity and IFN-γ production by human γδT cells in response to hepatocellular carcinomas was DNAM-1-dependent (47). In concert with our data on murine NK cells, CD155 was involved in DNAM-1–dependent γδT cell functions, and no role for CD112 was ascertained.

One recently published study has evaluated the ability of adoptively transferred NK cells to target melanomas expressing DNAM-1 ligands (48). Human melanoma cell lines derived from lymph node metastases express ligands for NCRs and DNAM-1,
but not NKG2D. In mice, DNAM-1 and NCR ligands were also found on spontaneous melanomas and melanoma cell lines. Inhibition of DNAM-1 and NCRs by Ab blockade or genetic disruption reduced the NK cell-mediated killing of melanoma cells in vitro (48). The authors used a xenogeneic model of cell therapy to show that lymph node melanoma metastases were more susceptible to NK cell-mediated cytotoxicity compared with metastases taken from other sites. They also used an adoptive transfer of DNAM-1+ or DNAM-1− NK cells into RAG-2−/−IL-2r−/− mice to treat RET melanoma cells in the peritoneum or lung, and they illustrated that clearance by WT NK cells was greater (48). Interestingly, these authors concluded that lung clearance of RET melanoma cells was more NKp46-dependent than DNAM-1-dependent. These data are not consistent with our own, but we
have used cytokine administration directly to WT mice rather than employing adoptively transferred IL-2–activated NK cells.

IL-2 remains one of the few cytokine immunotherapies with demonstrable clinical activity in patients with advanced melanoma, yet relatively little is known about why a small proportion of patients respond while the majority do not. Clearly, DNAM-1 is an important pathway in IL-2 therapy when the B16 melanoma lacks activating NKG2D ligands or other potential costimulatory molecules, such as CD70 and CD80. An assessment of DNAM-1 ligand (CD155) and other ligand (CD70, CD80, MICA/B) status and lung metastases response to IL-2 in patients beckons. IL-21 has been examined clinically in phase I trials in patients with malignant melanoma and renal cell carcinoma and has a superior safety profile compared with IL-2 (49, 50). Although IL-21 has now entered various phase II trials (5), the importance of DNAM-1 and their tumor ligands in the NK cell effector function and antitumor activity of this cytokine in humans remains unknown.

An important but insufficiently investigated finding presented in this study is the lack of a requirement for DNAM-1 in ADCC. It has become apparent in recent years that the role of the low-affinity FcγRIII (CD16) and ADCC may be key in the therapeutic activity of several important mAb in routine clinical use (e.g., Trastuzumab, RituximAb) (51–53). Our in vitro data would suggest that DNAM-1 is not essential for ADCC mediated by resting or IL-2–activated NK cells, but given that monocytes may also mediate ADCC, it will be important in the future to determine whether DNAM-1 plays a key role in antitumor activity of therapeutic mAbs in representative mouse models of cancer.

Currently, the adoptive transfer of tumor-infiltrating T cells reactive with melanoma Ag into preconditioned humans (receiving either nonmyeloblative therapy or total body irradiation) is a most promising clinical approach to the treatment of human advanced malignant melanoma. Many of the principles of this approach have been established using the mouse B16 melanoma and pMel TCR transgenic T cells (reactive with the human gp100 that cross-reacts with mouse gp100) that express DNAM-1 (data not shown). We are currently evaluating, using the same clinical conditioning methods, whether DNAM-1 might be essential in the therapeutic activity of such adoptive transfer against B16 expressing various ligands.

Thus far, a role for the closely related receptor to DNAM-1, CD96, in NK cell recognition and cytotoxicity of tumors has not been revealed (47). It is possible that the lack of DNAM-1 is at least in part compensated by CD96. Notably, two recent papers have described a potential inhibitory function of T cell Ig and

**FIGURE 8.** DNAM-1 is not required for NKG2D-mediated and ADCC. IL-2–activated NK cells from mice of the indicated genotypes were tested for ex vivo cytotoxicity against parental B16 targets and variants expressing different ligands in a standard 4-h $^{51}$Cr-release assay. IL-2–activated NK cell-mediated cytotoxicity of (A) B16 in the presence of cIg, anti–DNAM-1 or anti-CD155; (B) B16-Rae-1ε; (C) B16-CD70; and (D) B16-CD80. Splenocytes (E) or IL-2–activated NK cells (F) were also tested for ADCC against NP-labeled B16 targets in the presence (+) or absence (−) of anti-NPAb at several E:T ratios as shown. Each data point is the mean ± SEM of two independent experiments done in triplicate for each effector/target ratio.
ITIM domain (TIGIT) when expressed by NK cells and T cells and interacting with CD155 (54, 55). It is not clear yet what effect TIGIT binding to CD155 might have on DNAM-1 functions in the tumor settings we have examined, but clearly anti-CD155 resulted in a metastatic profile akin to that observed in the absence of DNAM-1. Anti-CD155 had no obvious effect in DNAM-1–deficient mice in the presence or absence of IL-2. Furthermore, the cytotoxicity of B16F10 cells by IL-2-activated NK cells incubated with anti-CD155 was also reduced. Future studies will be necessary to determine the relative contributions of all three receptors (DNAM-1, TIGIT, and CD96) in NK cell stimulation and suppression of tumorigenesis. Lastly, some tumors overexpress CD155 and release it as a soluble molecule (56) that might bind to DNAM-1 and prevent immune recognition of tumor cells. Our data suggest that in some circumstances this evasion mechanism could be an effective means for tumors to escape immune surveillance or cytokine-based immunotherapies.

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Disclosures

The authors have no financial conflicts of interest.

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