Mouse models of human cancer serve as essential experimental systems, and genetically defined immunodeficient mouse strains constitute a valuable tool for studying tumorigenesis. Athymic (nude) mice have been the standard for establishing xenograft tumor recipients. Improved tumor engraftment rates have been reported in the nonobese diabetic (NOD)-scid, NOD-scid β2mnull, and NOD-scid IL2Rγnull strains as xenograft tumor recipients. The absolute NK deficiency in NOD-scidβ2mnull mice. NK cells were detected in all strains except NOD-scid IL2Rγnull, and in vitro activated scid, NOD-scid, and NOD-scid β2mnull NK cells kill human melanoma lines and primary melanoma cells. Expression of human NKG2D ligands MHC class I chain–related A and B molecules renders melanoma susceptible to murine NK cell–mediated cytotoxicity and killing is inhibited by antibody blockade of murine NKG2D.

Conclusions: Murine NKG2D recognition of MICA/B is an important receptor-ligand interaction used by NK cells in immunodeficient strains to limit engraftment of human tumors. The absolute NK deficiency in NOD-scid IL2Rγnull animals makes this strain an excellent recipient of melanoma and potentially other human malignancies.
Translational Relevance

Immunodeficient mice are widely used in cancer research to study human cancer biology and evaluate new therapeutics. Although the athymic nude mouse has served as the standard recipient for over 40 years, new immunodeficient mouse strains have been developed that possess well-defined defects in adaptive and innate immunity. In this article, we examined the influence of residual innate immunity on the development of pulmonary metastases using the human A375-RhoC melanoma. The integration of small animal imaging with a clinically relevant lung metastases model reveals the inherent variability among the severe combined immunodeficiency (SCID), nonobese diabetic (NOD)-SCID, and NOD-SCID β2m null strains. Genetic ablation of the IL2Rγ chain leads to an absolute natural killer deficiency in the NOD-SCID IL2Rγnull strain and results in consistent engraftment of human melanoma. Our observation expands the potential to study human melanoma and establishes a new standard to evaluate novel agents in a clinically relevant animal model.

upon activation (11). The NOD-SCID IL2Rγ null strain was developed by introduction of the IL2Rγnull mutation into the NOD-SCID strain (12). Absence of IL2Rγ, the common cytokine-receptor γ-chain shared by the interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors leads to impaired NK cell development due to absence of IL-15 signaling (13). Improved engraftment of human cord blood CD34+ cells has been reported in NOD-SCID β2mnull and NOD-SCID IL2Rγnull highlighting the potential use of these strains as recipients of tumor xenografts (14).

Animal models of human melanoma are limited. Clark et al. (15) generated highly metastatic human melanoma (A375) cells through in vivo selection of lung metastasis in nude mice. A genomic analysis of metastatic A375 variants showed that RhoC, an RAS-related guanosine triphosphatase, is an important determinant of tumor cell invasion. Further studies have shown that RhoC plays a role in cytoskeleton organization and is essential for tumor metastasis (16). In a recent series of studies, Beige-SCID mice were used to evaluate therapeutics for human melanoma A375-RhoC pulmonary metastasis (17, 18). However, NK cell function was not studied and because no other mouse strains were evaluated, the relative effect of residual innate immunity remains undefined in the A375-RhoC model.

In the present study, we evaluate human melanoma (A375-RhoC) pulmonary metastases in four immunodeficient mouse strains: SCID, NOD-SCID, NOD-SCID β2mnull, and NOD-SCID IL2Rγnull strains. Tumor growth was monitored noninvasively by imaging using bioluminescence (BLI) and magnetic resonance imaging (MRI) combined with histopathologic assessment. BLI allowed for accurate, real-time serial in vivo quantitation of tumor burden, whereas MRI permitted three-dimensional structural imaging of tumor. Our results show that NOD-SCID IL2Rγnull mice are highly permissive for engraftment of human melanoma metastases, whereas endogenous NK activity in the other three strains delayed or, in certain instances, completely prevented the formation of pulmonary metastases. Importantly, we show that expression of human NKG2D ligands MHC class I chain–related A (MICA) and B (MICB) molecules by melanoma confers susceptibility to murine NK-mediated cytotoxicity.

Materials and Methods

Mouse strains. CB17-Prkdcsid/J (SCID), NOD.CB17-Prkdcsid/J (NOD-SCID), NOD.Cg-Prkdcsid B2mnull;Luc;J (NOD-SCID β2mnull), and NOD.Cg-Prkdcsid;IL2Rγnull;Luc;Scid (NOD-SCID IL2Rγnull) mice were obtained from The Jackson Laboratory and bred and housed according to the guidelines of Washington University, Division of Comparative Medicine. Strain background is BALB/c (H-2d) for SCID and NOD (H-2b) for all other strains. The animal ethics committee approved all experiments. All mice used were between ages 7 and 14 wk.

Melanoma cell lines. The human melanoma cell line A375P-RhoC-GFP (15) was transduced with a retrovirus expressing a click beetle red luciferase (cbr-luc)/enhanced yellow fluorescence protein (eYFP) fusion gene. A stable cell line was selected by flow cytometry on a MoFlo sorting for cells expressing high GFP (for RhoC expression) and eYFP (for cbr-luc expression) levels. Stable A375 RhoC-luciferase–expressing cell line is called A375RC-Luc. Human melanoma lines DM6 and LOX (American Type Culture Collection) are previously described (19). CG mel is a primary human melanoma (S100+ HMB45+) generated in our laboratory from a resected lymph node metastases. DM6, LOX, and CG lines stably expressing high GFP (for RhoC expression) and eYFG (for cbr-luc expression) were used for in vivo and ex vivo imaging and functional assays.
cbr-luc expression) levels were generated as described above for A375.

M14 melanoma cells (20) were transfected with MICA and MICB cDNA (21) using lipofectamine as directed by manufacturer’s instructions (Invitrogen). Clones were selected using G418 at 1 mg/mL and stable expression of MICA and MICB confirmed by flow cytometry using anti-MICA/B monoclonal antibody (mAb) 6D4 (Biolegend; ref. 22). Expression of ULBP1-3 by melanoma cell lines was determined by flow cytometry using antibodies (23) obtained from Axxora.

In vivo imaging. For serial analysis of tumor growth, mice were injected i.v. with 2.5 × 10⁶ RhoC/luciferase-expressing tumor cells in 200 μL PBS. After tumor inoculation (2-3 h), mice were injected i.p. with 150 mg/kg D-luciferin (Biosynth)/PBS and imaged 10 min later. Imaging was done using a charge-coupled device camera (IVIS 50; Caliper Corporation; exposure time, 1-30 s; binning, 8; field of view, 12; f/stop 1, open filter) at the Molecular Imaging Center (Washington University, St. Louis) as previously described (25, 26).

Histopathology. After MRI on day 28 (for NOD-scid IL2Rγnull) or day 35 (all other strains) tumor growth was monitored by MRI imaging. Representative slides at x10 and x100 magnifications are shown. Mean tumor burden is estimated as percentage of total lung parenchyma; tumor burden in each strain (average of 2 mice) as follows: scid, 7%; NOD-scid, 35%; NOD-scid β2mnull, 62%; and NOD-scid IL2Rγnull, 95%. One representative MRI coronal image per mouse per strain is shown.
Additional experiments were done as described (29). Multiparameter flow cytometry analysis was done using a BD Biosciences FACScan flow cytometer modified with a second, 633 nm 25 mW laser, and 2 additional detectors with bandwidths at 660 and 760 nm. Staining with murine (mu) NKG2D-biotin (28). Antibodies were obtained from eBioscience or Biolegend. Cells were incubated with anti-CD32/CD16 for 5 min at 4°C, followed by specific antibodies, washed, and, in the case of anti-NKG2D, followed by SA-APC. Staining with murine (mu) NKG2D and control tetramer was done as described (29).

Results

Human melanoma pulmonary metastases show different rates of engraftment in immunodeficient mouse strains. Human melanoma cells expressing RhoC (A375-RhoC) exhibit lung tropism after i.v. transplantation in athymic mice (15). To evaluate the effect of residual murine innate immunity on the development of human melanoma pulmonary metastases, we assessed A375-RhoC growth in scid, NOD-scid, NOD-scid β2mnull, and NOD-scid IL2Rγnull mice. Tumor growth was monitored by BLI: (A) NOD-scid (n = 14), (B) NOD-scid β2mnull (n = 28), and (C) NOD-scid IL2Rγnull (n = 32). Summary scatter dot plots with each dot representing one mouse are shown. Data represents four independent experiments. Horizontal line, median value; top and bottom of the whiskers plot, 75% and 25% percentile, respectively. D, survival curve of tumor-bearing NOD-scid β2mnull and NOD-scid IL2Rγnull. BLI and MRI imaging modalities were used to monitor rates of tumor engraftment (25, 31). A375-RhoC cells stably expressing a luciferase reporter (A375RC-Luc, 2.5 × 10⁶) were administered i.v. with 2.5 × 10⁶ A375RC-luc cells and monitored weekly by BLI (n = 10 mice per strain). Mice were sacrificed if weight dropped below 20% of initial. Curves were compared using the log-rank test. One of three independent experiments is shown.
Based on total lung parenchyma, quantitative tumor burden for each strain (n = 2 mice per strain) was assessed microscopically in a blinded manner: scid, 7%; NOD-scid, 35%; NOD-scid β2mnull, 62%; and NOD-scid IL2Rγnull, 95%. MRI detection of pulmonary metastases is less sensitive than BLI; in scid mice, BLI shows clear signal on day 35, although no solid tumor is detected by MRI. In contrast, innumerable pulmonary metastases are visualized in both NOD-scid β2mnull and NOD-scid IL2Rγnull strains (Fig. 2B, right column). Although several lung nodules are evident in the NOD-scid animals, the tumor burden is substantially less and is consistent with the BLI results.

**Human melanoma shows variable engraftment in NOD-scid β2mnull.** We did additional experiments to further characterize the kinetics of tumor growth in the three most permissive strains: NOD-scid, NOD-scid β2mnull, and NOD-scid IL2Rγnull. Figure 3A to C shows a summary of tumor growth rates as determined by BLI for individual animals at weekly time points. BLI signal is reproducibly detected in all animals within 2 to 3 hours after i.v. injection of A375RC-luc cells. In spite of brisk tumor growth in both NOD-scid β2mnull and NOD-scid IL2Rγnull, tumor engraftment is highly variable in NOD-scid β2mnull as shown by the larger interquartile range among mice in the NOD-scid β2mnull strain (Fig. 3B). Between day 28 and 35, 60% of NOD-scid IL2Rγnull mice died due to tumor progression. In contrast, no deaths were seen before day 35 in any tumor-bearing NOD-scid and NOD-scid β2mnull animal; NOD-scid mice show significantly delayed tumor growth compared with NOD-scid β2mnull and NOD-scid IL2Rγnull mice. Survival curves of NOD-scid β2mnull and NOD-scid IL2Rγnull in a representative experiment (n = 10 mice per cohort) are shown in Fig. 3D. The median survival was 42 days for NOD-scid β2mnull and 32 days for NOD-scid IL2Rγnull, in contrast, all NOD-scid mice survived past 48 days (data not shown).

**Rapid engraftment of multiple human melanoma lines in NOD-scid IL2Rγnull mice.** Due to the consistent and reproducible growth of A375RC-luc observed in NOD-scid IL2Rγnull, we selected this mouse strain to evaluate the growth of three additional Rho-C/luciferase expressing human melanoma cell lines. DM6 (19) and Lox are well-characterized cell lines, whereas CG mel is a primary (low passage) melanoma line isolated in our laboratory. NOD-scid mice (strain background NOD: H-2bγ) were used as the comparator strain with NOD-scid IL2Rγnull mice as these strains share identical genetic background (10). Average BLI values for mice (n = 3) at each time point is shown in Fig. 4A to D. Each melanoma studied exhibited a distinct rate and level of engraftment in NOD-scid and NOD-scid IL2Rγnull mice as assessed by BLI. Rates and levels of engraftment of A375, Lox, and CG were higher in NOD-scid IL2Rγnull relative to NOD-scid mice. Interestingly, despite similar BLI signal at day 0 in DM6-bearing NOD-scid and NOD-scid IL2Rγnull, no BLI signal was detected after day 7 in NOD-scid mice, suggesting complete rejection of DM6 in this strain. In agreement with this finding, no evidence of tumor was observed upon autopsy of DM6-bearing NOD-scid lungs (day 50 after tumor inoculation; data not shown). Altogether, these results show that NOD-scid IL2Rγnull mice are a permissive host for engraftment of human melanoma pulmonary metastasis.

**NK characterization and function in immunodeficient strains.** The superior engraftment of human melanoma in NOD-scid IL2Rγnull, a strain devoid of NK cells, implicated murine NK cells as the effector population responsible for tumor rejection. Flow cytometry analysis confirms the presence of NK cells in scid (48.9%), NOD-scid (27.3%), and NOD-scid β2mnull.
Spleen CD45+ populations as determined by the coexpression of CD49b (DX5) and NKG2D. No reactivity of NOD-scid IL2Rγnull spleen cells was observed with anti-CD49b or anti-NKG2D, indicating that NK cells were absent in this mouse strain as previously noted (Fig. 5A and B; ref. 13).

To assess NK effector function, spleen cells from the various strains were activated in vitro with IL-15 + IL-18 and, 48 hours later, assessed for their capacity to recognize human melanoma cells as targets in a 51Cr-release assay. NOD-scid IL2Rγnull spleen cells cultured in IL-15 + IL-18 yielded no appreciable numbers of NK cells and displayed no killing against human melanoma or YAC cells, confirming the complete absence of NK cells in this strain (data not shown). Only one cell line, DM6, expressed all three ULBP molecules. No correlation was observed between ULBP expression and the ability of murine NK cell to recognize human melanoma. In contrast, seven of nine melanoma cell lines expressed MICA/B as detected by the 6D4 mAb (22). Interestingly, low levels of MICA/B expression in M14 and M17 cells correlated with low susceptibility to murine NK cell cytoxicity (Figs. 6A-B and 7A), suggesting a potential role for MICA/B in murine NK cytotoxicity against human melanoma cells.

MICA and B expression by human melanoma confers susceptibility to muNKG2D-mediated cytotoxicity. To investigate MICA/B-muNKG2D interactions and its functional consequences, M14 cells were transfected with MICA or MICB and evaluated for binding of muNKG2D tetramer (29). As shown in Fig. 6B, M14 cells showed no reactivity with MICA/B mAb and exhibit low levels of muNKG2D tetramer binding. This finding is consistent with low level expression of ULBP-2 by M14 because muNKG2D

Expression of NKG2D ligands MICA, MICB, and UL-16 binding proteins 1 to 3 by human melanoma. MICA and MICB and UL-16 binding proteins (ULBP) 1 to 3 are the human ligands for NKG2D, a NK activating receptor (32). Several reports provide evidence that muNKG2D can bind human NKG2D ligands such as MICB, ULBP-1, and ULBP-2 (33, 34). To further investigate the mechanism of murine NK recognition of human melanoma, we determined the expression of NKG2D ligands on selected melanoma cell lines, including M17 a melanoma cell line obtained from the European Searchable Tumor Cell Line and Data Bank and reported as MICA/B negative (35). As shown in Fig. 6A, ULBP-1, ULBP-2, and ULBP-3 expression among melanoma cell lines was heterogeneous. Most lines were negative for ULBP-1, and expressed variable levels of ULBP-2 and ULBP-3. Only one cell line, DM6, expressed all three ULBP molecules. No correlation was observed between ULBP expression and the ability of murine NK cells to recognize human melanoma. In contrast, seven of nine melanoma cell lines expressed MICA/B as detected by the 6D4 mAb (22). Interestingly, low levels of MICA/B expression in M14 and M17 cells correlated with low susceptibility to murine NK cell cytotoxicity (Figs. 6A-B and 7A), suggesting a potential role for MICA/B in murine NK cytotoxicity against human melanoma cells.
tetramer can bind ULBP-2 as shown by Sutherland et al. (34). M14 cells were transfected with cDNA encoding MICA or MICB and expression was confirmed using the 6D4 mAb. Moreover, expression of MICA and MICB in M14 cells results in increased binding of muNKG2D tetramer as shown in Fig. 6B. In cytotoxicity assays, neither M17 nor M14 were susceptible to lysis by activated NK cells (Fig. 7A), whereas A375 and DM6 were killed in a dose-dependent manner at the indicated E/T ratios.

Expression of MICA and MICB rendered M14 cells sensitive to murine NK lysis as shown at multiple E/T ratios (Fig. 7B) compared with control M14 cells. In multiple independent experiments (n = 6), the expression of either MICA or MICB was sufficient to render M14 melanoma sensitive to NOD-scid NK cell lysis (Fig. 7C). The involvement of muNKG2D receptor in melanoma recognition was examined using the antagonistic anti-muNKG2D mAb C7 (28). Pretreatment of activated NK

**Fig. 6.** Expression of NKG2D ligands, MICA/B, and ULBP1 to 3, by human melanoma cell lines. A, melanoma cell lines were stained using anti-ULBPs and the 6D4 anti-MICA/B mAbs and analyzed by flow cytometry. ULBPs and MICA/B expression (thick line) and the isotype control (thin line) are shown in each histogram. Data are representative of three independent experiments. B, M14, M14-MICA, and M14-MICB cells were stained with anti-MICA/B 6D4 (top; thick line), isotype control (top; thin line), or muNKG2D tetramer (bottom; thick line), or control tetramer (bottom; thin line). Data are representative of two independent experiments.
cells with anti-NKG2D mAb resulted in a significant decrease in MICA and MICB recognition with no statistically significant effect on control M14 cells (Fig. 7D). Altogether, these data support the finding that MICA and MICB are capable of binding to and activating murine NK cells through interaction with their NKG2D receptor.

**Discussion**

In the present study, we compared scid, NOD.scid, NOD.scid-β2mnull, and NOD.scid IL2Rγnull mice as recipients for human melanoma. Engraftment of human melanoma pulmonary metastasis in NOD.scid IL2Rγnull mice is brisk and reproducible relative to the other strains examined. Despite multiple attempts to isolate and grow NK cells from NOD.scid IL2Rγnull mice, we were unsuccessful and, thus, confirm the original observation that ablation of the common cytokine-receptor γ-chain (IL2Rγ) confers an absolute NK cell deficiency (13). Our results also suggest that murine NK cells use NKG2D to recognize MICA/B, and we propose that this mechanism accounts for the increased xenograft rejection observed in scid, NOD.scid, and NOD.scid-β2mnull mice.

We initially observed dramatic differences in human melanoma engraftment among NOD.scid β2mnull littermates and were perplexed by this inconsistency. Although NK cells from NOD.scid β2mnull mice are present in similar percentages as scid and NOD.scid animals, it seems that NK cells that develop in a MHC class I−deficient environment are functionally impaired (11, 36). However, our findings suggest the presence of residual/partial NK cell activity in NOD.scid β2mnull mice because some animals clearly have delayed tumor engraftment (Fig. 3B). The variable engraftment rates of human CD34+ stem cells in NOD.scid β2mnull animals supports this conclusion (37). In support of NK cells retarding the growth of xenografts, administration of anti-CD122 antibody to deplete endogenous NK cells in NOD.scid mice has been shown to improve engraftment of human hematopoietic stem cells (38) as well as solid tumor stem cells (39). The inconsistent tumor growth seen is likely to reflect variable levels of NK function among mouse strains and not heterogeneity in tumor sample as similar results were obtained using tumor lines or clones obtained by limiting dilution (data not shown).

The inherent advantage of using the NOD.scid IL2Rγnull strain is that gene ablation of the IL2Rγ chain leads to absolute NK deficiency (12, 13) and results in remarkable consistency of human melanoma engraftment (this study). Additionally, NOD.scid IL2Rγnull do not develop thymic lymphoma with age, thus allowing for long-term studies in these mice. In contrast, NOD.scid and NOD.scid β2mnull animals have a high incidence of thymic lymphomas that are fatal (10).

**Fig. 7.** Human melanoma recognition by NOD-scid NK cells correlates with MICA/B expression and is blocked by antimuNKG2D antibody. A, NOD-scid spleen cells were activated with IL-15 + IL-18 for 48 h, harvested, and incubated with 51Cr-labeled targets for 4 h as described in Materials and Methods (n = 3 experiments). MICA/B expression by M14, M17, A375, and DM6 is shown in Fig. 6. B, NOD-scid NK cells were tested at the indicated E/T ratios in 51Cr-release assay using M14, M14-MICA, and M14-MICB transfectants (n = 3 experiments). C, recognition of M14-MICA and M14-MICB transfectants by NK cells from NOD-scid mice. Columns, mean value at a 30:1 E/T ratio is shown from 6 independent experiments; bars, SD. The lower killing of M14-MICB (compared with MICA) likely reflects the lower antigen expression level on the M14-MICB target cell population. D, recognition of M14-MICA and M14-MICB by NOD-scid NK cells is blocked by antimuNKG2D mAb. NOD-scid spleen cells were cultured in IL-15 + IL-18 for 48 h, harvested, and incubated with anti-muNKG2D mAb (black bars) or isotype control (white bars) for 1 h (30 ug/mL), 51Cr-labeled target cells added and assay done as described in Materials and Methods. E/T ratio is 30:1 (n = 3 experiments). Data in C and D was analyzed by repeated measure one-way ANOVA followed by Tukey’s analysis. P values of <0.05 are statistically significant.
NK function is regulated by inhibitory and activating cell surface receptors (40). NKG2D, a C-type lectin-like molecule, is a major NK activating receptor that interacts with a diverse array of ligands (41, 42). NKG2D ligands include retinoic acid early transcript 1 proteins and minor histocompatibility protein H60 and MULT1 in mice and MICA, MICB, and ULBPs in human (32). Recent data in both NKG2D-deficient mice and gain-of-function studies using a novel chimeric NKG2D construct support the critical role of NKG2D in tumor surveillance (43, 44). Human melanomas and various carcinomas have been reported to express MICA/B and/or ULBPs and both ligands have been shown to be involved in human NK recognition of tumors (20, 45). Interestingly, NKG2D from one species can bind ligands from another; for example muNKG2D has been previously shown to bind human ligands MICB, ULBP-1, and ULBP-2 (33, 34). Several previous studies have examined NK cell function in NOD mice and shown some degree of impairment in target cell recognition (compared with the C57BL/6 strain; refs. 46, 47); however, little direct information regarding NOD-scid NK cell function in the context of xenograft recognition is available. Our results show that MICA/B in muNKG2D interaction leads to recognition of human melanoma, which may, in turn, result in decreased tumor engraftment in certain immunodeficient strains. The beige-scid mouse has also been reported to be permissive to human melanoma xenografts and as shown by Elsner and colleagues (48), MICA/B seems to be the tumor associated rejection ligand recognized by muNKG2D. Interestingly, beige-scid mice have large numbers of DX5+ NK cells that express low levels of NKG2D (data not shown). Thus, our results are consistent with the observation that murine NKG2D recognition of MICA/B and ULBPs may prevent/delay engraftment of many human solid tumors in immunodeficient mouse strains.

Small animal imaging provides significant advantages to monitor tumor growth in experimental models (49). Whole body BLI with luciferase reporters is remarkably sensitive, facile to execute, serially, and allows detection of early metastases in various tissues. MRI is less sensitive but has inherent advantages including superior spatial resolution and no requirement for reporter constructs. A recent report evaluating positron-emission tomography, X-ray computed tomography, and BLI modalities documents the ability for detecting human melanoma lung metastases (A375-M) in scid mice at day 45 (50). In our model, 18F-FDG-PET imaging was done on several animals, which could, in fact, detect A375-RhoC lung metastases as early as day 30 (data not shown).

In summary, our study highlights muNKG2D recognition of MICA/B as an important receptor-ligand interaction used by NK cells in immunodeficient strains to limit engraftment of human tumors. Because NOD-scid IL2Rγc mice have an absolute NK cell deficiency as well as additional innate and adaptive defects, this strain seems to be the best recipient for human melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Richard Hynes, John DiPersio, Timothy Graubert, Marco Colonna, and Thomas Spies for reagents; Julie Ritchey and Matthew Holt for excellent technical assistance; Siteman Cancer Center High Speed Cell Sorter Core and Small Animal Imaging Core facilities for expert assistance; and Wayne Yokoyama and Todd Fehniger for advice.

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