A shed NKG2D ligand that promotes natural killer cell activation
and tumor rejection

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Running Title: Soluble MULT1 stimulates tumor rejection
Immune cells, including natural killer (NK) cells, recognize transformed cells and eliminate them in a process termed immnosurveillance. It is thought that tumor cells can evade this by shedding ligands that bind to the NKG2D activating receptor on natural killer (NK) cells, thereby inhibiting it. In contrast, we show that in mice, tumor cells shed the NKG2D ligand MULT1, which binds NKG2D with high affinity and causes NK cell activation and tumor rejection. Recombinant soluble MULT1 stimulated tumor rejection in mice. Soluble MULT1 functions by competitively reversing a global desensitization of NK cells imposed by engagement of membrane NKG2D ligands on tumor-associated cells. The results overturn conventional wisdom that soluble ligands are inhibitory, and suggest a new approach for cancer immunotherapy.

Main text

Many tumor cells release soluble NKG2D ligands through proteolytic shedding, alternative splicing, or exosome secretion (1, 2). Numerous reports conclude that excreted NKG2D ligands can modulate NKG2D from the cell surface and that receptor downregulation is associated with desensitization of the immune cells (3, 4), although a functional impact of soluble NKG2D ligands is not always observed (5-8). To study shed NKG2D ligands in a controlled setting, we focused on the mouse ligand MULT1. MULT1 is the most common ligand upregulated in primary tumors in two spontaneous cancer models(9), and is a transmembrane protein, similar to human ligands MICA, MICB, ULBP4 and ULBP5 (10).

Fibroblasts with very little endogenous MULT1 (11) were transduced with N-terminally HA-tagged full length MULT1 (“HA-MULT1”). Concentrated culture supernatants from the cells contained a 23 kD species after deglycosylation, representing the cleaved extracellular domain (Fig. 1A). Conversely, cell lysates of fibroblasts with C-terminally tagged MULT1-HA contained a 24 kD membrane “stub” fragment that was absent in control samples, in addition to full length MULT1-HA (around 42 kD) (Supplementary Fig. 1). Inhibiting matrix metalloproteinases blocked MULT1 shedding (Supplementary Fig. 2).
Concentrated supernatants of HA-MULT1-transduced fibroblasts contained nearly 8-fold more shed MULT1 than samples from untransduced fibroblasts (Supplementary Fig. 3). WEHI-7.1 C1498, YAC-1 but not 293T cell lines secreted MULT1. We detected serum MULT1 (mean concentration ~250 ng/ml) in most tumor-bearing Eμ-myc transgenic mice, which frequently develop MULT1+ tumors (9), but not in most non-transgenic littermates (Fig. 1B). Very high concentrations of soluble MULT1 were also detected in sera of Apoe-/- mice fed a high fat diet (Fig. 1B). Given that NKG2D function is necessary for the atherosclerosis and liver inflammation that develop in such mice (12), this seemed inconsistent with the notion that MULT1 inhibited NK cell function. Thus, MULT1 is released from cell lines that naturally or ectopically express MULT1, and accumulates in sera of animals with spontaneous tumors and NKG2D-dependent inflammatory disease.

Purified shed HA-MULT1 bound to NKG2D with high affinity (average K_D of 13 nM±3.8 nM) (Fig. 1C), similar to the affinity reported for recombinant MULT1(13). In parallel, we engineered fibroblasts to secrete an ectodomain fragment of HA-MULT1 (which we call secMULT). SecMULT1 bound to NKG2D with similar affinity as shed MULT1 (19 nM±4.3 nM) (Fig. 1C).

To test the function of soluble MULT1, we engineered two NKG2D ligand-negative B6 strain tumor cell lines that secreted secMULT1. Surprisingly, both cell lines were rejected by syngeneic B6 mice compared to cells transduced with empty vector (Fig. 1D, Supplementary Fig. 4A), despite the absence of cell surface MULT1 (Supplementary Fig. 4B). Tumor cells transduced with full-length MULT1 (mutated in the cytoplasmic tail to optimize cell surface expression (14), Supplementary Figure 4B) were also rejected (Fig. 1D). B16-secMULT1 cells were still rejected in B6 hosts that had been depleted of CD8+ cells but grew progressively in B6 and Rag1-/- hosts that had been depleted of NK1.1+ cells (Fig. 1E, Supplementary Fig. 5). Hence, NK cells but not CD8+ cells participate in the rejection of B16-secMULT1. B16 cells with inducible secMULT1 (Supplementary Fig. 6) were also partially rejected (Fig. 1F). In this case, the secMULT1 lacked an epitope tag, ruling out the possibility that the tag played a role in
rejection. To test whether secMULT1 functions extrinsically, we injected subcutaneously a
mixture of control B16 and B16-secMULT1 cells. Remarkably, even $2 \times 10^3$ B16-secMULT1
cells, when mixed with $2 \times 10^4$ B16 cells, caused rejection of the B16 cells in the same tumor
mass (Fig. 1G). These data ruled out the possibility that rejection was due solely to cell intrinsic
mechanisms, such as targeting of cells expressing stress-induced ligands associated with
secMULT1 overexpression. Instead, the data suggested that secMULT1 mobilizes or activates
anti-tumor effector cells.

To address whether tumor cells secreting secMULT1 activate NK cells we employed an
established short-term in vivo induction protocol by injecting irradiated tumor cells
intraperitoneally in normal mice (15, 16). Injection of B16-secMULT1 or B16 cells induced
similar modest increases in the percentages of NK cells in the peritoneal washes 3 days later
(Supplementary Fig. 7), but the B16-secMULT1 cells induced more potent ex vivo killing activity
against NK-sensitive YAC-1 tumor cells (Fig. 2A). Similar results were obtained with mice
injected with RMA-secMULT1 cells (Supplementary Fig. 8A). Furthermore, peritoneal NK cells
recovered from mice receiving B16-secMULT1 cells were more responsive in terms of IFNγ
production than those from mice receiving B16 cells, when stimulated with YAC-1 tumor cells
(Fig. 2B) or with immobilized antibodies against NKp46 or NKRP1C activating receptors (Fig.
2C, Supplementary Fig. 8B). Therefore, soluble MULT1 induced increases in NK cell functional
capacities, including tumor cell killing and cytokine production in response to activating receptor
engagement.

To allow the recovery and analysis of intratumoral NK cells at relatively early times after
subcutaneous transfer, we implanted $3-5 \times 10^5$ tumor cells mixed with matrigel. Seven days
later, NK cells extracted from B16-secMULT1 exhibited stronger IFNγ responses than NK cells
extracted from B16 tumors, when stimulated with immobilized antibodies specific for activating
receptors (Fig. 2D). Hence, secMULT1 induced NK cell functional capacity, in both subcutaneous and peritoneal tumors.

To address directly whether soluble MULT1 protein enhances NK responses in tumors, we employed recombinant MULT1 (rMULT1), which is of nearly identical size as shed MULT1 and secMULT1. Tumors exposed intratumorally to rMULT1 (Supplementary Fig. 9A) were significantly smaller than control tumors after seven days (Fig. 2E, Supplementary Fig. 9B), and NK cells extracted from the tumors exhibited heightened responsiveness to immobilized receptor antibodies (Fig. 2F). The rMULT1 sample was devoid of endotoxin or other PAMPs that activate macrophages. These data established that soluble MULT1 causes tumor rejection, likely by activating NK cells.

Activating receptor crosslinking is typically necessary for activation of lymphocytes. However, secMULT1 and shed MULT1 are monomeric (Supplementary Fig. 10A-C), and should therefore be incapable of crosslinking NKG2D. Moreover, monomeric rMULT1 failed to stimulate IFN-γ production when incubated in vitro with peritoneal NK cells for 4 hours (Supplementary Figure 11A). We considered the possibility that soluble MULT1 forms a multivalent array in vivo, perhaps by binding to some structure in the tissues, but preliminary staining analyses failed to detect such arrays (data not shown). These data argue against the notion that soluble MULT1 directly stimulates NK cells.

The presence of target cells bearing membrane NKG2D ligands, including MULT1, causes downregulation of cell surface NKG2D, presumably by aggregating the receptor and triggering receptor endocytosis (7, 17) (Fig. 3B). We found that secMULT1 production by tumor cells instead resulted in NKG2D upregulation on NK cells (Fig. 3, A and B). NKG2D upregulation was probably due to a decrease in downregulation from the membrane, as it occurred without increases in NKG2D mRNA (Supplementary Fig. 12) or intracellular NKG2D protein (not shown).
Based on these findings, we hypothesized (Fig. 4E) that untransformed host cells express membrane NKG2D ligands; that these cells persistently engage NK cells, cause NKG2D downregulation, and globally desensitize the NK cells as the tumors progress; and that soluble MULT1 enhances responsiveness and cell surface NKG2D expression by blocking these interactions. Consistent with the hypothesis, host cells (CD11b^+ F4/80^+ myeloid cells) associated with either peritoneal or subcutaneous B16 or B16-secMULT1 tumors displayed increased RAE-1 (but not MULT1) on the cell surface (Figure 3C, D). RAE-1 upregulation was also observed on macrophages infiltrating sarcomas using the spontaneous “KP” model of cancer (18) (Thompson and Raulet, unpublished data). Monocytes in patients with several types of cancer also express NKG2D ligands (19). Hence, normal myeloid cells display low amounts of RAE-1, whereas tumor-associated CD11b^+ F4/80^+ cells display elevated amounts, consistent with a previous report (20). We further demonstrated that rMULT1 competitively blocks binding of RAE-1^ε-Fc fusion protein to NKG2D on NK cells (Fig. 3E), confirming another prediction of the hypothesis.

To test whether RAE-1 expressed on endogenous cells caused NK cell inactivation, we employed the CRISPR/Cas9 method to disrupt both B6 strain RAE-1 genes, Raet1d and Raet1e. Peritoneal NK cells from Raet1d^-/-Raet1e^-/- mice exhibited significant increases in membrane NKG2D expression as well as functional responses (Fig. 3F-H). Greater differences occurred when the mice were injected with irradiated B16 tumor cells for 3 days, suggesting that tumors induce RAE-1 expression on host cells in WT mice, resulting in greater NK desensitization and NKG2D downregulation. Notably, NK cells in Raet1-deficient mice exhibited greater responses to both NKG2D-dependent stimuli (e.g. to YAC-1 cells, Fig. 3G) and NKG2D-independent stimuli (e.g. to anti-NKp46, Fig. 3H), consistent with published reports that persistent stimulation by cells expressing NKG2D ligands results in a global desensitization of NK cells (17, 21). In mice injected with B16-secMULT1 tumor cells, smaller differences were observed, as predicted if secMULT1 blocks NKG2D interactions with RAE-1-expressing cells.
Indeed, addition of rMULT1 to cultures of peritoneal wash cells resulted in increased functional responses of NK cells to stimulation, when tested 8-20hrs later, but not after 4 hrs (Supplementary Figure 11B, C). Together, these data indicate that interactions of NK cells with RAE-1 molecules on non-tumor cells cause NKG2D downregulation and functional desensitization, and that this is accentuated in tumor-bearing mice.

Our model further predicts that NKG2D receptor deficiency, or blocking with antibody, should have a similar effect as soluble MULT1. Confirming recent findings (22, 23), NK cells in NKG2D-deficient (Klrk1−/−) mice on a Rag2−/− background exhibited increased functional activity when compared to wildtype NK cells (Fig. 4A). A similar, if smaller, effect was evident in NKG2D-deficient NK cells on a Rag2+/− background (Supplementary Fig. 13A). Similarly, i.p. injections of B6 mice with F(ab’)2 fragments of blocking NKG2D antibody resulted in enhanced functional responses ex vivo (Fig. 4B). Most remarkably, NKG2D-deficient Rag2−/− mice exhibited a strongly enhanced rejection response against B16 and B16-secMULT1 tumors, compared to the responses of Rag2+/− mice (Fig. 4C, D). Furthermore, incorporation of F(ab’)2 fragments of blocking NKG2D antibody into B16 tumors that were established in subcutaneous matrigel plugs resulted in partial tumor rejection and augmented responsiveness of NK cells within the residual tumors (Supplementary Fig. 13B, C). Thus, NKG2D deficiency, or blockade, results in enhanced NK cell responsiveness and tumor rejection. These data strongly support the proposed model (Fig. 4E).

The finding that NK cells persistently stimulated through NKG2D or other receptors are broadly desensitized is consistent with published data (21, 24) and may reflect a defect in MAPK/ERK signaling (24). Blocking or disabling NKG2D restores killing of B16 cells because NK cells use receptors distinct from NKG2D to target B16 cells. A more complex outcome should pertain with tumor cells that express membrane NKG2D ligands, because the NK cells, while more active, will be partly blocked in tumor cell recognition. Tumor cells often express multiple NKG2D ligands, suggesting that therapeutic efficacy may be maximized by blocking.
only the specific NKG2D ligands expressed by host cells, rather than blocking NKG2D altogether.

Our results are surprising as they show that soluble NKG2D ligands in vivo stimulate tumor rejection and greater amounts of cell surface NKG2D, whereas the literature suggests they should suppress tumor rejection and cause decreased NKG2D expression. It must be noted, however, that NKG2D downregulation is frequently not observed in patients with soluble MICA/MICB (5-8). Moreover, MULT1 and MICA/MICB ligands differ in a key respect: affinity. Soluble MULT1 is a high affinity (K_D ~ 10 nM) monomeric ligand. Soluble MICA and MICB are low affinity ligands (K_D ~ 1 µM), which are present in patient sera at concentrations below 1 nM (3, 25), meaning that NKG2D occupancy is predicted to be extremely low. This consideration suggests that systemic effects of soluble MICA and MICB may be indirect, or that the active form of soluble MICA or MICB is actually a multimeric exosome form (26, 27), which can bind and crosslink the receptors despite a low affinity and low concentration. Binding and crosslinking are conditions known to cause modulation of other immune receptors from the cell surface (28). Consistent with these considerations, B16 cells secreting the low affinity MICA ligand, when injected i.p., failed to induce significant NKG2D upregulation or increased NK functional activity (Supplementary Figure 14). In conclusion, the results identify an unexpected mechanism of immune activation and support efforts to evaluate the potential of soluble NKG2D ligands or antibodies that block NKG2D or its ligands, for immunotherapy of cancer. Studies suggest that engagement of other NK activating receptors, such as NKp46, may also lead to NK cell desensitization, suggesting that multiple targets exist for amplifying NK function (29).

Acknowledgments.

We thank H. Nolla and A. Valeros for help with cell sorting, C. Kang for targeting eggs for creating the Raet1 knockout mice, Q. Yan for assistance with Biacore assays, and T. Trevino, L. Bai, S. Li for technical assistance. We thank Raulet Lab members and R. Vance for useful
discussion and comments on the manuscript. The data presented in this manuscript are tabulated in the main paper and in the supplementary materials. D.H.R. filed a patent (US 6821522) describing the use of soluble ligands for immunotherapy of cancer. WD was supported by a Cancer Research Institute postdoctoral fellowship. BG was supported by a National Science Foundation Graduate Research Fellowship and the Hirth Chair Graduate Fellowship of UC Berkeley. LW and AI were supported by Leukemia and Lymphoma Society Fellowships, and SL was supported by an NIH-IMSD grant. This work was supported by NIH grant R01 CA093678 to DHR.

Author Contributions.

WD designed, executed and analyzed the experiments. BG led the effort to generate the Raet1 knockout mice. LZ, LW, SL, AI, JX assisted with experiments. NX prepared the sera samples from Apoe−/− mice. TR prepared the 1D6 MULT1 antibody. DHR conceived of the study, and with WD, designed and interpreted the experiments. The manuscript was prepared by WD and DHR. All authors critically read the manuscript.
Figure Legends

Figure 1. NK cell promote the rejection of tumors that shed MULT1. (A) The extracellular domain MULT1 fragment (“shedMULT1”) detected in supernatants of HA-MULT1-transduced fibroblasts after HA immunoprecipitation, treatment with PNGaseF to remove N-glycans, and blotting with HA antibody. (B) ELISA detection of soluble MULT1 in sera from tumor bearing Eμ-Myc mice, nontransgenic littermates, and diseased Apoe−/− mice fed a Western diet (n=6-8). Each point represents a different mouse. (C) Surface plasmon resonance analysis of binding of shed MULT1 and secreted MULT1 (secMULT1) to chip-bound recombinant NKG2D. ShedMULT1 and secMULT1 (1, 2, 5,10, 20 nM) were sequentially analyzed for binding to SPR flow cells derivatized with biotin-anti-CD19 (negative control) or biotin-NKG2D. (D) Comparison of growth of 2 x 10⁴ subcutaneously transferred B16 melanoma tumor cells transduced with secMULT1, full length MULT1 or empty vector, in WT B6 mice. Rejection was usually partial but was complete in some animals in some experiments (n=4). (E) Subcutaneous growth of B16-secMULT1 tumors in B6 mice (2 x 10⁴ cells were inoculated) treated with control IgG, NK1.1 antibody or CD8 antibody (n=13, pooled from 3 experiments). (F) After inoculation of 2 x 10⁴ B16 cells transduced with pFG12-secMULT1 mice were treated or not with doxycycline starting from the time of tumor implantation. (n=6). (G) Mice received 2 x 10⁴ B16 cells alone, or 2 x 10⁴ B16 cells mixed with 2 x 10³ B16-secMULT1 cells. (n=6). Experiments in panel A, C, D, E and G were performed at least 3 times while the experiment in panel F was performed twice with similar results. Tumor volumes ± SE are shown. Statistical comparisons of secMULT1 tumors to vector-transduced tumors were performed using 2 way ANOVA.

Figure 2. Soluble MULT1 amplifies NK cell responses and causes tumor rejection. (A-C) B6 mice were injected i.p. with 5 x 10⁶ irradiated B16 or B16-secMULT1 cells, or PBS. Peritoneal wash cells were recovered three days later and tested for killing of YAC-1 target cells (A) or tested for
intracellular IFNγ after stimulation with YAC-1 cells (B) or immobilized NKp46 antibody (C). (D) B6 mice were injected subcutaneously with 3-5 x 10⁵ B16 or B16-secMULT1 cells in 100 μl matrigel. The tumors were dissociated 7 days later, and gated NK cells were tested for responses to immobilized NKp46 or NKR1C Abs. (E-F) Subcutaneous tumors were established with 3-5 x 10⁵ B16 cells in 100 μl matrigel. The tumor cells in one group were mixed with 1 μg of recombinant MULT1 (rMULT1). After 4 days, an additional 1 μg of rMULT1 was injected into each matrigel/tumor for that group. Tumors in the control group received PBS. On day 7, tumors were extracted, weighed (E), dissociated, and the tumor cells were counted (E). The immune cells within the tumor were stimulated with immobilized NKp46 and NKR1C Abs, and the IFNγ responses of gated NK cells were determined (F). The experiment in panel A was performed twice with similar results, while experiments in the other panels were performed at least 4 times.

**Figure 3. Mechanisms of immune activation by soluble MULT1.** (A, B) NKG2D expression levels after exposure to secMULT1 in subcutaneous (A) or intraperitoneal (B) tumors. (C) Expression of NKG2D ligand RAE-1 by gated CD11b⁺F480⁺ peritoneal macrophages in mice injected i.p. 3 days before with PBS or 5 x 10⁵ irradiated B16 or B16-secMULT1 tumor cells. Cells were stained with biotin-pan-RAE-1 Ab (blue). The staining was specific as it could be blocked by including an excess of unconjugated pan-RAE-1 antibody in the reaction (red). Grey shows isotype control staining. (D) Expression of RAE-1 by gated CD11b⁺F480⁺ intratumoral macrophages in mice injected SC with 2 x 10⁴ B16 or B16-secMULT1 tumor cells 20 days before. (E) rMULT1 and NKG2D antibody (M16 clone, in F(ab′)₂ form) block RAE-1 binding to NKG2D on NK cells. The MFI of RAE1ε-Fc staining of NK cells was used to calculate % inhibition. (F-H) Peritoneal NK cells from Raet1d⁺Raet1e⁻ mice (KO) exhibit increased amounts of cell surface NKG2D (F) and increased functional responses ex vivo to YAC-1 tumor cells (G) or NKp46 antibody stimulation (H). The effects were larger when the mice were injected 3 days earlier with irradiated B16 tumor cells, but
smaller when they were injected with B16-secMULT1 tumor cells. Panel B is combined data from 14 experiments. The remaining experiments were performed at least three times.

**Figure 4. Tumor rejection in NKG2D-deficient mice.** (A) NKG2D-deficient (Klrk1−/−) NK cells exhibit increased functional activity. Splenic NK cells from Rag−/− and Rag−/− Klrk1−/− mice were stimulated *ex vivo* with immobilized NKp46 or NKRP1C Abs, and the IFN-γ responses of gated NK cells were determined. (B) B6 mice were injected i.p. with 50 µg MI6 (anti-NKG2D) F(ab')2 or F(ab')2 of rat IgG on days 0, 3 and 6. On Day 8, peritoneal NK cells were stimulated *ex vivo* with immobilized NKp46 Abs, and the IFNγ responses of gated NK cells were determined. (C, D) Increased tumor rejection responses in NKG2D-deficient mice. Growth of B16-secMULT1 (C) or B16 (D) tumor cells in Rag2−/− or Rag2−/−Klrk1−/− mice. Panels C and D are from separate experiments. Separate, direct comparisons showed retarded growth of B16-secMULT1 vs B16 tumors in Rag2−/− mice. (E) Model of secMULT1 action. Persistent NKG2D engagement by endogenous RAE-1-expressing cells associated with the tumor desensitizes NK cells. Soluble MULT1 competitively blocks the NKG2D receptor, preventing NK cell desensitization and therefore augmenting tumor rejection. All experiments were performed three times.
References

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Intratumoral NK cells (S.C. model)

- **NKG2D**
  - B16: ****
  - B16-secMULT1: ns
- **DNAM-1**
  - B16: ****
  - B16-secMULT1: ns

Gated peritoneal NK cells

- **NKG2D MFI**
  - PBS: ***
  - B16: ****
  - B16-secMULT1: ****

Peritoneal Wash Macrophages

- **RAE1**
  - PBS: Block first, a-RAE-1-biotin
  - B16: Block first, a-RAE-1-biotin
  - B16-secMULT1: Block first, a-RAE-1-biotin

Intratumoral macrophage (CD11b+F4/80+)

- **RAE1**
  - a-RAE-1-biotin

Gated peritoneal NK cells

- **NKG2D MFI**
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****

%IFN-γ+ NK cells

- **PBS**
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****

- **YAC-1**
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****

%IFN-γ+ NK cells

- **PBS**
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****

- **α-NKp46**
  - WT KO: ****
  - WT KO: ****
  - WT KO: ****

Fig. 3
**Fig. 4**

A. Tumor elimination

- **Rag2^-/-Klrk1^+/+**
- **Rag2^-/-Klrk1^-/-**

B. Intraperitoneal Injection

- **Rat F(ab')2**
- **MI6 F(ab')2**

C. PBS anti-NKp46 anti-NKRP1C

D. %IFNγ+ NK cells

E. NKG2D NK desensitization

- RAE1

- Restored NK cell

- Soluble MULT1

- Tumor elimination

- Activating Receptor

- Tumor associated macrophage

- Initial tumor
Materials and Methods

Cells, antibodies and reagents

Cells culture was performed at 37°C in humidified atmosphere containing 5% CO₂. Fibroblasts and B16 cells were cultured in complete DMEM, consisting of DMEM (Invitrogen), 10% fetal calf serum (Omega Scientific), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 0.2 mg/ml glutamine (Sigma-Aldrich), 10 µg/ml gentamycin sulfate (Lonza), 20 mM Hepes (Thermo Fisher Scientific), and 50 µM 2-mercaptoethanol (EMD Biosciences). RMA and YAC-1 cells were cultured in similarly supplemented RPMI medium.

Antibodies against NK1.1 (PK136), CD3ε (145-2C11), CD45.2 (104), IFN-γ (XMG1.2), CD107a (1D4B), NKG2D (MI6), NKp46 (29A1.4), CD11b (M1/70) and F4/80 (BM8) were purchased from eBioscience. Monoclonal HA antibody (clone 16B2) was from Covance. Anti–panRAE-1 (clone 186107), anti-MULT1 antibody (clone 237104), RAE1-Fc and recombinant MULT1 were from R&D Systems. NK1.1 (PK136) and CD8 (2.43) antibodies for in vivo depletions were prepared in the laboratory or purchased from BioXCell.

Mice and in vivo procedures.

All mice were bred at the University of California, Berkeley, in compliance with institutional guidelines. C57BL/6J (hereafter called B6), B6-\(E_{\mu}\)-Myc transgenic and B6-\(Rag2^-\)- mice were purchased from the Jackson Laboratory. The B6-\(Klrk1^-\) (NKG2D KO) strain (1) is available at the Jackson Laboratory Repository (JAX Stock No. 022733). B6-\(Rag2^-\) and B6-\(Klrk1^-\) mice were intercrossed two generations to generate B6-\(Rag2^-\)\(Klrk1^-\) mice (2). NKG2D-deficient mice were screened for the \(Klrk1\) deletion by genomic PCR. All mice were used between 6 and 12 weeks of age.
Conventional subcutaneous tumors were generated by injecting cells subcutaneously in 100 µl PBS in the right flank. Tumor growth was monitored by measuring the tumor size twice weekly with a metric caliper. For short-term experiments, tumor cells were injected S.C. in the right flank after resuspending in 100 µl matrigel, matrix growth factor reduced (BD Bioscience), and adding recombinant proteins. In this case, tumor size was determined as the weight of the tumor plug or tumor cell numbers (after dissociation). The tumor experiments typically included 3-15 (usually 4-6) mice per group.

When indicated, NK cells and CD8+ T cells were depleted in vivo by intraperitoneal injection of 200 µg NK1.1 (PK136), CD8 (2.43) or control IgG antibody at days -1, 1, 8, 15, 22. Depletions were confirmed by flow cytometric analysis of spleen cells 3 weeks after tumor challenge using non-competing antibodies.

**Generation of Raet1 KO mice**

*Raet1* mutant mice were generated using the CRISPR/Cas9 system. We chose a shared Cas9 target site present in the second coding exon of the highly related *Raet1d* and *Raet1e* genes. *Cas9* mRNA and the *Raet1* sgRNA were *in vitro* transcribed and injected into single-cell embryos as described (3). *F₀* mice carrying frame-shift mutations in both *Raet1d* and *Raet1e* genes were identified by PCR and were back-crossed to C57BL/6 mice, and the resulting *Raet1* heterozygous *F₁* mice were intercrossed to produce the mice used in this study. *Raet1⁺⁻* mice from one founder lines were used in this study. *Raet1* genomic target sequence with protospacer-adjacent motif (PAM, underlined): 5'-TAGGTGCAACTTGACCATCAAGG-3'. Oligonucleotides used to clone the *Raet1* sgRNA: Fwd: 5'-CACCCGGTAGTGCAACTTGACCATCA-3', Rev: 5'-AAACTGATGGTCAAGTTGCACTACC-3'. The underlined GG dinucleotide, which is
not present in the coding sequences, was added to the 5’ end of the sgRNA sequence to allow for better transcription from the T7 promoter.

Constructs and transduction of cells.

Segments encoding epitope tagged full length MULT1 (a mutant version (KR) in which the six lysine residues in the cytoplasmic tail were mutated to arginines in order to optimize cell surface expression (4) and secMULT1 (amino acids 1-211) and secMICA (amino acids 1-307) were cloned into the pMSCV2.2-IRES-GFP retroviral vector. Retroviral supernatants were generated by cotransfecting 293T cells with plasmids encoding VSV gag/pol and env and pMSCV retroviral constructs using Lipofectamine 2000 (Invitrogen). Culture supernatants collected 48h after transfection were added directly to actively proliferating cells, and transduced cells were sorted based on GFP expression. A segment encoding secMULT1 (no tag) was also cloned into a Tet-inducible lentiviral vector pFG12-TRE-UbC-rtTA-Thy1.1. For in vitro experiments, cells were cultured in 100 ng/ml doxycycline (Sigma-Aldrich) in complete DMEM medium. For induction in vivo, the drinking water was supplemented with 0.2 mg/ml doxycycline in 0.5% sucrose solution, which was kept in light-protected bottles and refreshed every week.

Immunoprecipitation and Immunoblot Analysis

Transduced fibroblasts were cultured in serum-containing medium (5%) for 48 hours and switched to serum-free medium for 24 hours. Supernatants were collected and concentrated with 10 KDa Amicon Ultra-15 Centrifugal filter Units (Millipore). Protease inhibitor mixture tablets (Roche, Basel, Switzerland) were added to the
supernatants. Immunoprecipitation, PNGaseF treatment and immunoblot analysis of supernatants and cell lysates were performed as previously described (5).

**MULT1 sandwich ELISA**

In brief, plates were coated with 2 µg/ml of MULT1 capture antibody (clone 237104, R&D) overnight at 4°C. After blocking with 2% BSA-PBST, plates were washed with 0.05% Tween 20 in PBS. Samples, or recombinant MULT1 (R&D) (which served as a standard), were diluted in 0.05% Tween 20 in PBS and incubated overnight at 4°C. After washing, biotinylated MULT1 detecting antibody (clone 1D6) was added at a concentration of 1 µg/ml. After incubation and washing, HRP-conjugated streptavidin (BD Pharmingen, diluted 1/2000) was applied. Plates were washed extensively before adding the peroxidase substrate TMB (Sigma). HRP activity was stopped by addition of H₂SO₄, and absorbance was measured at 450 nm. Male Apoe⁻/₋ mice were fed on a high fat diet for two months, starting at the age of six weeks, before their sera were collected, as previously described (6).

**Purification of MULT1 protein and MI6 F(ab')₂**

Shed MULT1 and secMULT1 were purified from concentrated supernatants of fibroblasts transduced with HA-MULT1(KR) and HA-secMULT1, by HA immunoprecipitation followed by elution with HA peptide (Sigma-Aldrich). The HA peptide was removed with 10 kD Amicon Ultra-15 Centrifugal filter units. MI6 F(ab')₂ fragments were prepared using immobilized pepsin (Pierce) according to the manufacturer’s instructions. After purification of F(ab')₂ fragments with 50 kD Amicon Ultra-15 Centrifugal filter units, no Fc fragments or intact antibody were
detected by SDS-PAGE. Rat IgG F(ab')2 (Jackson Immunoresearch) was used as a control.

**Surface plasmon resonance (SPR) assay**

All experiments were performed on a BIACore 2000 (Biacore) at 25°C using HBS-P (Fisher Scientific) as flow buffer. Biotin-NKG2D or biotin-CD19 were affixed to SA sensor chip (GE) with the desired level of Rmax (300-400RU). ShedMULT1 and secMULT1 (1, 2, 5,10, 20 nM) were sequentially analyzed for binding to SPR flow cells derivatized with biotin-anti-CD19 (negative control) or biotin-NKG2D. Flow rates were 10 µl/min and regeneration was performed for 30s with 100 mM NaOH. Steady-state experiments were performed in triplicate over two separate surfaces. Raw data were analyzed and graphed using BIAeval 3.1 (BIACore) and Prism Software.

**In vivo and ex vivo analyses**

In preparation for ex vivo analyses, 5 x 10^6 irradiated tumor cells (120 Gy) were injected intraperitoneally as described (7). Peritoneal cells were collected after 3 days and were examined by flow cytometry or used to sort NK cells and prepare RNA for RT-qPCR analysis. Peritoneal wash cells were pooled for cytotoxicity and IFN-γ assays.

Fresh tumor tissues or matrigel plugs were excised from mice, weighed, minced, and dissociated using the gentleMACS Dissociator (Miltenyi Biotec). Dissociated tumor samples were further digested in RPMI containing 200 µg/ml Collagenase IV (Roche) and 20 µg/ml DNase I (Sigma-Aldrich) at 37°C for 30 min. After filtering through a 40 mm nylon mesh, single cell suspensions were counted and used for experiments.

**Antibody staining and flow cytometry analysis**
Dead cells were excluded by staining with Live-Dead fixable dead cell stain kit (Molecular Probes, Grand Island, NY) following manufacturer’s instructions. Before staining, cells were preincubated for 20 min with 2.4G2 hybridoma supernatant to block FcrRII/III receptors. Cells were stained with the specified antibodies in 50 µl of PBS supplemented with 2% FCS (FACS buffer). Intracellular staining was performed after surface staining with the Cytofix/Cytoperm kit (BD Bioscience) according to manufacturer’s instructions. Multicolor flow cytometry was performed on a cytometer (LSRII; BD), and data were analyzed with FlowJo software (Tree Star, Inc.).

**Cytotoxicity and IFNγ assays**

NK cell cytotoxicity was determined with a standard 4-h $^{51}$Cr-release assay, with a pool of cells from three mice, tested in quadruplicate (2). The spontaneous release was, in all cases, <20% of the maximum release. The percentage of specific $^{51}$Cr release was calculated according to the following formula: % specific lysis = 100 × (experimental release − spontaneous release)/(detergent release − spontaneous release). In some experiments, LIVE/DEAD fixable stain kits (Invitrogen) were used to measure the killing of target cells (GFP$^+$) (8). NK cells and the target cells were incubated for 2h and the total cells were stained with LIVE/DEAD fluorescent dye to detect dead cells. The percentages of violet cells (dead cells) in the GFP$^+$ population were used to calculate the percentages of specific lysis according to the following formula: % specific lysis = 100 x (%dead cells in experimental sample - %dead cells in spontaneous control sample)/(100% - %dead cells in spontaneous control sample).

For assaying IFNγ producing cells, 2x10$^5$ – 1x10$^6$ cells were stimulated in flat-bottomed, high protein-binding plates (Thermo Fisher Scientific, Waltham, MA) for 4h in the presence of 1 µg/ml GolgiPlug (BD) and 1000 U/ml recombinant human IL-2.
(National Cancer Institute), before staining the cells for intracellular IFNγ. For some of
experiments, CD107a staining was also assayed. For those experiments, an additional 1
µg/ml GolgiStop (BD) and CD107a antibody were added to the wells before stimulation.
As indicated, the wells were coated with 5 µg/ml NKG2D, 50 µg/ml NKRP1C, or 5 µg/ml
NKp46 antibodies. In other cases the wells contained an equal number of tumor target
cells. Between 3 and 12 (usually 4-5) replicate samples per group were compared.

**Quantitative RT-PCR**

Total RNA was isolated using Trizol LS (Invitrogen), followed by digestion of
contaminating DNA using DNA-free (Ambion) according to the manufacturer's protocol.
RNA was reverse transcribed to cDNA using iScript reverse transcription system (Bio-
Rad Laboratories) according to the manufacturer's instructions. Quantitative real-time
PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using SSO-Fast
EvaGreen Supermix (Bio-Rad Laboratories). 18S rRNA and RPL19 were used as
references. The primers used for qRCR analyses are listed in Extended Data Table 1.

**Gel filtration FPLC**

Concentrated supernatants of Fibro-MULT1 and RMA-secMULT1 were
fractionated by Gel filtration chromatography with a Superdex 200 column on an FPLC
system. Pools correspond to >85 kD (1-10), ~65-85 kD (11-20), ~50-65 kD (21-30), ~35-
50 kD (31-40), ~20-35 kD (41-50), <20 kD (51-60).

**Statistical analysis.**

Statistical comparisons were performed with the Prism software (GraphPad
Software). P-values were determined as follows:

One-way ANOVA Kruskal-Wallis test with Dunns multiple comparisons: Supplementary Figure 7.

One-way ANOVA, after Kolmoogorv-Smirnov normality test, with Bonferroni’s multiple comparison tests: Supplementary Figures 14A,B.

Mann-Whitney tests: Figure 2E and Supplementary Figures 12 and 13B.

Differences between groups were considered significant for P-values <0.05. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
Supplementary Table 1 List of forward and reverse primers used in Q-RT-PCR and the amplicon size for each target

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (F) and reverse (R) primers (5’-3’)</th>
<th>Amplicon size</th>
</tr>
</thead>
</table>
| *Klrk1* | F: ACAGGATCTCCCTTCTCTGCT  
R: AAGTATCCCACCTTTGCTGGCT | 75bp          |
| *Rn 18s* | F: GTCTGTGATGCCCCTTAGATG  
R: AGCTTATGACCCGCACCTAC | 177bp        |
| *Rpl19* | F: AGCCTGTGACTGTCCATTCC  
R: GGCAGTACCCTTCTCTTCC | 98bp         |
| *Il1b*  | F: CAACCAACAAGTGATATTCTCCATG  
R: GATCCACACTCTCCAGCTGCA | 155bp        |
| *Il6*   | F: CTGCAAGAGACTTCCATCCAGTT  
R: GAAGTAGGGAAGGCCGTGG | 70bp         |
Supplementary Figure 1

C-terminal MULT1 “stub” detected in lysates of MULT1-HA-transduced fibroblasts (middle lane after HA immunoprecipitation, treatment with PNGaseF, and blotting with HA antibody. The band at 25 kD was a background band.

Supplementary Figure 2

Shedding of MULT1 is inhibited by Matrix metalloproteinase (MMP) inhibitors. HA-MULT1 transduced fibroblasts were treated with DMSO, the broad-spectrum inhibitor of MMPs and other metalloproteinases BB-94 (5 µM), the broad spectrum MMP inhibitor GM6001 (10 µM), a cysteine/serine/threonine protease inhibitor leupeptin (10 µM), or an ADAM17 inhibitor TAP-1 (10 µM) for 24 hours in serum-free medium. Cell lysates and concentrated supernatants were subjected to HA immunoprecipitation and PNGase F treatment followed by HA immunoblotting.

Supplementary Figure 3

MULT1 is released from cell lines that naturally or ectopically express MULT1. ELISA detection of soluble MULT1 in 20x concentrated culture supernatants of transduced fibroblasts, or the indicated unconcentrated supernatants of untransduced cell lines that do or don’t express MULT1 (as indicated).

Supplementary Figure 4

RMA tumor cells expressing secMULT1 are rejected. (A) Comparison of subcutaneously transferred RMA lymphoma tumor cells transduced with secMULT1, full length MULT1 or
empty vector, in WT B6 (syngeneic) mice. (B) RMA cells or B16 cells (before injection) were stained with MULT1 Abs. Grey shows isotype control staining.

**Supplementary Figure 5**

**NK cells participate in the rejection of B16-secMULT1 cells.** Subcutaneous growth of B16-secMULT1 tumors in B6-\(\text{Rag}^1\) mice (1 x 10^4 cells were inoculated) treated with control IgG or NK1.1 antibody (PK136) to deplete NK cells.

**Supplementary Figure 6**

**Inducible expression of secMULT1 in B16 tumor cells.** B16-pFG12-secMULT1 or B16-pFG12-GFP cells were cultured with or without 100 ng/ml doxycycline (Sigma-Aldrich) in complete DMEM medium for 4 days, at which time culture supernatants were concentrated and subjected to MULT1 immunoprecipitation and gel analysis.

**Supplementary Figure 7**

**Increased NK cell percentages among peritoneal wash cells in mice injected i.p. with irradiated B16 or B16-secMULT1 tumor cells.** B6 mice were injected i.p. with 5 x 10^6 irradiated (120Gy) B16 or B16-secMULT1 cells, or with PBS. Peritoneal wash cells were recovered three days later. Percentages of NK (NK1.1^+CD3^-) cells were determined by flow cytometry.

**Supplementary Figure 8**
Exposure to secMULT1 expressing RMA or B16 cells induces NK activity. (A) B6 mice were injected i.p. with $2 \times 10^6$ irradiated (120 Gy) RMA or RMA-secMULT1 cells. 3 days later, peritoneal wash cells were harvested and tested for killing of RMA-MULT1 (left) or RMA (right) target cells in vitro. For both target cell types, RMA-secMULT1-induced killing was significantly higher than RMA-induced killing. (B) B6 mice were injected i.p. with $5 \times 10^6$ irradiated (120 Gy) B16 or B16-secMULT1 cells, or with PBS. Peritoneal wash cells were recovered three days later and stimulated with immobilized NKR-P1C for 4 hours and tested for intracellular IFNγ.

Supplementary Figure 9

Recombinant MULT1 causes tumor rejection. (A,B) Subcutaneous tumors were established with 3-5 x 10^5 B16 cells in 100 µl matrigel. The tumor cells in one group were mixed with 1 µg of recombinant MULT1 (rMULT1). After 4 days, an additional 1 µg of rMULT1 was injected into each matrigel/tumor for that group. Tumors in the control group received PBS. On day 7, tumors were extracted for experiments.

Supplementary Figure 10

Soluble MULT1 is monomeric. (A) Shed MULT1 and secMULT1 exhibit similar apparent molecular weights (~35 kD), based on Western blots of HA immunoprecipitates of samples from fibroblasts transduced with full length MULT1 and secMULT1. Lanes from the same gel were spliced together for simplicity. (b, c) Shed MULT1 and secMULT1 are monomeric. Concentrated shed MULT1 (B) or secMULT1 (C) were fractionated by S200 gel filtration FPLC, and fraction pools were immunoprecipitated and blotted with HA Abs. Pools
correspond to >85 kD (1-10), ~65-85 kD (11-20), ~50-65 kD (21-30), ~35-50 kD (31-40),
~20-35 kD (41-50), <20 kD (51-60).

Supplementary Figure 11

Sustained exposure of peritoneal NK cells to rMULT1 in vitro amplifies NK functionality. Peritoneal wash cells, which include NK cells and RAE-1+ myeloid cells, among other cell types, were incubated for 4, 8 or 20 hours with the addition of 10 µg/ml rMULT1 or PBS to the cultures. For the 4 hr timepoint cells were incubated with rMULT1 during the stimulation assay. For the 8 hr timepoint, cells were incubated with rMULT1 for 4hrs before addition to stimulation wells for an additional 4 hrs. For the 20 hr timepoint, cells were incubated with rMULT1 for 16 hrs before addition to stimulation wells for an additional 4 hrs. Note that NK cell activity diminishes after prolonged in vitro culture, and therefore the control responses for different time points cannot be directly compared.

Supplementary Figure 12

No increase in NKG2D mRNA after exposure to secMULT1 in intraperitoneal tumors. Mice were injected i.p. with PBS, 5 x 10⁶ irradiated B16 or B16-MULT1 cells, and peritoneal wash cells were harvested 3 days later and used to sort NK cells and prepare RNA for RT-qPCR analysis.

Supplementary Figure 13

Blockade or absence of NKG2D result in increased NK functional activity. (A) Splenic NK cells from B6 WT and NKG2D-deficient mice were stimulated ex vivo with immobilized
NKp46 or NKRP1C Abs, and the IFN-γ responses of gated NK cells were determined. (B) Subcutaneous tumors were established with 5 x 10⁵ B16 cells in 100 µl matrigel, to which was added either 10 µg of MI6 F(ab')₂ or F(ab')₂ of rat IgG. After 4 days, an additional 10 µg of F(ab')₂ (or control F(ab')₂) was injected into the matrigel/tumors. On day 7, tumors were extracted and weighed (B). The tumors were then dissociated, the cells within the tumor were stimulated with immobilized NKp46 Abs, and the IFN-γ responses of gated NK cells were determined (C).

Supplementary Figure 14

secMICA fails to restore NK responses. B6 mice received i.p. injections of 5 x 10⁶ irradiated B16 cells, B16-secMULT1 cells or B16-secMICA cells. Three days later peritoneal wash cells were harvested and either stained with NKG2D antibody (A) or stimulated in vitro with YAC-1 tumor cells before performing staining for intracellular IFN-γ (B). The secMICA fragment corresponded to amino acids 1-307 of MICA.

References


Supplementary Fig. 1

Cell Lysates

<table>
<thead>
<tr>
<th>Flag</th>
<th>MULT1-HA</th>
<th>HA-MULT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector</td>
<td></td>
<td></td>
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<tr>
<td>Flag-MULT1-HA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA-MULT1</td>
<td></td>
<td></td>
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</tbody>
</table>

(kD)

50

37

MULT1
(full length)

MULT1
TM/Cyto stub

IP: α-HA
PNGase F
IB: α-HA
Supplementary Fig. 2

<table>
<thead>
<tr>
<th>Cell lysates</th>
<th>Culture Supernatants</th>
</tr>
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<tbody>
<tr>
<td>Fibro</td>
<td>DMSO</td>
</tr>
<tr>
<td>HA-MULT1</td>
<td>DMSO</td>
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</tbody>
</table>

Ratio: 1 0.3 1 0.003 0.8

kDa: 50 37 25 20

IP: α-HA
PNGase F
IB: α-HA

MULT1
shed MULT1
Supplementary Fig. 3

![Graph showing Soluble MULT1 (ng/ml)]

- **Fibroblasts (20X conc)**
  - None HA-MULT1
  - MULT1

- **Soluble MULT1 (ng/ml)**
  - 0
  - 50
  - 100
  - 150
  - 200
  - 250
  - 300
  - 350
  - 400
  - 450
  - 500
  - 550
  - 600
  - 650
  - 700
  - 750
  - 800
  - 850
  - 900
  - 950
  - 1000

- **Cell Lines**
  - WEHI7.1
  - C1498
  - YAC-1
  - 293T

- **MULT1**
  - MULT1
  - MULT1

- **Values**
  - MULT1
  - MULT1
  - MULT1
  - MULT1
  - MULT1
  - MULT1
  - MULT1
  - MULT1
Supplementary Fig. 4

A

Tumor volume (mm$^3$)

Days after implantation

RMA lymphoma 2 x 10$^5$/mouse

RMA

***** RMA-secMULT1

**** RMA-MULT1

B

B16

RMA

B16-MULT1

RMA-MULT1

B16-secMULT1

RMA-secMULT1

MULT1
Supplementary Fig. 5

![Tumor volume graph](image-url)
Supplementary Fig. 6

B16 Supernatants

<table>
<thead>
<tr>
<th></th>
<th>sec MULT1</th>
<th>GFP</th>
<th>sec MULT1</th>
<th>GFP</th>
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<tbody>
<tr>
<td>pFG12- DOX</td>
<td>-DOX</td>
<td>+DOX</td>
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</tbody>
</table>

IP: α-MULT1
IB: α-MULT1
Supplementary Fig. 7

% NK Cells

Mice PBS B16 B16-

Inj with: secMULT1

* ns

PBS      B16      B16-

secMULT1

% NK Cells

Mice PBS B16 B16-

Inj with: secMULT1

* ns
Supplementary Fig. 8

A

Target: RMA-MULT1

% Specific Lysis

target: RMA-MULT1

Effector:Target

% Specific Lysis

target: RMA

Effector:Target

B

Control

anti-NKRP1C

%IFN^γ^ NK cells

Mice

Inj with:

PBS B16 B16-secMULT1

* ****
Supplementary Fig. 9

A

3-5x10^5 B16 S.C. + Matrigel + rMULT1 (Day 0 and 4, intratumoral)

Monitor tumor growth and NK cell function

7 days

B

control + rMULT1

14 ± 1.7  4.9 ± 1.6

Cell # x 10^6
Supplementary Fig. 12
Supplementary Fig. 13

A

%IFNγ⁺ NK cells

Kirk1+/+

Kirk1−/−

PBS anti-NKp46 anti-NKRP1C

B

Tumor weight (mg)

Rat F(ab′)2 MI6 F(ab′)2

intratumoral injection

C

%IFNγ⁺ NK cells

Rat F(ab′)2 MI6 F(ab′)2

intratumoral injection

PBS anti-NKp46
Supplementary Fig. 14

(A) NKG2D Intensity

(B) Response to YAC-1 stimulation

IFNγ⁺ NK cells (%)

B16  B16-secMULT1  secMICA

NKG2D MFI

B16  B16-secMULT1  secMICA

NS

***  **

****  ****

Supplementary Fig. 14