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Antibody-mediated inhibition of MICA and MICB shedding promotes NK cell–driven tumor immunity

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MICA and MICB are expressed by many human cancers as a result of cellular stress, and can tag cells for elimination by cytotoxic lymphocytes through natural killer group 2D (NKG2D) receptor activation. However, tumors evade this immune recognition pathway through proteolytic shedding of MICA and MICB proteins. We rationally designed antibodies targeting the MICA α3 domain, the site of proteolytic shedding, and found that these antibodies prevented loss of cell surface MICA and MICB by human cancer cells. These antibodies inhibited tumor growth in multiple fully immunocompetent mouse models and reduced human melanoma metastases in a humanized mouse model. Antitumor immunity was mediated mainly by natural killer (NK) cells through activation of NKG2D and CD16 Fc receptors. This approach prevents the loss of important immunostimulatory ligands by human cancers and reactivates antitumor immunity.

The stress proteins MICA and MICB are expressed by many human cancers as a consequence of genomic damage, enabling elimination of cancer cells by cytotoxic lymphocytes expressing the natural killer group 2D (NKG2D) receptor. However, tumors evade this immune recognition pathway through proteolytic shedding of MICA and MICB proteins. We rationally designed antibodies targeting the MICA α3 domain, the site of proteolytic shedding, and found that these antibodies prevented loss of cell surface MICA and MICB by human cancer cells. These antibodies inhibited tumor growth in multiple fully immunocompetent mouse models and reduced human melanoma metastases in a humanized mouse model. Antitumor immunity was mediated mainly by natural killer (NK) cells through activation of NKG2D and CD16 Fc receptors. This approach prevents the loss of important immunostimulatory ligands by human cancers and reactivates antitumor immunity.

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(10–12). The membrane-proximal MICA and MICB α3 domain is the site of proteolytic shedding, whereas the membrane-distal α1 and α2 domains bind to the NKG2D receptor (Fig. 1A) (9, 21, 22). We hypothesized that shedding could be inhibited in a highly specific manner, with antibodies binding to key epitopes on the MICA and MICB α3 domain required for initiation of shedding and that such antibodies would not interfere with NKG2D binding. We further reasoned that the Fc segment of such antibodies could contribute to therapeutic efficacy by engaging activating Fc receptors. We immunized mice with the recombinant MICA α3 domain and identified three monoclonal antibodies (mAbs) (7C6, 6F11, and 1C2) that bound to the α3 domain and the full-length MICA extracellular domain (Fig. 1B and fig. S1, A, B, and D). MICA and MICB genes are polymorphic, but the α3 domain is more conserved than the α1 and α2 domains, explaining why these antibodies bound to all tested MICA variants and also MICB (fig. S1, B and C).

Functional studies showed that MICA and MICB α3 domain-specific antibodies strongly inhibited MICA shedding by a diverse panel of human tumor cell lines, resulting in a substantial increase in the cell surface density of MICA (Fig. 1, C and D, and fig. S2, A and B). By contrast, the previously reported 6D4 mAb (23) bound outside the MICA α3 domain and did not inhibit MICA shedding (Fig. 1, B to D, and fig. S2B). The α3 domain–specific antibodies also reduced MICA and MICB shedding by murine tumor cell lines expressing cDNAs encoding full-length human MICA or MICB under the control of a lentiviral vector (figs. S2C, S3, A to C, and S4D) but did not affect amounts of secreted MICA by cells expressing only the MICA extracellular domain (fig. S4, C and D). These antibodies minimally affected detection of recombinant soluble MICA by enzyme-linked immunosorbent assay (ELISA) (fig. S4, A and B) and did not interfere with NKG2D binding to MICA (fig. S5, A to C). Antibody-mediated targeting of the MICA and MICB α3 domain could thus specifically inhibit proteolytic shedding of these NKG2D ligands.

We selected mAb 7C6 for further experiments because it was most effective in stabilizing MICA and MICB on the surface of tumor cells (figs. S3, A and C). NKG2D is an important receptor for NK cell–mediated cytotoxicity, and we found that the 7C6 mAb (with human immunoglobulin G1 Fc region hIgG1) enabled strong NK cell–mediated killing of human tumor cells, including tumor cell lines not targeted by NK cells in the absence of this antibody (Fig. 1E and figs. S6A and S7).

Although only primates have MICA and MICB genes (1), human MICA is recognized by the murine NKG2D receptor (fig. S9), which enabled preclinical testing in syngeneic, fully immunocompetent mouse tumor models. We introduced the MICA cDNA with a lentiviral vector into murine B16F10 melanoma and CT26 colon cancer cell lines and tested the activity of the 7C6 antibody in lung metastasis models. Treatment with 7C6 antibody (mouse immunoglobulin G2a Fc region, mlgG2a) strongly reduced the number of lung metastases formed by B16F10-MICA tumor cells (Fig. 2A and fig. S10). Notably, MICA concentrations were high in sera of mice treated with an isotype control antibody but became undetectable in mice treated with 7C6 antibody (Fig. 2C). The 7C6 mAb also demonstrated efficacy in a lung metastasis model with CT26 cells expressing MICA (Fig. 2D) and could be detected on the surface of B16F10-MICA cells in subcutaneous tumors (fig. S11A and B). Also, increased MICA expression was detected on the surface of B16F10-MICA tumor cells when mice were treated with 7C6-mlgG2a compared to isotype control antibody (fig. S12). Interestingly, endogenous anti-MICA antibodies naturally arose in mice inoculated with MICA-expressing tumor cells (fig. S11D). Murine IgG1 was the predominant isotype for these antibodies (fig. S11E), an isotype associated with poor antitumor activity (25). These endogenous antibodies did not affect detection of recombinant MICA by ELISA (fig. S1F), did not slow tumor growth (fig. S1G), and moderately inhibited detection of shed MICA in serum samples (fig. S11H). Accordingly, 7C6 treatment inhibited growth of subcutaneous B16F10-MICA melanomas more effectively in B cell–deficient mice (Igκ−/−) that were unable to mount such an antibody response (fig. S11, I and
The therapeutic efficacy was lost in perforin (metastases (Fig. 2F and fig. S12B). Furthermore, MICA and MICB shedding had antitumor activity. Results demonstrate that a mAb that inhibited clearance of secreted MICA protein (fig. S12A). These ligands (Fig. 2E). Furthermore, 7C6 mAb inhibited domain of MICA or that lacked these NKG2D.

The efficacy of the 7C6 mAb was restricted to tumor cell apoptosis and a substantial reduction in the number of lung metastases while enhancing infiltration of lung tissue by activated NK cells (fig. S14, A to D). We also identified ILC1 in lung tissue of naive mice that had not been injected with tumor cells (fig. S19, A and B), indicating that ILC1 originated from a lung-resident cell population. Taken together, these data indicated that treatment with this MICA antibody resulted in a notable activation of tissue-infiltrating NK cells and expression of cytotoxicity genes.

Using flow cytometry, we validated key findings from the single-cell RNA-seq study. Lung-infiltrating NK cells were identified using EOMES and CD49b as markers, whereas lung-resident ILC1 were positive for CD49a, CD226, CXCR3, and CXCR6 (fig. S20A). Staining for GZMA allowed identification of activated NK cells that also expressed EOMES and CD49b (fig. S20A). Quantification of EOMES’ GZMA’ cells demonstrated an approximately fourfold increase of these activated NK cells (adjusted for tumor burden) on days 4, 7, and 11 in 7C6-mIgG2a-treated mice compared to isotype control antibody–treated mice with lung metastases (Fig. 3C and fig. S20C).

The presence of lung metastases increased absolute numbers of tissue-resident NK cells and ILC1 as shown by comparison of naïve mice and isotype control antibody–treated mice with lung metastases (figs. S16B and S20B). ILC1 expressed higher amounts of NKG2D at the protein level, but not at the mRNA level, compared to NK cells (Fig. 3B and fig. S20A). Also, surface levels of NKG2D were higher among tissue-infiltrating NK cells than blood NK cells (figs. S16C and S21A). However, NKG2D surface levels were substantially reduced among tissue-resident NK cells and ILC1 in tumor-bearing mice compared to naïve mice, even when tumor cells did not express NK cells are regulated by multiple activating (such as transforming growth factor–β) and inhibitory receptors, and both NKG2D and CD49b modulate NK cell functions (29). We introduced two mutations into the 7C6-hIgG1 and mIgG2b heavy chains [asparagine to alanine at position 265 (D265A) and asparagine to alanine at position 297 (N297A), or DANA] to abrogate binding to activating Fc receptors (25). 7C6-DANA mutant antibodies did not bind to the activating Fc receptor expressed by NK cells (CD16a) but retained MICA.

The efficacy of the 7C6 mAb was restricted to subcutaneous tumors that expressed full-length MICA or MICB; no therapeutic effect was observed for tumors that secreted the extracellular domain of MICA or that lacked these NKG2D ligands (Fig. 2E). Furthermore, 7C6 mAb inhibited MICA and MICB shedding but did not promote clearance of secreted MICA protein (fig. S22A). These results demonstrate that a mAb that inhibited MICA and MICB shedding had antitumor activity in fully immunocompetent mouse models. Antibody-mediated depletion revealed that NK cells, but not CD8 T cells, were essential for the therapeutic activity of 7C6 mAb against lung metastases (Fig. 2F and fig. S12B). Furthermore, therapeutic efficacy was lost in perforin (Prf1)– but not IFN-γ (Ifng)–deficient mice, indicating that NK cell–mediated cytoxicity represented an essential mechanism (Fig. 2G and fig. S12C). Treatment with 7C6 mAb was associated with tumor cell apoptosis and a substantial reduction of tumor cell load within lung tissue (fig. S13, A to E). The MICA antibody also had activity against established metastases. Treatment was delayed until day 7, when metastases were detectable, and 7C6-mIgG2a reduced serum MICA concentrations and the number of lung metastases while enhancing infiltration of lung tissue by activated NK cells (fig. S14, A to D).

We next examined the changes in gene expression by NK cells induced by MICA antibodies. Human NK cells cocultured with 7C6-hIgG1–pretreated human A375 melanoma cells upregulated genes associated with NK cell activation and effector functions (fig. S15, A and B). It was previously reported that tumors are infiltrated by group 1 innate lymphoid cells (ILCs), which are composed of NK cells and innate lymphoid cells 1 (IIC1) (26). We sorted group 1 ILCs from metastatic lung tissue by flow cytometry for single-cell RNA sequencing (RNA-seq); these tissue-infiltrating group 1 ILCs expressed CD69, a tissue residency marker, whereas blood group 1 ILCs (likely NK cells) were low in CD69 (fig. S16A). Single-cell RNA-seq demonstrated major differences in the composition and activation state of group 1 ILCs between 7C6-mIgG2a and isotype control treatment groups. In 7C6-mIgG2a–antibody treated mice, most group 1 ILCs (63.2%) were NK cells with a gene expression signature associated with activation and cytotoxicity, including expression of eomesodermin (EOMES), granzyme A (GZMA), granzyme B (GZMB), and perforin 1 (PRF1) (Fig. 3, A and B, and fig. S17, A and B). By notable contrast, a large fraction of cells (49.4%) in isotype control antibody–treated mice were ILC1 with a gene expression signature associated with cytokine and chemokine signaling and inflammation, including expression of the CXCR3 and CXCR6 chemokine receptors and lymphotixin β (LTB) (Fig. 3, A and B, and fig. S18, A and B).
binding (fig. S22, A to D). The 7C6-mIgG2b-DANA mutant antibody inhibited MICA shedding to the same extent as nonmutated mlgG2b and mlgG2a forms (fig. S23, A and B). Pretreatment of A375 cells with the 7C6-hIgG1-DANA mutant mAb induced killing by human NK cells, and this effect was blocked with an NKG2D-blocking mAb. This result demonstrates that inhibition of MICA shedding could induce NK cell–mediated cytolysis in the absence of Fc receptor engagement (Fig. 3D and fig. S23C). The 7C6-DANA mutant antibody also had therapeutic activity in the absence of Fc receptor engagement (fig. S23, D and E). These results demonstrate that inhibition of MICA and MICB shedding by 7C6 restored NKG2D-mediated tumor immunity.

Engagement of multiple activating receptors enhances NK cell effector functions (29, 30). 7C6 mAb with a fully functional Fc region (hlgG1) triggered stronger cytolysis by human NK cells than the 7C6-hlgG1-DANA mutant (Fig. 3D). We also addressed the contribution of NKG2D and CD16 Fc receptors to NK cell functions in vivo by transfer of wild-type (WT) or mutant NK cells into Rag2\(^{-/-}\)Il2rg\(-/-\) mice that were T cell and NK cell deficient. The most consistent reduction in the number of lung metastases was observed after transfer of WT NK cells. The therapeutic effect was maintained (but more variable) after transfer of NK cell deficient in either NKG2D (Klrk1\(-/-\)) or CD16 (Fcgr3a\(-/-\)). By notable contrast, antibody treatment was ineffective after transfer of NK cells that lacked both NKG2D and CD16 receptors, although shed MICA was still reduced (fig. 3E and fig. S12D). NKG2D and CD16 Fc receptors were also both required for optimal inhibition of subcutaneous tumor growth (fig. S24, A and B). These data demonstrate that 7C6 mAb activated NK cells through two important receptors, the NKG2D and CD16 Fc receptors.

In the syngeneic tumor models described above, MICA and MICB gene expression was induced by a heterologous promoter. However,
in human cancers, MICA and MICB gene expression is endogenously activated in response to malignant transformation (7). To test this therapeutic concept with human cancer cells and NK cells, NOD-scidIL2Rgnull (NSG) mice were reconstituted with human NK cells, followed by injection of human A2058 melanoma cells (Fig. 4A). IL-2 was injected every other day for a week to support NK cell survival. Inoculation of A2058 cells by an intravenous route resulted not only in lung metastases but, surprisingly, also widespread metastases in many other organs (Fig. S25C). Significantly fewer lung metastases were present in mice reconstituted with human NK cells and treated with 7C6-hlgG1 mAb (Fig. 4B and fig. S25A). Antibody treatment also reduced the spread of metastases to many other organs (fig. S25, C to E). Metastases were particularly
prominent in the liver and caused liver damage, as measured by a serum biomarker (alanine transaminase activity). Interestingly, 7C6-hIgG1 treatment substantially reduced the number of liver metastases and prevented liver damage even without NK cell transfer (Fig. 4C and fig. S26A). Liver-resident F4/80<sup>hi</sup> macrophages (Kupffer cells) that expressed activating Fc receptors (fig. S26B) had higher surface levels of the CD80 activation marker in 7C6-hIgG1 treated mice (fig. S26C). Macrophage depletion with clodronate liposomes abrogated the therapeutic activity of 7C6-hIgG1 antibody against liver metastases (Fig. 4D) but had no negative effect on therapeutic efficacy in the lung metastasis model in immunocompetent mice (fig. S27). Human macrophages cultured in vitro express MICA and MICB, and treatment with acetylated low-density lipoproteins, a model of foam cells present in atherosclerotic lesions, increased MICA and MICB expression (33). Treatment with 7C6-hIgG1 antibody inhibited MICA shedding and increased MICA and MICB surface levels on macrophages (fig. S28A to C). These mechanisms account for the significant survival benefit of 7C6-hIgG1 treatment in this humanized metastasis model (Fig. 4E). These data demonstrate the therapeutic activity of a MICA α3 domain–specific antibody in a humanized metastasis model by activating NK cells and macrophages in an organ-dependent manner.

We found that MICA and MICB α3 domain–specific antibodies substantially increased the density of the stimulatory MICA and MICB ligands on the surface of tumor cells, reduced shed MICA amounts, and induced NK cell–mediated tumor immunity. This therapeutic strategy restores the function of an activating immune pathway that promotes clearance of stressed and transformed cells (Fig. 4F). We propose that the association between MICA and MICB shedding and cancer progression is primarily due to the loss of immunostimulatory NK2D ligands on the tumor cell surface, although shed MICA may also be a relevant contributing factor. Interestingly, shedding of the high-affinity murine MULT-1 ligand of NK2D enhances antitumor immunity by inhibiting chronic NK2D engagement of intratumoral NK cells by myeloid cells that express RAE-1, a murine NKG2D ligand (32). Soluble MICA and MICB have a substantially lower affinility for the NKG2D receptor than MULT-1, which may explain why shed MICA and MICB do not have such a stimulatory function (7). Given that MICA and MICB are widely expressed in human cancers, MICA and MICB antibodies may hold promise for both solid and hematological malignancies (14, 33–36). Such antibodies could be used in combination with established therapies that induce or enhance MICA and MICB expression through genomic damage pathways, including local radiation therapy or antibody-drug conjugates that deliver toxic payloads to tumor cells (4). MICA antibodies are also of considerable interest as a combination partner with other immunotherapies to activate NK cells and enhance cytotoxic T cell function for protective antitumor immunity.

**REFERENCES AND NOTES**

Author contributions: K.W.W. and G.D. conceived the study; L.F.d.A., G.D., and K.W.W. designed experimental approaches and interpreted the data; L.F.d.A. characterized antibodies reported in this study and performed therapy as well as mechanistic experiments; R.E.T., Y.I., and L.F.d.A. performed flow cytometry experiments; D.P. analyzed RNA-seq data; S.B. generated some of the recombinant MICA proteins and the BISF3D-MICB cell line; A.M.L., D.T., and G.C.Y. generated single-cell RNA-seq data; B.F. and K.F.M. isolated the first MICA antibody; C.J.H. contributed to early stages of this project; S.K. performed in vivo studies; J.W.P. purified recombinant MICA and MICB proteins; C.Y. provided primary human melanoma cell lines and helped with the design of in vitro functional assays; F.S.H. contributed clinical expertise; and L.F.d.A. and K.W.W. wrote the paper.

Competing interests: The technology has been licensed by the Dana-Farber Cancer Institute to a pharmaceutical company. B.F., K.F.M., C.J.H., J.W.P., F.S.H., G.D., and K.W.W. are inventors on the relevant patent applications (WO 2014/144791 A3, WO 2015/179627 A1). Data and materials availability: Materials reported in this study are available through a materials transfer agreement with the Dana-Farber Cancer Institute and by contacting K.W.W. RNA-seq data are deposited in the Gene Expression Omnibus database under accession number GSE109542.

SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/359/6383/1537/suppl/DC1
Materials and Methods
Figs. S1 to S28

References
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K.W.W. and G.D. conceived the study; L.F.d.A., G.D., and K.W.W. designed experimental approaches and interpreted the data; L.F.d.A. characterized antibodies reported in this study and performed therapy as well as mechanistic experiments; R.E.T., Y.I., and L.F.d.A. performed flow cytometry experiments; D.P. analyzed RNA-seq data; S.B. generated some of the recombinant MICA proteins and the BISF3D-MICB cell line; A.M.L., D.T., and G.C.Y. generated single-cell RNA-seq data; B.F. and K.F.M. isolated the first MICA antibody; C.J.H. contributed to early stages of this project; S.K. performed in vivo studies; J.W.P. purified recombinant MICA and MICB proteins; C.Y. provided primary human melanoma cell lines and helped with the design of in vitro functional assays; F.S.H. contributed clinical expertise; and L.F.d.A. and K.W.W. wrote the paper.

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Helping NK cells find their way

MICA and MICB proteins can be expressed on tumors and act as "kill me" signals to the immune system. But tumors often disguise themselves by shedding these proteins, which prevents specialized natural killer (NK) cells from recognizing and destroying the cancer. Ferrari de Andrade et al. engineered antibodies directed against the site responsible for the proteolytic shedding of MICA and MICB (see the Perspective by Cerwenka and Lanier). The approach effectively locked MICA and MICB onto tumors so that NK cells could spot them for elimination. The antibodies exhibited preclinical efficacy in multiple tumor models, including humanized melanoma. Furthermore, the strategy reduced lung cancer metastasis after NK cell–mediated tumor lysis.

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