NKG2D-independent suppression of T cell proliferation by H60 and MICA

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The activating receptor NKG2D recognizes a wide range of different ligands, some of which are primarily expressed in "stressed" tissues or on tumor cells. Until now, similar stimulatory effects on natural killer and CD8$^+$ T cells have been described for all NKG2D ligands, and the NKG2D receptor/ligand system has therefore been interpreted as a sensor system involved in tumor immune surveillance and activation of immune responses. We show here that the NKG2D ligands H60 and MIC class 1 chain-related protein A (MICA) can also mediate strong suppressive effects on T cell proliferation. Responsiveness to H60- and MICA-mediated suppression requires IL-10 and involves a receptor other than NKG2D. These findings might provide explanations for the observation that strong *in vivo* NKG2D ligand expression, such as that on tumor cells, sometimes fails to support effective immune responses and links this observation to a distinct subgroup of NKG2D ligands.

immunology | NKG2D ligands

Innate immune cells, such as macrophages, neutrophils, and natural killer (NK) cells, respond to stimuli with rather invariable character and mediate the discrimination of self vs. foreign tissue ("missing self"), the recognition of foreign microbial substances ("microbial nonself"), and the rejection of tumor cells ("altered self") (1). These stimulatory mechanisms are tightly counter-regulated or modulated by inhibitory receptors, many of which recognize "self" MHC–peptide complexes (2). Interestingly, the immunomodulatory receptors commonly found on innate immune cells are also expressed on cells of the adaptive immune system, especially on CD8$^+$ T lymphocytes. These observations suggest a general role of immunomodulatory receptor–ligand interactions for the regulation of immune responses. In this context, the stimulatory receptor NKG2D and its ligands have attracted substantial attention over the past years, because this receptor/ligand system seems to play a central role in the complex regulatory network (3–5).

NKG2D is a homodimeric receptor that is variably expressed on the surface of NK cells, CD8$^+$ T cells, and a subset of potentially autoreactive CD4$^+$ T cells (6). Signal transduction via NKG2D is mediated through two main adaptor molecules, DAP10 and DAP12 (4). Different splice variants of NKG2D, NKG2D-L (long), and NKG2D-S (short), determine the association with intracellular DAP molecules (7, 8). Differential expression of NKG2D splice variants and differences in DAP recruitment have been demonstrated to determine stimulatory vs. costimulatory activities mediated by NKG2D ligation in mice. NK cells can be directly activated via NKG2D, whereas only costimulatory NKG2D-mediated activities, which depend on additional TCR-mediated signals as well as the presence of DAP10, have been described for CD8$^+$ T cells.

A variety of NKG2D ligands, which are distantly related to MHC molecules, have been identified. Human NKG2D binds to MIC class 1 chain-related protein A (MICA) and MICB (5) as well as to molecules belonging to the UL-16-protein (ULBP) family (9). Ligands for murine NKG2D are H60, members of the RAE1 family (10, 11), and MULT1 (12). It is likely that even more ligands for NKG2D exist (13). All NKG2D ligands identified so far share the characteristic that protein expression on the cell surface is almost undetectable in normal tissues, but expression can be induced by a variety of different forms of cell stress, such as heat shock (MICA) (14) or inflammation (15). In addition, NKG2D ligands are expressed on many tumor cells (3), and expression is heavily modulated by chronic virus infections like murine cytomegalovirus (9, 16–19). Based on these findings, the NKG2D receptor/ligand system has been interpreted as a sensor system specifically for the recognition of "stressed" cells and tumors. The NKG2D-mediated stimulatory activities described for the different ligands are very similar; however, because the binding affinities to NKG2D can differ substantially between ligands, it has been suggested that different expression levels of individual ligands might be an important parameter regulating the strength and outcome of stimulation (20).

Almost all *in vitro*-cultured tumor cell lines, as well as most endogenous tumors studied *in situ*, express at least one member of the large family of NKG2D ligands (11). In humans, the very high expression of MIC molecules on epithelial tumors is well documented (21). Overexpression of murine NKG2D ligands on tumor cell lines leads to rapid NK cell-mediated rejection of these tumor cells *in vivo* (22, 23) and induces, in some cases, effective tumor-reactive CD8$^+$ T cell responses that protect against a secondary challenge with tumor cells that lack (or have significantly reduced) surface expression of NKG2D ligands (22).

It is still a matter of debate why so many different ligands exist for the same receptor. In the context of infections, the multitude of structurally diverse ligands could be explained as a strategy to prevent rapid immune escape by pathogens through interference with NKG2D ligand expression. Very little is known about the mechanisms of NKG2D ligand induction and expression, but several studies suggest that the regulation of individual NKG2D ligands differs substantially, indicating that the heterogeneity of NKG2D ligands does not simply reflect redundant expression of molecules with identical functions. Even more puzzling is the strong expression of NKG2D ligands on endogenous tumor cells; why do most tumor cells not undergo selection for loss of these potent immunostimulatory molecules?

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Abbreviations: NK, natural killer; CFSE, carboxyfluorescein diacetate succinimidyl ester; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; TR cells, antigen-driven regulatory T cells; MICA/B, MIC class 1 chain-related protein A/B; APC, allophycocyanin.

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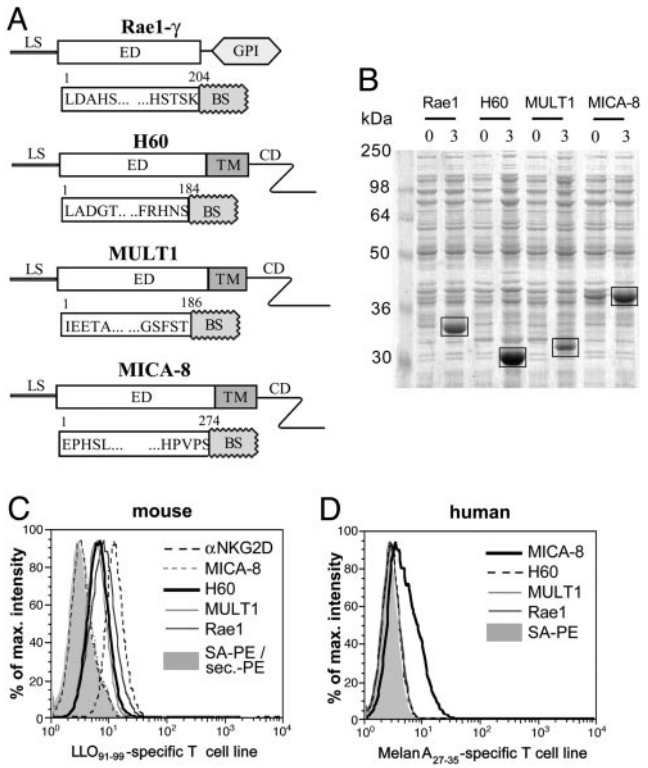

Fig. 1. Generation of soluble NKG2D ligands. (*A*) Schematic structure of NKG2D ligands from N to C terminus showing leader sequence (LS), extracellular domain (ED), transmembrane region (TM), and cytosolic domain (CD) or glycosylphosphatidylinositol-anchor (GPI). cDNAs were mutagenized at the positions indicated in single-letter amino acid code, and a biotinylation sequence (BS) was added. (*B*) Expression of recombinant proteins after transformation of expression vectors into BL21(DE3) hosts. Samples from each culture before (0) and 3 h after (3) isopropyl β-D-thiogalactoside induction were run over a 10% SDS/PAGE gel. Murine (*C*) and human (*D*) antigen-specific $CD8^+$ T cell lines were stained with multimers of NKG2D ligands, as indicated in the legends. Gray-filled histograms show background control staining using streptavidin PE (SA-PE), and dotted gray lines represent control staining with the PE-conjugated isotype-specific mAb used to visualize anti-NKG2D (mAb C7) staining on murine cells.

The exact physiological role of the heterogeneity of NKG2D ligands is unknown. We therefore addressed the question whether different NKG2D ligands trigger different functional activities. Indeed, we found that, in addition to their known stimulatory effects, distinct NKG2D ligands can also induce strong inhibitory effects, such as the suppression of T cell proliferation. This inhibition is almost absent in IL-10-deficient mice and involves binding to a receptor different from NKG2D.

Materials and Methods

Generation of Multimers. A C-terminal biotinylation tag was added to the extracellular domains of Rae1γ, H60 (cDNA kindly provided by N. Shastri), MULT1, and human MICA-8 by using standard PCR techniques on full-length cDNA (fusion protein design and exact flanking protein sequences shown in Fig. 1*B*). Recombinant biotinylated proteins were generated by using pET27b or pET3a expression vectors (Novagen) and subsequent *in vitro* refolding procedures as described (24, 25). For multimerization, PE- and allophycocyanin (APC)-conjugated streptavidin (Molecular Probes) was used.

Mice, Cell Lines, Antibodies, and Flow Cytometry. BALB/c and C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany). $DAP10^{-/-}$ mice were housed at the animal facility at Washington University; spleens from $TCR\gamma/\delta^{-/-}$ and $CD1^{-/-}$ mice were obtained from H. W. Mittrücker (Max Planck Institute for Infection Biology, Berlin); $L9.6/RAG^{-/-}$ (26) were generously provided by Eric Pamer (Memorial Sloan–Kettering, New York). MHC $II^{-/-}$ ($I\text{-}A\alpha^{-/-}$), $TLR4^{-/-}$, $MyD88^{-/-}$, and $IL\text{-}10^{-/-}$ mice were derived from in-house breeding at the Technical University Munich under specific pathogen-free conditions. Polyclonal LLO_{91-99}- and SIINFEKL-specific T cell lines were expanded by *in vitro* peptide stimulation, as described (27).

Purification of $CD8^+$ and $CD4^+$ T cells from spleens was achieved either by staining with fluorescence-labeled mAb [anti-CD8–phycoerythrin (PE) or anti-CD4-PE] and subsequent cell sorting on a MoFlo Cytometer (DakoCytomation, Glostrup, Denmark) or enrichment by standard protocols for magnetic cell sorting (MACS, Miltenyi, Bergisch Gladbach, Germany); only purities of >95% were considered as highly purified. All fluorescence-conjugated mAbs were purchased from Pharmingen. Flow cytometry was performed on a FACSCalibur (Becton Dickinson) and by using FLOJO software (Treestar, Ashland, OR).

Cytotoxicity Assays. Antigen-specific cytolytic activity of T cells was tested in standard 4-h ^{51}Cr-release assays performed in triplicate. ^{51}Cr-labeled targets (1×10^4; EL4 cells) were added per well of a 96-well plate. T cells (2×10^5 per well) from a SIINFEKL-specific T cell line were added together with different peptide concentrations and 10-μg/μl Rae1 or H60 multimers. The percentage of specific lysis was calculated as described (27).

Proliferation Assays. For carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assays, 2×10^6 cells were labeled with CFSE and stimulated with 0.2 μg/ml anti-CD3 mAb in the presence or absence of 10 μg/ml NKG2D-ligand multimer (streptavidin fluorochrome conjugate only as control) or monomer. Enriched $CD8^+$ T cells, enriched $CD4^+$ T cells, or human peripheral blood mononuclear cells (PBMC) (1×10^6 cells per well) were cultured in 24-well plates and stimulated with plate-bound anti-CD3. NKG2D blocking experiments were performed by adding mAb A10 (10 μg/ml) or C7 (10 μg/ml) to the cell cultures (28). IL-10 neutralizing experiments were performed by daily addition of mAb JES5–2A5 (5–10 μg/ml) to the cultures, as isotype control rat IgG1 (clone R3–34) was used; TGFβ blocking was done by adding mAb 1D1 (50 μg/ml). After 65 h, cells were stained with anti-CD8-PE or anti-CD4-APC and analyzed on a FACSCalibur.

RT-PCR. For the PCR experiments, total RNA of FACS-sorted NK cells (>96% $DX5^+$ cells) and $CD8^+$ (>92%) or $CD4^+$ (>98%) T cells was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen). The same amounts of cDNA were subjected to PCR. Primers for the unique 5′ UTR of the two NKG2D splice variants and the common 3′ site were used as described (7).

Human PBMC (hPBMC). hPBMC were obtained by Ficoll separation of blood samples from healthy individuals. FACS staining, CFSE labeling, and proliferation assays were performed as described above for murine splenocytes.

Generation of Stably Transfected Cell Lines Overexpressing NKG2D Ligands. Full-length coding sequences for H60 and RAE1e were ligated into the BamHI and XhoI sites of plasmid pMX-IRES-GFP. Vectors were transfected into the PLAT-E packaging line (courtesy of Toshio Kitamura, University of Tokyo, Tokyo). PLAT-E supernatants were used to transduce RMA cells, which were further sorted to purity by using a MoFlo (DakoCytomation), as assessed by unimodal log-normal GFP fluorescence intensity. Expression of ligands was verified by staining by using NKG2D tetramers.

***In Vivo* Tumor Challenge Model.** *In vivo* tumor challenge experiments were performed by transplantation of a thy1.1-positive tumor into thy1.1-negative recipients. *In vivo* application of biotinylated anti-thy1.1 mAb and streptavidin allows building of a bridge to target other biotinylated molecules directly to the tumor cell surface (so-called "three-step technology") (29). C57BL/6 mice (thy1.2 positive, $n = 20$, in four groups of five) were inoculated, with 5×10^4 RMA-S cells (thy1.1 positive) in the right flank. At day 2 after tumor implantation, mice were treated with sequential administrations of biotinylated antibodies, streptavidin, and biotinylated NKG2D ligands: step 1, 40 μg of biotinylated-mAb 19E12 (anti-thy1.1; i.p.); step 2 (after 19 h), 60 μg of streptavidin (to create the streptavidin–biotin bridge, i.p.); step 3 (24 h later), 40 μg of biotinylated H60 or Rae1. Control mice did not receive streptavidin in step 2. For 3 days, starting from day 4, 2,000 units of IL-2 were administered i.p. to each mouse. All mice were monitored every day for tumor growth by measuring two perpendicular diameters with a caliper. Mice were killed when tumor size exceeded 600 cubic millimeters. The data set was analyzed with an ANOVA model for repeated measures; variance analysis on single-day measurements was performed by using Student's *t* test (EXCEL, Microsoft).

Results

Generation of Recombinant NKG2D Ligands. To design experimental settings to precisely study interactions of defined NKG2D-ligand molecules with their receptor(s), we generated recombinant soluble molecules for all main groups of murine NKG2D ligands. Similar to the recently published generation of soluble murine NKG2D (24), the extracellular domains of Rae1γ, H60, and MULT1, as well as the human NKG2D ligand MICA allele 8 (MICA-8), were expressed in bacterial hosts (Fig. 1 *A* and *B*) and subsequently refolded and C-terminally biontinylated *in vitro* (25). To verify that all NKG2D ligands were correctly refolded and functional, we stained NKG2D-expressing cell lines with fluorochrome-labeled NKG2D ligand multimers. As summarized in Fig. 1*C*, a murine NKG2D-expressing $CD8^+$ T cell line (expression confirmed by staining with the NKG2D-specific mAb C7) bound multimers of all murine NKG2D ligands with similar staining intensity. In contrast, no binding of human MICA-8 to murine NKG2D-expressing cells (10 days after the last stimulation) was detected (Fig. 1*C* and data not shown). Staining of a human $CD8^+$ T cell line with the recombinant ligands demonstrated the opposite pattern: only the human ligand bound to human NKG2D-expressing cells (Fig. 1*D*). These data indicate that all ligands are correctly refolded and specifically bind to their species-matched NKG2D receptor molecules. Although it has been demonstrated that murine NKG2D can bind to some human NKG2D ligands, especially for MIC alleles, substantially different binding qualities to NKG2D have been described (30). According to our data, the MICA allele 8 does not bind strongly enough to murine NKG2D to result in detectable multimer staining.

NKG2D Ligands Demonstrate Moderate Costimulatory Activity on Cytotoxicity. In addition to the direct activation of NK cells, NKG2D ligands have been described to provide costimulatory signals to activated $CD8^+$ T cells, resulting in an increase of antigen-specific target cell lysis as well as an elevated release of effector cytokines (31). To address the question of whether these costimulatory effects differ depending on the NKG2D ligand, we performed cytotoxic T lymphocyte (CTL) assays with *SIINFEKL*-specific T cell lines in the presence or absence of defined NKG2D ligands. As shown in Fig. 2, the presence of NKG2D ligands slightly increased the sensitivity of SIINFEKL-specific CTL, which responded with more effective specific lysis to lower amounts of presented epitope (same results for MULT1; data not shown). Similar results have been described for human $CD8^+$ T cell lines (31). We did not observe any differences in this moderate costimulatory activity between the diverse ligands, and increasing concentrations of soluble NKG2D ligands did not further enhance these effects. We also did not observe significant differences in IFN-γ, IL-2, or TNF-α release into supernatants of murine CTL cultures short term (4 h) exposed to different NKG2D ligands (data not shown). Such effects have been described before for human $CD8^+$ T cells (31), but, consistent with our observations, were not found to be as strong for murine $CD8^+$ T cell lines (32, 33). Thus, the NKG2D ligands H60, Rae1, and MULT1 equivalently demonstrate weak costimulatory influences on $CD8^+$ T cell effector function.

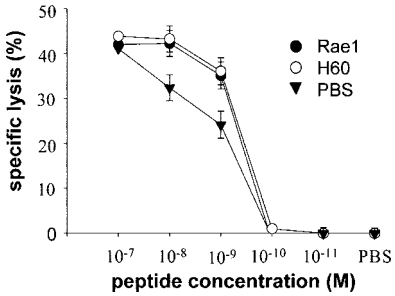

Fig. 2. Influence on different NKG2D ligands on effector function. SIINFEKL-specific T cells were incubated in the presence of target cells (EL-4) and different SIINFEKL peptide concentrations (*x* axis) with or without addition of 10 μg/ml soluble Rae1 (filled circles) or H60 (open circles) multimers. Specific lysis was determined in a standard chromium release assay. Filled triangles indicate control values in the absence of any soluble NKG2D ligand. Data are representative of at least three independent experiments. Assays were done in triplicate, and standard deviations are indicated.

Distinct NKG2D Ligands Interfere with T Cell Proliferation. We next examined the effects of NKG2D ligands on lymphocyte proliferation by CFSE-labeling experiments. Bulk cultures of anti-CD3-stimulated splenocytes (Fig. 3*A*) or SIINFEKL-specific polyclonal $CD8^+$ T cell lines (data not shown) were incubated in the presence or absence of NKG2D ligand multimer. In the absence of an additional TCR-mediated stimulus, soluble NKG2D ligands had no effect on T cell proliferation (data not shown). In combination with anti-CD3 stimulation, the presence of Rae1 or MULT1 did not significantly affect the number of cell divisions and the extent of proliferation. In contrast, the presence of H60 almost completely blocked $CD8^+$ T cell division (identical results were obtained when additional CD28 stimulation was provided, and increasing the H60 concentration did not result in further enhancement of inhibitory effects, data not shown). Even more surprising, $CD4^+$ T cell division was also strongly and selectively inhibited by H60 in the same bulk cultures (Fig. 3*B*). NKG2D expression has been described only for rare subsets of T helper cells (6), and the absence of NKG2D on conventional $CD4^+$ T cells was confirmed by extensive PCR analysis for mRNA expression of the known splice variants of NKG2D (Fig. 3*E*). We performed further CFSE-based proliferation assays on highly purified primary $CD8^+$ and $CD4^+$ T cells by using stimulation with plate-bound anti-CD3. As shown in Fig. 3 *C* and *D*, highly purified $CD8^+$ and $CD4^+$ T cells were still selectively inhibited in the presence of H60.

Because all experiments so far were performed by using recombinant proteins, we further included cell lines overexpressing NKG2D ligands into our studies. As shown in Fig. 3*F*, similar to the data using recombinant proteins, H60 inhibits T cell proliferation, whereas Rae1 does not, strongly suggesting that our findings are directly transferable to native proteins.

In summary, these data show that NKG2D ligands can mediate different functions and identify H60 as a potent suppressor of T cell proliferation. The suppressive effects of H60 are maintained in cultures containing highly purified T cells, even when populations without any detectable NKG2D expression are used.

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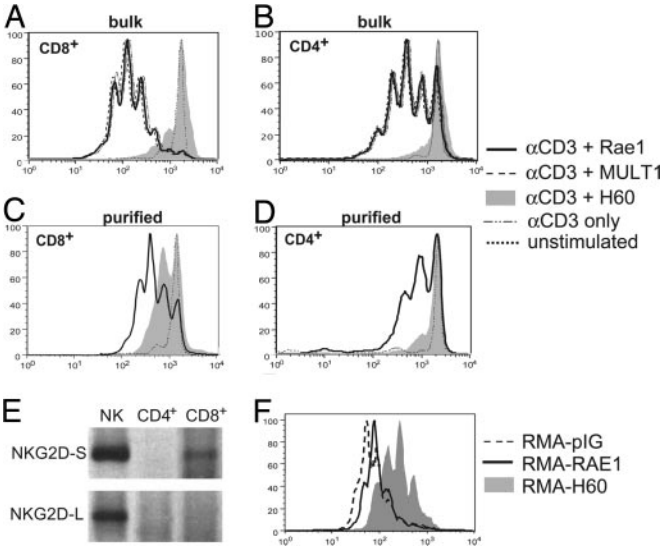

Fig. 3. H60-specific inhibition affects $CD8^+$ as well as $CD4^+$ T cells. (*A* and *B*) CFSE-labeled splenocytes (BALB/c) were analyzed 65 h after stimulation with anti-CD3 in the presence or absence of different soluble NKG2D ligand multimers. For analysis, cells were gated on $CD8^+$ (*A*) or $CD4^+$ (*B*) T cells. Fluorescence profiles represent cell divisions in the presence of Rae1 (bold line), MULT1 (dashes; sometimes hidden behind the bold line), H60 (filled gray), or no additional ligand as a control; the dotted line indicates the CFSE profile in the absence of any stimulation. (*C* and *D*) Highly purified (>95%) $CD8^+$ (*C*) and $CD4^+$ (*D*) T cells were stimulated with plate-bound anti-CD3 under the same experimental setting as in *A* and *B*. (*E*) Expression of NKG2D-S and -L splice variants was examined in highly purified NK cells and $CD8^+$ and $CD4^+$ T cells by RT-PCR. (*F*) Splenocytes from C57BL/6 mice were CSFE-labeled and stimulated with anti-CD3 in the presence of irradiated RMA cells overexpressing H60 (gray) or Rae1(black line); mock-transfected cells (RMA-pIG) served as negative controls (dashed line).

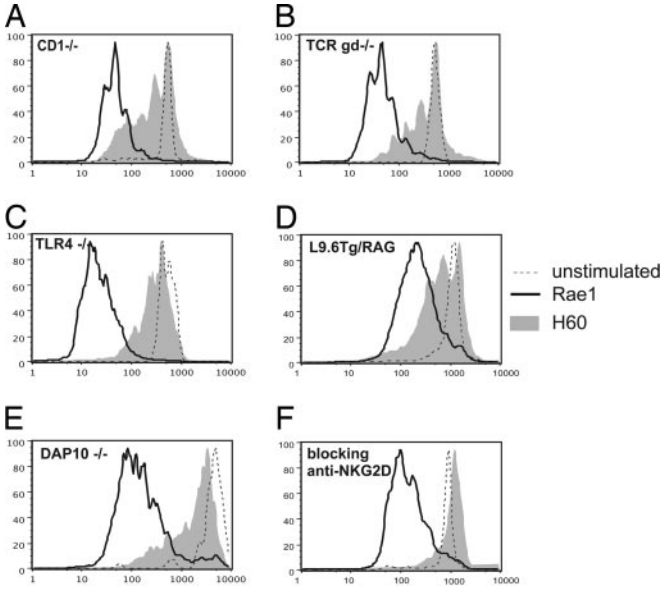

Fig. 4. H60-mediated T cell inhibition is independent of naturally arising Tregs and NKG2D. CFSE proliferation profiles of $CD8^+$ T cells upon anti-CD3 (*A–C*, *E*, and *F*) or peptide stimulation (*D*) in the presence or absence of soluble NKG2D ligands were determined as described for Fig. 3 (same symbols used for ligands and controls). Splenocytes were derived from different gene-deficient (*A–C* and *E*) or transgenic mice (*D*), as indicated near the top of each histogram. L9.6/$RAG^{-/-}$ cells (*D*) were stimulated in the presence of 1 μM $p60_{217–225}$ peptide. (*F*) Summary of data obtained for splenocytes derived from wild-type BALB/c mice stimulated in the presence of high concentrations of blocking NKG2D mAb (clone C7).

H60-Mediated T Cell Inhibition Is Independent of Naturally Arising T_{regs} and NKG2D. Suppression of T cell proliferation is a characteristic effector function of so-called regulatory T cells (T_{reg}). Different types of T_{reg} have been described in the past, including naturally arising $CD25^+/CD4^+$ T_{reg} (34), antigen-driven regulatory T cells [TR cells (35, 36)], as well as subsets of NK T cells (37) and γ/δ T cells (38). We tried to test all these candidate populations for selective binding or responsiveness to H60 and examined whether the suppressive effects of H60 were affected in bulk cultures depleted of lymphocyte subpopulations or in knockout mice ($CD1d^{-/-}$, TCR$\gamma/\delta^{-/-}$, and MHC $II^{-/-}$) with deficiencies in these subpopulations (Fig. 4 *A* and *B* and data not shown). In summary, we did not find any correlation of H60-specific inhibition of proliferation with naturally arising regulatory lymphocyte populations, CD1d-restricted NK T cells (Fig. 4*A*), or γ/δ T cells (Fig. 4*B*). We further excluded that potential endotoxin contaminations (measurements indicated levels below 0.1 ng/ml) contributed to inhibition of T cell proliferation by using TLR4-deficient mice (Fig. 4*C*) or MyD88 knockout mice (data not shown). Even T cells from *Listeria*-specific TCR-transgenic mice backcrossed on the $RAG^{-/-}$ background (L9.6tg/$RAG^{-/-}$, kindly provided by Eric Pamer) and stimulated with their cognate epitope ($p60_{217–225}$, Fig. 4*D*) were strongly and selectively inhibited by H60, indicating that the regulatory effects can be directly derived from antigen-specific T cell populations. This was further confirmed by assays using FACS-purified L9.6/$RAG^{-/-}$ $CD8^+$ T cells stimulated with artificial APCs (beads conjugated with purified H2-K^d/$p60_{217–225}$ complexes); in this system, antigen-specific T cells are specifically activated in the absence of any other cell populations, and even in this maximally reduced setting proliferation is still inhibited by H60 (data not shown). Furthermore, $CD8^+$ and $CD4^+$ T cells from $DAP10^{-/-}$ mice, which have been shown to lack NKG2D-mediated costimulation as well as NKG2D expression on $CD8^+$ T cells (7, 8), were also still strongly inhibited in the presence of H60 (Fig. 4*E*). Especially this last observation indicates that a receptor different from NKG2D is involved in H60-mediated inhibition of T cell proliferation, a notion further supported by the observation that H60-mediated inhibition was not affected by the presence of blocking NKG2D-specific mAb clones C7 (Fig. 4*F*) or A10 (data not shown) (28).

IL-10 Is Required for Responsiveness to H60-Mediated Suppression. We further analyzed the effects of a human NKG2D ligand on proliferation of human T cells. As shown in Fig. 5*A*, the presence of MICA-8 multimers during anti-CD3 stimulation of human PBMC substantially reduced T cell proliferation. Even more surprising, the murine NKG2D ligand H60 was also able to inhibit proliferation of human T cells. The same could be observed when incubating murine splenocytes with human MICA-8 (Fig. 5*A*). As demonstrated above (Fig. 1), we found no evidence that human MICA-8 binds stably to murine NKG2D or that the murine NKG2D ligands H60, Rae1, and MULT1 can bind to human NKG2D.

The generation of TR cells requires IL-10, and they can mediate their suppressive effects via both a poorly defined cell contact-dependent mechanism and by secretion of inhibitory cytokines such as IL-10 and TGFβ (39). Because our data point toward antigen-induced TR cells, we tested whether H60-mediated inhibition of lymphocyte proliferation depends on IL-10. Indeed, the effect of H60 on T cell proliferation was almost completely lost on splenocytes derived from $IL\text{-}10^{-/-}$ mice (Fig. 5*B*). Addition of neutralizing anti-IL10 antibodies to *in vitro* stimulation assays, however, had only minor affects on H60-mediated suppression (Fig. 5*B*), suggesting that IL-10 is essential for the generation or responsiveness of H60-triggered TR cells but is not the only effector mechanism of inhibition. Addition of blocking anti-TGFβ antibodies to the cul-

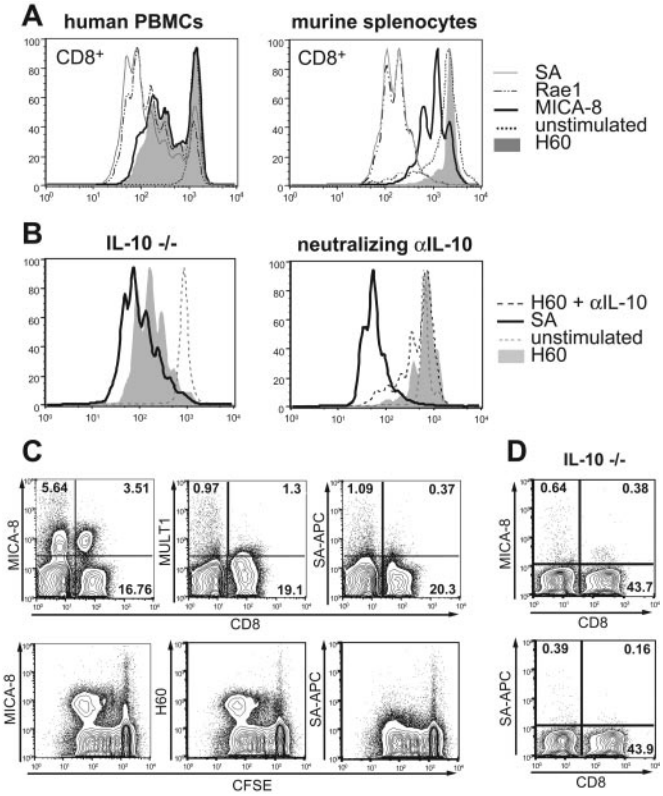

Fig. 5. IL-10 is required for H60-mediated inhibition. (*A Left*) Freshly isolated CFSE-labeled human PBMC were stimulated with anti-CD3 (OKT3) in the presence or absence of soluble MICA (allele 8; bold line) or murine NKG2D ligands (H60, filled gray; Rae1, thin dark line), as described in Fig. 4 legend. As controls, cells stimulated only with anti-CD3 (thin gray line) or maintained in the absence of any stimulation (dotted line) are shown. (*Right*) Murine (BALB/c) splenocytes were used as responder cells under the same experimental settings. (*B*) Splenocytes derived from IL10$^{-/-}$ (*Left*) or wild-type (*Right*) mice were stimulated in the presence or absence of H60; neutralizing anti-IL10 antibody was added to wild-type cultures, as indicated. (*C Upper*) Splenocytes from wild-type mice were analyzed after short-term *in vitro* stimulation with anti-CD3. Dot plots of live lymphocytes are shown, with staining for CD8 (*x* axis) and NKG2D ligand/streptavidin-APC multimers (*y* axis, as indicated). As a control, cells were stained with streptavidin-APC and CD8. (*Lower*) Under the same experimental setting as in *A*, splenocytes were labeled with CFSE to monitor their proliferation profile. The dot plots (gated on live lymphocytes) show the CFSE profile (*x* axis) of cells stained with MICA multimers (*y* axis, *Left*), H60 (*Center*), or streptavidin-APC as control (*Right*). (*D*) Splenocytes derived from IL-10$^{-/-}$ mice were treated as described under *C*, stainings for CD8 vs. MICA-8 (*Upper*) or streptavidin-APC control (*Lower*) are shown.

tures also slightly diminished H60-mediated inhibition but never completely blocked the activity (data not shown). Furthermore, supernatants from H60-inhibited cell cultures did not mediate detectable inhibitory effects (data not shown), indicating that also cell-contact-dependent mechanisms contribute to H60-mediated suppression of proliferation.

Because MICA-8 mediates similar inhibitory effects on murine T cells as H60 via a so-far-unknown receptor but does not stably bind to murine NKG2D, we asked whether we could stain short-term activated T cells with MICA-8 multimer reagents. Most impressively, a substantial fraction (up to 10% in some experiments) of CD8$^+$ and CD4$^+$ T cells became strongly positive for MICA-8 multimer staining (Fig. 5*C*; the same population also stained with H60 multimers), and this staining could not be abolished in the presence of blocking NKG2D-specific mAbs (data not shown). MULT1 demonstrated a different staining pattern on cells from the same cultures, binding to activated (NKG2D-expressing) CD8$^+$ T cells. Combination of these assays with CSFE staining clearly revealed that most of the MICA- and H60-binding cells are found in cell fractions that have divided several times (Fig. 5*C*). The absolute number of MICA-8-binding T cells was highest early (day 3) after stimulation and almost undistinguishable from background levels at later time points (>day 7; Fig. 1*D* and data not shown), indicating that the unknown receptor is regulated during T cell activation or that the MICA-8-binding populations poorly survive in the cell cultures. Depletion of the MICA-8-binding subpopulation out of purified CD8$^+$ or CD4$^+$ T cells before *in vitro* stimulation did not significantly reduce H60-mediated inhibition (data not shown), further indicating that H60-responsive regulatory T cells are generated during T cell stimulation and expansion. Because in IL-10 knockout mice H60 or MICA-8-mediated effects were almost absent (Fig. 5*B*), we performed MICA-8 multimer stainings on *in vitro* stimulated IL10$^{-/-}$ splenocytes (Fig. 5*D*). We were never able to detect MICA-8 multimer staining above background levels in cultures derived from IL10$^{-/-}$ mice, strongly suggesting that the MICA-8-positive populations are directly linked to our functional observations.

Suppressive Effects of H60 Are Detectable Under *in Vivo* Conditions. To test whether H60 exerts inhibitory effects also *in vivo*, we used an experimental system where molecules can be targeted to the surface of tumor cells by means of avidin-biotin bridging [three-step technology (29, 40); for details, see also *Materials and Methods*]. Mice were treated with biotinylated H60 or Rae1, respectively, and both treatments significantly affected tumor growth ($F_{2,17} = 5.49$; $P = 0.015$) along with a time variation ($F_{10,85} = 2.01$; $P = 0.042$) (Fig. 6). Most strikingly, the two NKG2D ligands mediated completely opposite effects on *in vivo* tumor growth; whereas Rae1 reduced tumor growth as compared with the control group, in H60-treated mice, the tumors grew even faster. This difference was statistically highly significant ($P = 0.001$). These data impressively demonstrate that NKG2D ligands can mediate very different effects also under *in vivo* conditions.

Discussion

In this report, we demonstrate that not only are NKG2D ligands characterized by redundant immunostimulatory effects, but distinct ligands can also trigger inhibitory mechanisms (*in vitro* and *in vivo*), such as suppression of T cell proliferation by an induced regulatory lymphocyte subset. We identified H60 and MICA-8 as NKG2D ligands capable of triggering this inhibitory function, but other NKG2D ligands may also belong to this group of "bifunctional" molecules. Suppression of T cell proliferation by H60 and MICA-8 was independent of NKG2D and crossreactive between human and mouse, indicating that the as-yet-unknown receptor mediating these suppressive effects must be highly conserved between species.

It has been difficult to explain why tumor cells so often express high levels of NKG2D ligands, although the members of this heterogeneous group of molecules have been described as potent stimulators of innate and adaptive immune cells. Shedding and systemic flooding with soluble ligands has been discussed as one possible mechanism to explain this discrepancy (41, 42). In some cancer patients, chronic stimulation with soluble NKG2D ligands indeed results in loss of NKG2D surface expression on human lymphocyte subsets that otherwise constitutively express this receptor and reduced responsiveness of adaptive immune responses. Our data demonstrate that, in addition to these "exhausting" effects by NKG2D-mediated overstimulation, distinct NKG2D ligands can also specifically induce immunosuppressive mechanisms. This finding adds an interesting aspect to the consequences of NKG2D ligand expression or shedding of soluble molecules from tumor cells, because strong expression of NKG2D ligands with inhibitory capacity could certainly be a selective advantage and a potent immune evasion mechanism for individual tumor cell clones *in vivo*.

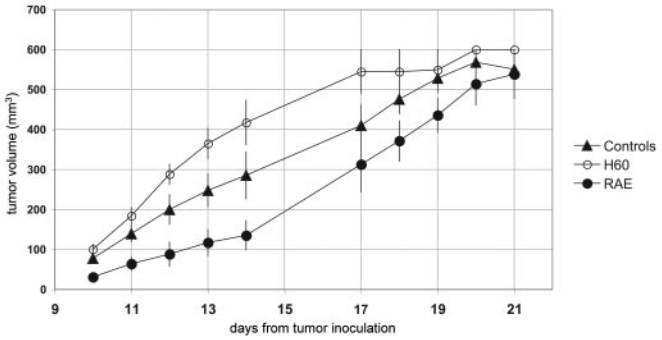

Fig. 6. Inhibition by native H60 expression and *in vivo* effects of NKG2D ligand application on tumor growth. Cohorts of mice were s.c. inoculated with aggressively growing tumor cells and subsequently treated with a three-step therapy to target H60 or Rae1 *in vivo* to the tumor cells (for details see *Materials and Methods*). The volumes of growing tumors were monitored over time.

This interpretation is supported by the finding that NKG2D ligand expression is often enhanced during tumor progression (41, 43).

Several lines of evidence indicate that the receptor mediating the H60- and MICA-8-specific effects is different from NKG2D: (*i*) Proliferation of purified $CD4^+$ T cells, which are negative for NKG2D surface expression as well as NKG2D mRNA, is still potently and selectively inhibited by H60, whereas another high-affinity murine NKG2D ligand (MULT1) is not capable of mediating this effect; (*ii*) addition of blocking NKG2D-specific antibodies did not affect H60-mediated inhibitory effects; (*iii*) $CD8^+$ T cells from DAP-$10^{-/-}$ mice, which have been described to lack NKG2D surface expression (8) and are unresponsive to NKG2D-mediated stimulation, remain responsive to H60-mediated inhibition; and (*iv*) human MICA-8, which does not stably bind to murine NKG2D, inhibits the proliferation of murine T cells to a similar extent as H60, and the same crossreactivity was observed for H60 on human T cells. Also, in a recent report by others (44), the potential existence of additional receptors for NKG2D ligands has been suggested. The identity of the receptor for H60 and MICA-8 is unknown. However, given the observed crossreactivity between man and mouse, the structure of the receptor must be substantially conserved between different species.

Responsiveness to H60-mediated inhibition of T cell proliferation was found to depend strongly on IL-10, thereby IL-10 seems to play a major role during the generation and/or expansion of H60-responsive TR cells. Our data indicate that, as has been shown in several studies characterizing effector functions of TR cells, other mechanisms including cell contact-dependent factors can contribute to inhibition of T cell proliferation (45). Inhibition of antigen-specific T cells by an induced regulatory cell subset could play a more general role in the regulation of antigen-specific immunity, such as the prevention of excessive immune responses or induction or maintenance of antigen-specific tolerance. However, before these possible connections to immune homeostasis and the regulation of immune responses can be experimentally challenged, it will be necessary to determine how and under which conditions the H60-responsive receptor is up-regulated on T cells (or other cell types), and where and when the ligands are expressed under physiological conditions or during immune responses.

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