Report

Human IgG1 antibodies antagonizing activating receptor NKG2D on natural killer cells

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Abbreviations: CDR, complementarity determining regions; CRTAM, class I restricted T cell associated molecule; DAP10/12, DNAX activating protein with 10 or 12 kDa; FACS, fluorescence activated cell sorter; FR, framework region; GM-CSF, granulocyte-macrophage colony-stimulating factor; h, human; IFN, interferon; Ig, immunoglobulin; IL-2, interleukin-2; kₐ, association rate constant; k₃, dissociation rate constant; mAb, monoclonal antibody; MHC, major histocompatibility complex; MIC A/B, MHC class I chain-related antigen A/B; NKG2D, natural killer group 2 member D of the lectin-like receptor family; NK cells, natural killer cells; NOD, nonobese diabetic; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; Rae-1, retinoic acid early inducible-1; scFv, single-chain fragment of variable domains; TNF, tumor necrosis factor; ULBP, UL16 binding proteins; VL, variable domain of light chain; VH, variable domain of heavy chain

Key words: NKG2D, NK cell, T cell, monoclonal antibody, human IgG1, humanization, phage display, autoimmune disease

NKG2D is a surface receptor expressed on NK cells but also on CD8⁺ T cells, γδ T cells, and auto-reactive CD4⁺/CD28⁺ T cells of patients with rheumatoid arthritis. Various studies suggested that NKG2D plays a critical role in autoimmune diseases, e.g., in diabetes, celidic disease and rheumatoid arthritis (RA), rendering the activating receptor a potential target for antibody-based therapies. Here, we describe the generation and characteristics of a panel of human, high-affinity anti-NKG2D IgG1 monoclonal antibodies (mAbs) derived by phage display. The lead molecule mAb E4 bound with an affinity (KD) of 2.7 ± 1.4 x 10⁻¹¹ M to soluble and membrane-bound human NKG2D, and cross-reacted with NKG2D from cytomolous macaque, indicating potential suitability for studies in a relevant primate model. MAb E4 potently antagonized the cytolytic activity of NKL cells against BaF/3-MICA cells expressing NKG2D ligand, and blocked the NKG2D ligand-induced secretion of TNFa, IFNγ and GM-CSF, as well as surface expression of CRTAM by NK cells cultured on immobilized MICA or ULBP-1 ligands. The antibody did not show a detectable loss of binding to NKG2D after seven days in human serum at 37°C, and resisted thermal inactivation up to 70°C. Based on these results, anti-human NKG2D mAb E4 provides an ideal candidate for development of a novel therapeutic agent antagonizing a key receptor of NK and cytotoxic T cells with implications in autoimmune diseases.

Introduction

In humans, the NKG2D receptor is expressed on all NK cells, CD8⁺ αβ T cells and γδ T cells.1-3 It is a homodimeric type II transmembrane protein with an extracellular C-type lectin-like domain, to which specific ligands bind in a Ca²⁺-independent manner.4,5 Ligands of NKG2D are very diverse and differ in their structure, expression pattern and binding affinity.1,6-8 The first human ligands described for NKG2D were MHC class I-related molecules A and B (MICA and MICB), which are closely related and highly polymorphic.9,10 Other ligands are UL16-binding proteins ULBP-1, -2, -3 and -4. These ligands are poorly expressed by healthy cells but frequently found upregulated in transformed cells after induction of the so-called DNA damage response pathway, as well as after infection with viruses or other pathogens.3,11-14 The expression of NKG2D ligands is important for the recognition of transformed or infected cells by NK cells. According to the “missing-self” hypotheses, as postulated by Ljunggren and Kärre, healthy cells express sufficient MHC class I molecules on their surface that protect them from NK cell-mediated cytolysis by interaction with inhibitory receptors on NK cells. On the other hand, reduced levels of MHC class I on transformed cells and the expression of alternative MHC class I-related surface molecules activate receptors on NK cells, which leads to elimination of the target cell by release of granzymes.
and perforin. The activating receptor NKG2D and its interaction with ligands have an essential function in tumor surveillance and immunity against pathogens.

NKG2D not only mediates the activation of NK but also of CD8⁺ T cells. While human NK cells can directly become activated through interaction of NKG2D and its ligands, CD8⁺ T cells need additional signals. For signaling and expression on the cell surface, NKG2D associates with adaptor molecules DAP10 in humans, and with DAP10 or DAP12 in mice. Ligand binding to the NKG2D/DAP10 receptor complex is leading to tyrosine phosphorylation within the YxxM motif in the cytoplasmic tail of DAP10 and induces cytotoxicity and cytokine release.

Dysfunction of NKG2D can lead to autoimmune diseases like diabetes mellitus type I, RA, celiac disease, and multiple sclerosis, and might also be involved in graft rejection. In a type I diabetes model in NOD mice, pancreatic tissue was found to express Rae-1, a ligand of murine NKG2D. NKG2D-expressing auto-reactive CD8⁺ T cells were observed to infiltrate the pancreas, leading to tissue destruction. Inhibition of the NKG2D/Rae-1 interaction by an antagonistic anti-NKG2D antibody could stop progression of the disease even if the antibody was administered to late prediabetic mice.

In celiac disease, an inflammation of the colon induced by wheat protein gluten leads to destruction of the epithelial layer of the colon due to adaptive and innate immune responses. Several studies suggested that interaction of MICA-expressing epithelial cells with NKG2D-expressing, intraepithelial lymphocytes caused destruction of the colon tissue.

NKG2D and its ligands also seem to be involved in the onset of rheumatoid arthritis. Proinflammatory factors like IL-15 and TNFα induce expression of NKG2D on auto-reactive CD8⁺ T cells, which recognize the MICA/MICB-expressing synovial tissue and infiltrate the synovium. These studies indicate that antibodies blocking interactions of NKG2D with ligands might have potential for the treatment of certain autoimmune diseases.

We here describe generation and characteristics of a new human monoclonal anti-NKG2D antibody called E4, which by several criteria appears ideal for development of a human therapeutic. E4 has high target affinity for NKG2D, is stable in serum and at elevated temperature, is of lowest possible immunogenicity by bearing a fully human variable light chain and humanized variable heavy chain, and shows the necessary antagonist activity for intercepting with biological activities of NKG2D.

**Results**

Generation of human anti-NKG2D mAb E4. Human single-chain antibodies (scFv) were generated by phage display and guided selection of human light chains (VL) and stepwise humanization of the heavy chains (VH) as outlined in Figure 1. ScFv-based phage libraries were prepared using VL and VH sequences derived from murine human NKG2D-binding and neutralizing monoclonal antibody (mAb) 6H7 (Micromet AG). Because guided selection failed to identify a human VH sequence that would preserve the biological activity of the lead antibody, stepwise humanization of the VH framework region (FR) was performed, while retaining the murine complementarity-determining regions (CDR). During this process, each chimeric scFv variant was expressed and tested for binding and neutralizing activity. By successive replacement of murine with human framework amino acids and after conversion into the human IgG1 antibody format, mAb E1 was obtained, which showed a significant loss in binding affinity and biological function. Affinity maturation of scFv from mAb E1 was performed by sequential randomization of the CDR3 domains of VL and VH domains. This resulted in five different anti-NKG2D scFv of increased affinity. All five human candidates were converted into human IgG1 by recombinant fusion with constant domains Cγ, CH1, CH2 and CH3 resulting in mAbs B1, E4, C3, C12 and H5.

Biological activity of human anti-NKG2D monoclonal antibodies. MAbs B1, E4, C3, C12 and H5 were investigated in a FACs-based assay for cell surface binding to NKL cells expressing human NKG2D (Fig. 2A). Except for mAb E1, all five affinity maturated candidates showed comparable binding to human NKG2D expressed on NKL cells, as detected by titration analysis using FACs. To determine association (kₐ) and dissociation rate constants (k₅) for NKG2D-specific binding, all mAbs were analyzed by surface plasmon resonance spectroscopy using soluble recombinant human NKG2D/Fc bound to the surface of a sensor chip. The resulting kₐ and k₅ values were used to calculate the equilibrium dissociation constant (K_D) (Table 1).

The two candidates with highest affinity for human NKG2D were mAb E4 and mAb B1 with association rate constants of kₐ = 3.53 ± 0.25 x 10⁸ M⁻¹ s⁻¹ and 4.30 ± 0.42 x 10⁸ M⁻¹ s⁻¹, dissociation rate constants of k₅ = 9.30 ± 4.53 x 10⁻⁶ s⁻¹ and 9.95 ± 2.90 x 10⁻⁶ s⁻¹, and K_D values of 2.70 ± 1.40 x 10⁻¹¹ M and 2.25 ± 0.35 x 10⁻¹¹ M, respectively. These two antibodies were selected for further functional and biophysical analyses. To test their inhibitory potential, mAbs E4 and mAb B1 were compared in a ⁵¹Cr-release cytotoxicity assay using NKL cells as effectors and BaF/MICA transfectants as target cells (Fig. 2B). In this assay, NKG2D receptor-positive NKL cells recognized MICA expressed on BaF cells leading to subsequent lysis of ⁵¹Cr-loaded target cells. While the non-optimized mAb E1 had an IC₅₀ value of 31 ± 15 nM, the two selected lead candidates showed approximately 100-fold improved IC₅₀ values of 0.23 ± 0.12 nM (mAb B1) and 0.22 ± 0.08 nM (mAb E4), indicating that the affinity maturation process was also successful in improving the biological activity of the two antibodies.

To analyze the activation potential of the mAbs, a redirected lysis experiment was performed with NKL cells as effectors and ⁵¹Cr-labelled Fcγ receptor expressing P815 cells (Fig. 2C). In this assay, the Fc part of anti-NKG2D antibodies binds to Fc receptors expressed on P815 cells, which induces the aggregation of NKG2D receptors leading to NKL cell-mediated target cell lysis. The EC₅₀ values observed were 21.20 ± 13.60 pM and 19.40 ± 11.80 pM for mAb B1 and mAb E4, respectively, and 1.30 ± 1.10 nM for mAb E1, again showing a 100-fold difference in potency.

Serum and thermostability of mAb E4 and mAb B1. The two selected antibodies E4 and B1, and parental mAb E1 were...
tested for their resistance to elevated temperatures as a means to determine overall stability. The antibodies were incubated for five minutes at various temperatures between 37°C and 100°C and tested after cooling for their ability to bind NKG2D expressed on NKL cells using FACS. All three antibodies preserved 100% of their binding capability up to a treatment at 70°C (data not shown). To determine stability and protease resistance in human serum, the three antibodies were incubated at 37°C for up to seven days and thereafter binding to NKG2D on NKL cells analyzed at various concentrations by FACS analysis (data not shown). None of the antibodies showed a detectable loss of binding to NKG2D after incubation in human serum for up to seven days at 37°C.

**Binding of mAbs E4 and B1 to NKG2D expressing human PBMC.** To verify that mAbs E4 and B1 bind to human cells expressing NKG2D, PBMC from two healthy donors were purified and stained with mAbs E4 and B1, as well as for the antigens CD4, CD8, CD14, CD16, CD19 and CD56 marking various cell populations of PBMC (Fig. 3). Like positive control mouse-anti-human NKG2D antibody 1D11, both antibodies bound to sub-populations of PBMC known to express NKG2D. MAbs E4 and B1 were found to selectively recognize the majority of human CD8⁺ (CTL), CD16⁺ and CD56⁺ cells (NK cells). In contrast, there was no evidence for binding to human CD4⁺ T cells, CD19⁺ B cells or CD14⁺ monocyte cell population, confirming the specificity of mAbs E4 and B1 for human NKG2D.

**Cross-reactivity of mAb E4 with NKG2D from cynomolgus monkeys (M. fascicularis).** For facilitated pre-clinical development, it can be a considerable advantage if antibodies cross-react with NKG2D from non-human species to allow for safety and efficacy studies in relevant animal models. To further assess the antibodies’ specificity and to analyze if mAbs E4 or B1 can bind to cells expressing NKG2D from *Macaca fascicularis*, PBMC were purified from whole blood of healthy animals and cells analyzed for antibody binding by co-staining for antigen markers CD3, CD8, CD16, CD19 and CD56 in a FACS-based assay (Fig. 4). Both mAbs E4 and B1 were found to equally well bind to macaque and human T cells and NK cells indicating binding of the mAbs to epitopes conserved between NKG2D receptors of human and macaque origin. This finding qualifies both antibody candidates for future safety and efficacy studies in non-human primates provided they can be produced in sufficient amounts. Because mAb E4 had shown higher expression levels than mAb B1, mAb E4 was finally selected as lead candidate for further development, and studied in more detail in the following.

**Immobilized mAb E4 induces cytokine release by NK cells.** In addition to cytolytic activity, it has been reported that interaction
Rapid internalization of mAb E4 after binding to NKG2D. Soluble NKG2D ligands as shed by tumor cells can trigger internalization of NKG2D on NK cells, which can reduce their killing activity. To determine if mAb E4 could also induce internalization of NKG2D, Alexa-Fluor 488-conjugated antibody (E4-488) was incubated for different time points with NKL cells and internalization of the bound antibody studied by confocal laser scanning microscopy (Fig. 7). Shortly after addition to cells, the labeled antibody evenly stained the cell surface (Fig. 7A), and a polarization of bound receptors was evident after five minutes (Fig. 7B). After 15 min, the antibody/NKG2D complex was...
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Table 1  Binding constants of affinity matured anti-hNKG2D mAbs in comparison with the human precursor mAb E1

| Human antibody | \( k_d \) (s\(^{-1}\)) | \( k_a \) (M\(^{-1}\)s\(^{-1}\)) | \( K_D = k_d/k_a \) (M) |
|----------------|-----------------|-----------------|-----------------|
| E1 mAb         | 6.1 ± 0.9 × 10\(^{-4}\) | 4.4 ± 0.6 × 10\(^{-4}\) | 1.7 ± 0.5 × 10\(^{9}\) |
| C3 mAb         | 1.0 ± 0.0 × 10\(^{-4}\) | 3.1 ± 0.1 × 10\(^{-5}\) | 3.3 ± 0.3 × 10\(^{11}\) |
| C12 mAb        | 2.1 ± 0.7 × 10\(^{-4}\) | 2.9 ± 0.1 × 10\(^{-5}\) | 7.3 ± 2.1 × 10\(^{11}\) |
| H5 mAb         | 1.5 ± 0.0 × 10\(^{-6}\) | 2.7 ± 0.5 × 10\(^{-5}\) | 5.8 ± 1.0 × 10\(^{11}\) |
| B1 mAb         | 9.9 ± 2.9 × 10\(^{-6}\) | 4.3 ± 0.4 × 10\(^{-5}\) | 2.3 ± 0.4 × 10\(^{11}\) |
| E4 mAb         | 9.3 ± 4.5 × 10\(^{-6}\) | 3.5 ± 0.3 × 10\(^{-5}\) | 2.7 ± 1.4 × 10\(^{11}\) |

Rates of association \((k_a)\) and dissociation \((k_d)\) of the different human anti-hNKG2D Abs were measured by surface plasmon resonance (SPR) and the equilibrium dissociation constant \(K_D\) \((M)\) was calculated from \(k_d/k_a\). The values shown are means ± standard deviation of three independent Biacore experiments.

Table 2  Characteristics of mAb E4

| Characteristic                  | Unit                   | E4 mAb                  |
|--------------------------------|------------------------|-------------------------|
| Isootype                       | -                      | Human IgG1κ             |
| Identity of human VL to human germline | -                        | 89% identity to human VL\(_{13-7}(A)1c\) |
| Identity of human germline     | -                      | Human VH 4-59           |
| Specificity                    | -                      | Human and macaque NKG2D |
| Binding affinity               | Equilibrium dissociation constant \((K_D)\) | 2.7 ± 1.4 × 10\(^{-11}\) M \((\text{Biacore})\) |
|                                | Association rate constant \((k_a)\) | 3.5 ± 0.3 × 10\(^{5}\) M\(^{-1}\)s\(^{-1}\) |
|                                | Dissociation rate constant \((k_d)\) | 9.3 ± 4.5 × 10\(^{-6}\) s\(^{-1}\) |
| Inhibition of NKG2D-dependent cytotoxicity | Half maximal inhibitory concentration \((IC_{50})\) | 0.22 ± 0.08 nM |
| Induction of NKG2D-dependent cytotoxicity | Half maximal effective concentration \((EC_{50})\) | 19.40 ± 11.80 pM |
| Serum stability at 37°C         | -                      | at least 7 days         |
| Heat stability                  | -                      | Up to 70°C              |
| Inhibition of cytokine release  | -                      | yes                     |
| Induction of cytokine release   | -                      | yes                     |
| Internalization by NKL cells    | -                      | yes                     |

The numbers are based on the experimental data shown in Figure 2B and C, and mentioned in the results. Standard deviations from triplicate determinations are given. EC\(_{50}\) and IC\(_{50}\) values from dose response curves shown in Figure 2B and C were calculated by curve fitting software GraphPadPrism4.

Discussion

Natural killer cells are receiving increasing attention as a therapeutic target because of their potential involvement in autoimmune and inflammatory diseases. A number of recent studies suggested a role for NKG2D receptor expressed on NK cells in rheumatoid arthritis, diabetes, celiac disease and multiple sclerosis.\(^{23-25,27}\) In order to control an aberrant activation of NK cells via NKG2D, two antibody-based approaches can be chosen: (1) inhibition of the activating receptor, (2) neutralization of its activating ligands. We developed human monoclonal antibodies that bind to the NKG2D receptor and prevent its interaction with activating ligands. We preferred this approach over ligand-neutralizing antibodies because of the variety of ligands potentially binding to NKG2D. Neutralization of just one ligand will not prevent NK cell activation by other ligands of NKG2D. NKG2D was selected as the target antigen because of its key role in activating NK cells and its monomorphic nature. NKG2D is also a costimulatory receptor on cytotoxic CD8\(^{+}\) T cells. The ability to also control auto-reactive CD8\(^{+}\) T cells would broaden the effect of a therapeutic antibody.

We here selected mAb E4 as a candidate for development of an antagonistic anti-NKG2D antibody. Antibody generation employed phage display guided selection using an antagonistic antibody of murine origin. A fully human light chain was identified that in combination with a humanized heavy chain and affinity maturation created a set of mAbs suitable for selection of a candidate.

A number of prerequisites need to be fulfilled for the development of a novel antagonistic anti-NKG2D as a potential drug candidate. These include lowest possible immunogenicity, high affinity and potency, high stability, cross-reactivity with test animals, defined biological activity, and in-vivo efficacy and therapeutic window. Apart from in-vivo performance, we have demonstrated that the selected mAb E4 fulfills all other requirements. Having a fully human variable light chain and a humanized variable heavy chain, E4 is as close to human germline sequences...
as other antibodies used for treatment of patients with inflammatory diseases. With a dissociation rate constant in the range of 30 pM and a very slow off-rate, mAb E4 should be of sufficient affinity to interact with its target antigen on NK cells at antibody doses equivalent to those of therapeutic antibodies. MAb E4 showed exquisite stability in human serum for one week at 37°C and could resist heating up to 70°C without loss of binding activity. Future in-vivo studies with mAb E4 will be facilitated by cross-reactivity of the antibody with target antigen from a non-human primate species. Antagonistic activities of mAb E4 were evident from several biological assays. Inhibition of NKG2D-mediated cytotoxicity by mAb E4 was observed as well as blockade of TNFα, IFNγ and GM-CSF release in response to stimulation of NKG2D with its ligand MICA. Half maximal inhibition of cytotoxicity was achieved at 0.22 nM mAb E4, which is close to the Kd value for NKG2D binding.

Under certain conditions, mAb E4 also exhibited agonistic activities. These can be explained by situations where NKG2D is extensively cross-linked by antibody, which is either immobilized on plastic or presented by cells bearing a high density of Fcγ receptors. Recently, Kwong et al. published a study which also describes the generation and affinity maturation of a human anti-hNKG2D antibody called KYK-2.0. Analysis of this human IgG1 antibody produced similar results as our human IgG1 antibody mAb E4. In solution, KYK-2.0 shows an antagonistic profile by interfering with the cytolytic activity of ex vivo expanded NK cells. In contrast, immobilized KYK-2.0 was found to strongly induce activation of human NK cells. We agree that the ambivalent characteristics of human anti-hNKG2D antibodies may translate into a potent therapeutic benefit. But for therapeutic application in autoimmune diseases, it will be necessary to generate non-depleting antibody formats like Fab, F(ab)2, or antibodies with mutated Fc-parts to disrupt Fc-receptor binding. Our data suggest that only cross-linking of mAb E4 appeared to stimulate NKG2D in the absence of ligands, while bivalent binding appears neutral and showed antagonistic effects in the presence of activating ligands. The neutralization potential of mAb E4 in a relevant species remains to be clarified in animal experiments where circulating cytokine levels may be a sensitive readout for potential agonistic activities.

**Materials and Methods**

**Cell lines and purification of cells.** The human NK cell line NKL was grown in RPMI 1640 medium supplemented with 20% heat inactivated fetal calf serum (h.f. FCS) (Biochrom), 2 mM L-glutamine, 100 μg/ml Streptomycin/Penicillin and 200 U/ml of recombinant human IL-2 (rhIL-2, Novartis). The pro-B cell line, BaF/3, mock-transduced or transduced with human MICA and the FcγR-positive murine mastocytoma cell line P815 were kindly provided by A. Cerwenka and C. Watzl (DKFZ, Heidelberg, Germany). BaF/3 and P815 cells were cultured in RPMI 1640 medium supplemented with...
Primary lymphocytes from two healthy cynomolgus monkeys (M. fascicularis) were isolated from peripheral blood using Leucosep® devices (Greiner) and Biocoll-Separating Solution (Biochrom). Cells were cultured for 4 days in petri dishes coated with anti-CD28 Ab (1 μg/ml, BD 556620) and anti-CD3 Ab (1 μg/ml, clone SP34, BD 557052) in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (h.i. FCS) (Biochrom), 2 mM L-glutamine, 100 μg/ml Streptomycin/Penicillin, 10 mM HEPES (Biochrom), 10x non-essential amino acids (Biochrom) and 5,000 U/ml of recombinant human IL-2 (rhIL-2, Novartis).

Generation of human anti-human NKG2D antibodies. Based on the identified mouse hybridoma cell line 6H7 (Micromet AG), which produced a monoclonal antibody (mAb) neutralizing hNKG2D, humanization of single-chain antibodies (scFv) was performed using phage display guided selection. For selection of human VL, scFv libraries were generated by cloning VLκ and VLλ pools via SacI/SpeI in phagemid vector pComb5BHis/N2 (Micromet AG) containing the sequence of parental murine VH. The selection of antigen-binding phages by panning was carried out according to the procedure as described in Raum et al.42 on recombinant soluble biotinylated NKG2D antigen immobilized on streptavidin coated microtiter plates (Nunc). For periplasmic expression of scFv, the pool of fragments obtained after four rounds of panning was subcloned into pComb5BF/HdelN2 (Micromet AG). The expression of multiple different clones using E.coli TG-1 cells and preparation of periplasmic extracts was performed as described elsewhere.43 ScFv were collected for screening by FACS. Because phage display guided selection method failed to select a human VH with designated binding activity, stepwise humanization of VH framework-region (FR) was performed while retaining the murine CDR-domains. The successive replacement of murine with human framework amino acids resulted, after conversion in IgG1 antibody format, in the construct mAb E1, which showed loss in binding and biological activity. Due to the fully human VL and the mostly human VH, the constructs will be referred to as ‘human’. Affinity maturation process of scFv E1 was performed by sequential randomization of the CDR3 domains of VL and VH, and selection of scFv with high affinity by method of in
solution-panning as described in Krinner et al.43 Here phage particles were incubated for 1 h with recombinant hNKG2D/Fc in a total volume of 0.5 ml in PBS/0.1% BSA. Then 30 μl of protein G beads (Sigma) were added for an additional hour. Elution of binding phages was performed according to the procedure described in Krinner et al.43 During phage display panning, the antigen concentration was reduced every round from 100 nM to 0.1 nM to select for scFv with high affinity. The affinity maturation process resulted in five different anti-hNKG2D scFv lead candidates with high affinity. All five human candidates were converted into IgG1 mAbs, designated mAb B1, mAb E4, mAb C3, mAb C12, mAb H5.

Expression and purification of human anti-NKG2D antibodies. For transient transfection of HEK293-F cells or stable expression of light and heavy chain in CHO cells, separate expression vector constructs were prepared as described before.42 For initial characterization of binding affinity of human anti-hNKG2D mAbs by FACS and Biacore, mAbs were expressed in transiently transfected human HEK293-F cells. Vectors encoding the heavy and light chain of mAbs were co-transfected in HEK293-F cells using the 293fectin reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions.

For 51Cr-release and cytokine assays human anti-hNKG2D mAbs were expressed in stably transfected CHO cells as described before.42

MAbs from cellular supernatants were purified by protein G affinity chromatography with HiTrap protein G HP-column according to standard protocols for the isolation of IgGs using an ÄKTAexplorer 100 Air (GE Healthcare).

Surface plasmon resonance (SPR). The SPR measurements were performed using a BIACore™ 2000 instrument (Biacore AB, Switzerland). The surface of the CM5 sensor chip (Biacore) was activated with NHS/EDC. Approximately 1,600 resonance units (RU) of hNKG2D were immobilized on the CM5 sensor chip by injection of 5 μg/ml hNKG2D/Fc (1299-NK, R&D Systems) in 10 mM sodium-acetate (pH 4). After immobilization of the antigen, the activated chip surface was blocked by 1 M Ethanolamine-HCl (pH 8.5). Control flow cells were prepared by carrying out the coupling reaction in the presence of coupling buffer alone. Control sensograms were automatically subtracted from sensograms obtained with immobilized hNKG2D/Fc. Binding assays were performed at 25°C in HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). Anti-hNKG2D antibodies were diluted 1:2 in HBS-EP and injected (1 min stabilization and 3 min injection) at several concentrations (1:2 serial dilution: mAbs H5, B1, E4, C3, C12 at 5-156.3 x 10⁻³ μg/ml or mAb E1 at 50-1.6 μg/ml) and a flow rate of 20 μl/min, followed by 5 min of buffer flow for dissociation. Regeneration was obtained with a 45 sec pulse of 100 mM glycine in 500 mM NaCl solution, pH 3. The association and dissociation rate constants (ka [1/Ms] and kd [1/s]) and the equilibrium dissociation constants KD were determined using the BIAevaluation software (3.2 RC, Biacore) with the Langmuir binding equation and global curve fitting. The equilibrium dissociation constant KD [M] was calculated from

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**Figure 5.** Cross-linking of NKG2D with immobilized mAb E4 is sufficient to activate human NK cells pre-stimulated with rHuIL-2 and induce increased cytokine release. (A–C) Plate-bound mAb E4 induces IFNγ (A), TNFα (B) and GM-CSF (C) production by human NK cells. Secreted cytokines were measured in supernatants after 20 h using ELSA (GM-CSF) or CBA array (IFNγ, TNFα). Error bars show standard deviation of eight different healthy donors. (***p < 0.001)
Figure 6. For figure legend, see page 124.
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Figure 6. Inhibitory effect of soluble mAb E4 on NKG2D-dependent expression of CRTAM on NK cells and cytokine release. (A and B) mAb E4 (5 μg/ml) reduced MICA/Fc (A) or ULBP-1/Fc (B)-induced expression of the NK cell activation marker CRTAM. Error bars show standard deviation of four healthy donors tested. No reduction was detectable with control mAb. (C–E) NK cells were pre-incubated with mAb E4 (5 μg/ml) before exposure to NKG2D-ligand MICA/Fc. The antibody reduced the release of cytokines TNFα (C), IFNγ (D) and GM-CSF (E) as measured in supernatants by CBA array or ELISA. Error bars show standard deviation of eight healthy donors. [***p < 0.001]

Figure 7. Internalization and subcellular localization of mAb E4. Confocal laser scanning microscopy of NKL cells incubated with mAb E4 conjugated to Alexa Fluor 488 (mAb E4-488, green). (A–D) Cells were incubated with mAb E4-488 and the nucleus labeled with DAPI (blue). The different panels show internalization of NKG2D-bound mAb E4-488 at time points 0 min (A), 5 min (B), 15 min (C) and after 4 h (D). The arrows indicate internalized anti-hNKG2D Ab vesicles. Optical sections of 0.2 μm thickness are shown. (E) Intracellular accumulation of mAb E4. The graph shows time-dependent intracellular accumulation of anti-hNKG2D antibody E4-488 (●) in NKL cells. The y-axis represents the percentage of MFI measured by flow cytometry of NKL cell population over time (x-axis) relative to total binding at 0 min. Antibody-bound cells were incubated at 37°C for 0, 5, 15, 30, 60, 120, 180 and 240 min. Each time point was replicated at least three times.

dkd/κa. The analytes were produced and purified and used to record at least three independent data sets.

FACS. Unless specified otherwise, all incubations were performed on ice in PBS-F (phosphate buffered saline, pH 7.4, 2% FCS). Routinely, washing was performed in three consecutive steps applying 200 μl washing buffer PBS-F per well each time.

(1) The binding activity of untreated human anti-hNKG2D mAbs was tested by flow cytometry on NKG2D expressing NKL cells. Per sample 2 x 10^5 NKL cells were seeded in a 96-well microtest plate and incubated with different anti-hNKG2D mAbs diluted 1:3 in PBS-F (10–5.6 x 10^-5 μg/ml) or with respective controls (human IgG1, Micromet AG) on ice. Cells were washed and incubated 30 min on ice with a goat anti-human IgG mAb conjugated to PE (Jackson/Dianova) 1:100 in PBS-F. After final washing, cell associated fluorescence was determined by FACS.

(2) The purity of enriched human naïve NK cells was determined by incubation of PBL and isolated NK cells with PE- or PE-Cy5-conjugated anti-CD3 (Becton Dickinson, BD), anti-CD14 (BD), anti-HLA-DR (BD), anti-CD16 (BD), anti-CD56 (BD), anti-NKG2D (BD) mAbs and respective isotype controls. Incubation was performed on ice for 30 min. After final washing, cell associated fluorescence was determined by FACS.

(3) After stimulation of naïve human NK cells with NKG2D-ligands in the presence or absence of anti-hNKG2D mAbs the expression of the surface protein CRTAM was analyzed. Two x 10^5 of stimulated human NK cells were seeded in a 96-well microtest plate and incubated for 30 min on ice with 0.25 μg of anti-CRTAM mAb (R&D Systems) or the respective IgG2b isotype control. Cells were washed and incubated on ice with a goat anti-mouse IgG mAb conjugated to PE (Jackson/Dianova) 1:100 in PBS-F. After final washing, cell associated fluorescence was determined by FACS. The mean and standard deviation were generated from four different healthy donors using the software package GraphPadPrism4.

(4) Cross-reactivity of human anti-hNKG2D mAbs was analyzed with PBMC obtained from the following species: H. sapiens and M. fascicularis. Per well 5 x 10^5 of human or macaque PBMC were seeded in 96-well U-bottom plates. To block Fcγ receptors cells were treated with 10% human AB serum (PAA) for 15 min at 4°C. Blocked PBMC were then incubated with 1 μg of the Alexa Fluor 488-conjugated human
anti-hNKG2D mAbs B1 (B-488) or E4 (E-488), or a human IgG1 isotype control (human IgG1-488 isotype control) (Micromet AG) in PBS-F. Incubation was performed for 30 min on ice. PBMC subpopulations were gated by co-staining with PE-Cy5- or APC-coupled mouse anti-human CD4, CD8, CD16, CD19, CD14 and CD 56 (all BD) for human cells and PerCp-, APC-, PE- or Alexa Fluor 647 coupled mouse anti-human CD3 (BD), CD19 (Beckman Coulter), CD8 (eBioscience) and CD56 (BD) for PBMC of *M. fascicularis*. PE-conjugated mouse anti-human NKG2D (1D11, BD) was used as positive control. After final washing, cell associated fluorescence was determined by FACS collecting 20,000 gated events for each sample. The stainings were analyzed for two different donors per species.

(5) Cytokine concentrations were measured using the CBA Th1/Th2 Cytokine Kit II (BD).

**Thermal stability.** For analysis of thermal stability of human anti-hNKG2D mAbs they were diluted to a final concentration of 1 μg/ml in 1x PBS/1% h.i. FCS/0.05% sodium azide. Aliquots (100 μl) were heated in a water bath at temperatures varying from 37°C to 100°C (37°C, 50°C, 60°C, 70°C, 80°C and 100°C). After 5 min incubation the samples were cooled on ice. The stability of mAbs at different temperatures was tested by binding to NKG2D expressing NKL cells by FACS analysis. NKL cells (2 x 10^5 per well) were seeded in a 96-well microtest plate and 30 μl of the incubated protein solution was added. After washing with 200 μl of 1x PBS/1% h.i. FCS/0.05% sodium azide for three times the binding of the human mAbs was detected using a PE-conjugated goat anti-human mAb (Jackson/Dianova, 1:100 in 1x PBS/1% h.i. FCS/0.05% sodium azide). Data were normalized by setting the MFI of untreated antibody (4°C) at 100% binding. This assay was performed in four replicates and repeated three times.

**Serum stability.** For analysis of serum stability, human anti-hNKG2D mAbs were diluted in PBS with 50% human AB serum (PAA) at a concentration of 60 μg/ml. After incubation for different time points (d0, d1, d2, d4 and d7) at 37°C the samples were frozen in liquid nitrogen (LN) and stored at -80°C. For FACS analysis of binding activity all samples were thawed simultaneously and binding of mAbs tested in 1:3 dilutions (dilution in PBS-F: 10–4.6 x 10^-3 μg/ml) on NKG2D-expressing NKL cells. NKL cells (2 x 10^5 per well) were seeded in a 96-well microtest plate and 100 μl of the respective sample were added. For detection a PE-conjugated goat anti-human Ab (Jackson/Dianova, 1:100 in PBS-F) was used. This assay was performed in duplicates and was repeated three times.

**51Cr chromium release assays.** Inhibition of NKG2D-dependent cytolytic activity in the absence or presence of different anti-hNKG2D antibodies was tested in a 4-h 51Cr-release assay. BaF3 target cells, which were mock-transduced or transduced with human MICA, were labeled for 1 hour with 100 μCi 51Cr (Hartmann Analytics) at 37°C. The human NKL effector cell line was added at an E:T ratio of 40:1. NKL cells was washed, resuspended in RPMI 1640 medium supplemented with 10% h.i. FCS, 2 mM L-glutamine, 100 μg/ml Streptomycin/Penicillin, and 200 U/ml rhIL-2. Cells were then pre-incubated for ca. 30 min with 1:3 diluted samples (mAb E1 and hIgG1 isotype control: 20 μg/ml-1.1 x 10^-4 μg/ml or mAb B1 and mAb E4: 2.2 μg/ml-1.2 x 10^-5 μg/ml) of different anti-hNKG2D mAbs or isotype controls.

To analyze Fc-part-mediated redirected lysis, a 6-hour assay was performed. NK cells were washed, resuspended in RPMI 1640 medium supplemented with 10% h.i. FCS, 2 mM L-glutamine, 100 μg/ml Streptomycin/Penicillin, and 200 U/ml rhIL-2. Cells were then pre-incubated for ca. 30 min with 1:3 diluted samples (mAb E1 and hIgG1 isotype control: 5 μg/ml-2.8 x 10^-5 μg/ml or mAb B1 and mAb E4: 0.6 μg/ml-3.1 x 10^-6 μg/ml) of different anti-hNKG2D mAbs or isotype controls. Briefly, P815 target cells were labeled with 100 μCi in medium for 1 hour at 37°C. Cells were washed three times in RPMI 1640 medium supplemented with 10% h.i. FCS, 2 mM L-glutamine, 100 μg/ml Streptomycin/Penicillin, and 200 U/ml rhIL-2. Cells were then pre-incubated for ca. 30 min at 37°C before adding the mixture to plate bound NKG2D-ligands. After incubation for 20 hours at 37°C cells were spun down and supernatants harvested. Cytokine levels in cell-free supernatants were determined by ELISA or CBA and the stimulated NK cells analyzed for surface expression of CRTAM by FACS.

**ELISA.** GM-CSF levels were determined using the OptEIA Human GM-CSF ELISA Set (BD Bioscience, Heidelberg, Germany) according to manufacturer’s instructions. In brief, 100 μl of capture mAb (BD) 1:250 diluted in immobilization buffer (0.1 M sodium-carbonate, pH 9) were coated on microtiter plates (Maxisorb, Nunc, Wiesbaden, Germany). After blocking of plates with PBS-T (PBS with 0.05% Tween 20) containing 10% FCS, cell culture supernatants and human GM-CSF standard (BD) were added in 1:2 dilutions and incubated for 2 hour at room temperature. Detection of bound human GM-CSF was accomplished by 1 hour incubation at room temperature using a mixture of biotinylated detection Ab and streptavidin-HRP (BD). The ELISA was developed with OPD substrate (Sigma) and absorbance was measured at 490/650 nm. All assays were performed in duplicates. The mean and standard deviation of 8 donors was calculated using the software package GraphPadPrism4. To analyze significance, data were transformed (Y = Y + K, K = 1) and tested with a ONE Way ANOVA test by using the software package GraphPadPrism4.
Cytometric bead assay. TNFα and IFNγ levels in cell-free supernatants were analyzed using the cytometric bead assay ‘Th1/Th2 Cytokine Kit II’ (BD) according to the manufacturer’s instructions. The mean and standard deviation of eight donors were calculated using the software package GraphPad Prism4. To analyze statistical significance, data were transformed [TNFα: Y = Y + K, K = 1; IFNγ: Y = Log(Y)] and tested with a ONE WAY ANOVA test by using the software package GraphPad Prism4.

Alexa fluor 488 labeling of mAb E4 and mAb B1. Chemical coupling of Alexa Fluor-NHS 488 (Invitrogen, A20000) to human anti-hNKG2D mAb E4, mAb B1 and IgG1λ isotype control (Sigma) was carried-out by a 30-fold molar excess of Alexa Fluor-NHS 488 over mAb E4 or mAb B1. The coupling reaction was performed in borate buffer containing 0.05 M boric acid (Sigma), 0.1 M sodium chloride (pH 8.5) and 5% DMSO at room temperature for 1 h. Unconjugated Alexa Fluor 488 was removed by gel filtration with PBS using a sephadex G-25 column (GE Healthcare Bio-Science, Uppsala, Sweden).

Confocal laser scanning microscopy. Internalization of Alexa Fluor 488-conjugated human anti-hNKG2D mAb E4 (mAb E4-488) was determined by kinetic studies using confocal laser scanning microscopy (CLSM). 6 x 10⁵ human NKL cells were seeded in 6-well round bottom plates and the Fc-receptors blocked with PBS/10% human AB serum (PAA) for 15 min at 4°C. After washing the cells were stained with 1 μg mAb E4-488 or human IgG1-488 isotype control for 45 min on ice. Cells were washed again and incubated at 37°C for 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h and 4 h. Cells were then placed on ice and stained intracellularly with ~500 nM DAPI at room temperature for 30 min using the Cytofix/Cytoperm Fixation/Permeabilization kit (BD). Cells were plated on coverslips and mounted in fluorescein-concentrating mounting medium (DakoCytomation) before analysis with a Leica TCS SP2 AOBS confocal laser scanning microscope. Optical sections (0.2 μm) were prepared using a HCX PL APO 1.4 oil 63x objective. Confocal microscopy images were acquired by use of Amira 3.1.1 (Visage imaging) software.

Analysis of in vitro internalization of mAb E4 by FACS. After blocking of NKL cells with 10% human AB serum for 15 min at 4°C mAb E4-488 or a human IgG1-488 isotype control were added to blocked cells (1 μg of antibody per 5 x 10⁵ cells in 200 μl of PBS-F for 30 min at 4°C). Then cells were pelleted, washed once with PBS-F and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (h.f.c.S) (Biochrom), 2 mM L-glutamine, 100 μg/ml Streptomycin/ Penicillin. Cells were transferred to a 37°C tissue culture incubator, and aliquots of 5 x 10⁵ cells removed at various time points (5 min, 15 min, 30 min, 60 min, 120 min, 180 min and 240 min). At specified time points, cells were immediately placed on ice and stored at 4°C. At each time point, one aliquot of cells was resuspended in 200 μl of 2% paraformaldehyde, a second aliquot was resuspended in 200 μl of 0.1% acetic acid wash (ultra pure water, 150 mM NaCl, HCl, pH 2.5) for 5 min at room temperature to remove non-immunized antibody prior to fixing in 2% paraformaldehyde. Fixed cells were then analyzed by FACS.

Statistical analysis. Differences between groups were analyzed using the One Way ANOVA test. Values of p < 0.05 were considered significant.

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