Published in final edited form as:

J Mol Biol. 2008 December 31; 384(5): 1143–1156. doi:10.1016/j.jmb.2008.09.008.

Generation, affinity maturation, and characterization of a human anti-human NKG2D monoclonal antibody with dual antagonistic and agonistic activity

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Summary

In humans, NKG2D is an activating receptor on NK cells and a costimulatory receptor on certain T cells and plays a central role in mediating immune responses in autoimmune diseases, infectious diseases, and cancer. Monoclonal antibodies that antagonize or agonize immune responses mediated by human NKG2D are considered to be of broad and potent therapeutic utility. Nonetheless, monoclonal antibodies to NKG2D that are suitable for clinical investigations have not been published yet. Here we describe the generation, affinity maturation, and characterization of a fully human monoclonal antibody to human NKG2D. Using phage display technology based on a newly generated naïve human Fab library in phage display vector pC3C followed by a tandem chain shuffling process designed for minimal deviation from natural human antibody sequences, we selected a human Fab, designated KYK-2.0, with high specificity and affinity to human NKG2D. KYK-2.0 Fab blocked the binding of the natural human NKG2D ligands MICA, MICB, and ULBP2 as potently as a commercially available mouse anti-human NKG2D monoclonal antibody in IgG format. Conversion of KYK-2.0 Fab to IgG1 resulted in subnanomolar avidity for human NKG2D. KYK-2.0 IgG1 was found to selectively recognize defined subpopulations of human lymphocytes known to express NKG2D, i.e. the majority of human CD8+, CD16+, and CD56+ cells as well as a small fraction of human CD4+ cells. In solution, KYK-2.0 IgG1 interfered with the cytolytic activity of *ex vivo* expanded human NK cells. By contrast, immobilized KYK-2.0 IgG1 was found to strongly induce human NK cell activation. The dual antagonistic and agonistic activity promises a wide range of therapeutic applications for KYK-2.0 IgG1 and its derivatives.

Keywords

NKG2D; NK cells; human monoclonal antibodies; phage display; antibody engineering

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Introduction

In humans, natural killer (NK) cells comprise 5–10% of circulating lymphocytes and constitute a key component of the innate immune system as they have the ability to immediately secrete interferon- γ , as well as to release cytotoxic granules containing perforin and granzymes upon target cell recognition. Unlike B cells and T cells of the adapted immune system, NK cells do not have antigen receptors that are diversified by somatic recombination and mutation events. However, based on their hematopoietic lineage, cell surface receptor repertoire, and killing mechanism, NK cells are more closely related to T cells than to cells of the innate immune system.¹ NK cells express two types of cell surface receptors referred to as *inhibitory receptors* and *activating receptors*. Both types of cell surface receptors consist of several different protein families. Inhibitory receptors recognize MHC class I ligands on target cells, whereas the activating receptors can recognize MHC class I, MHC class I-related, and non-MHC ligands on target cells. The integration of activating and inhibitory signals provided by the target cells ultimately controls NK cell reactivity through processes that remain incompletely understood. Tumor cells and virally infected cells often express activating receptors and downregulate inhibitory receptors, thus rendering them susceptible to NK cell mediated killing. Prominent NK cell surface receptors include CD16 (Fc γ RIIIA; an activating receptor that binds IgG and mediates antibody-dependent cellular cytotoxicity), CD56 (NCAM; a cell adhesion molecule), the polymorphic KIR protein family which recognizes conventional MHC class I ligands (HLA-A, HLA-B, and HLA-C), the polymorphic CD94/NKG2A/NKG2C/NKG2E/NKG2F protein family which recognizes nonconventional MHC class I ligands (HLA-E), the natural cytotoxicity receptors NKp30/NKp44/NKp46/NKp80 which are activating receptors whose ligands have remained largely elusive, and NKG2D which is an activating receptor that recognizes MHC class I-related ligands.

Despite its name, NKG2D^{2,3} is functionally and structurally distinct from the CD94/NKG2A/NKG2C/NKG2E/NKG2F protein family. NKG2D is a type II transmembrane glycoprotein with an N-terminal cytoplasmic domain (amino acids 1–51) followed by a transmembrane anchor (amino acids 52–72) and a C-terminal extracellular domain (amino acids 73–216) with three potential N-glycosylation sites. At the cell surface, NKG2D forms a homodimer stabilized by a disulfide bridge and associates with the adaptor protein DAP10, a type I transmembrane protein that exclusively mediates NKG2D signaling. The NKG2D homodimer associates with two DAP10 homodimers, thereby forming a hexameric structure.⁴ In the absence of DAP10, NKG2D is retained inside the cell.⁵ NKG2D recognizes transmembrane proteins MICA, MICB, and ULBP4 as well as GPI-anchored membrane proteins ULBP1, ULBP2, and ULBP3. Like MHC class I molecules but in striking contrast to their receptor NKG2D, these MHC I-related ligands are polymorphic. Unlike MHC class I molecules, however, MIC and ULBP proteins do not bind and display peptides and do not associate with β 2 microglobulin. Induced in stressed cells by the DNA damage response pathway⁶, NKG2D ligands are often expressed on tumor cells and virally infected cells but not on healthy cells. Through recognition of NKG2D ligands, NKG2D is a key molecule in mediating immunosurveillance by NK cells as evidenced by the findings that both tumors and viruses have developed molecular strategies to evade NKG2D immunosurveillance. For example, whereas tumors often secrete soluble MICA and MICB as decoys, cytomegalovirus (CMV) expresses a protein known as UL16 which prevents the cell surface expression of MICB, ULBP1, and ULBP2. In addition, the direct involvement of NKG2D in tumor immunosurveillance was recently shown in NKG2D-deficient mice that were crossed with transgenic mouse models of spontaneous malignancy.^{7,8} Expression of NKG2D ligands on healthy cells has been detected in several autoimmune diseases. In NOD mice, a mouse model of type I diabetes, mouse NKG2D ligand RAE-1 is expressed on pancreatic islet cells. A rat anti-mouse NKG2D monoclonal antibody (mAb) that did not deplete NKG2D⁺ NK cells and NKG2D⁺ T cells was shown to completely prevent type I diabetes in NOD mice.⁹

In humans, in addition to NK cells, NKG2D is also expressed on T cells, including CD8+ $\alpha\beta$ TCR+ T cells, $\gamma\delta$ TCR+ T cells, and NKT cells. The participation of NKG2D in fighting cancer and viral infections as well as in promoting autoimmune diseases likely involves both NKG2D + NK cells and NKG2D+ T cells. On T cells, however, rather than being an activating receptor NKG2D functions as a costimulatory receptor similar to CD28,¹⁰ albeit more restricted.¹¹ The central role that NKG2D plays in mediating immune responses in autoimmune diseases, infectious diseases, and cancer, makes NKG2D an attractive target for therapeutic intervention. This perception is further supported by the fact that a single receptor mediates recognition of a diverse and polymorphic group of ligands¹², singling out NKG2D as unique target for a broad range of applications. In particular mAbs that interfere with NKG2D receptor/ligand interactions and thereby, depending on the context, antagonize or agonize immune responses mediated by NKG2D could be of broad and potent therapeutic utility. Nonetheless, mAbs to NKG2D that are suitable for clinical investigations have not been published yet.

To facilitate clinical investigations of the therapeutic potential of NKG2D targeting, we here report the generation, affinity maturation, and characterization of a fully human anti-human NKG2D mAb. Taking into consideration that the immunogenicity of mouse, chimeric mouse/human, or even humanized anti-human NKG2D mAbs would likely interfere with their therapeutic potential, our efforts focused on the generation of a fully human mAb with minimal deviation from natural human antibody sequences. Over the past three decades, a number of different technologies have been developed for the generation of human mAbs. Arguably the most successful and accessible technology has been phage display. Not only does phage display provide a potent platform for the de novo generation of human mAbs to human antigens but also for their affinity maturation in vitro.^{13,14} Among the most widely applied phage display technologies for affinity maturation of human mAbs in Fab or scFv format are chain shuffling^{15,16} and CDR walking.¹⁷ Although both methods have their merits, chain shuffling provides access to a more diverse structural repertoire without introducing intentional synthetic mutations.

Results and Discussion

Design and generation of a naïve human Fab library in phage display vector pC3C

We previously reported the design of pC3C, which is based on phagemids from the pComb3 series¹⁸, for the generation and selection of Fab libraries with human constant domains.¹⁹ A key feature of pC3C is its ability to accommodate Fab libraries that were assembled in just 2 PCR amplification steps, thereby improving the Fab library complexity and ultimately the number, diversity, and affinity of selected Fab. Thus, pC3C might be particularly suited for the generation of a naïve human Fab library and its selection for rare human anti-human specificities. In particular ubiquitous and conserved human antigens like NKG2D, which is found on the cell surface of both NK cells and T cells, still impose considerable challenges for the selection of human antibodies by phage display.

Freshly harvested bone marrow from 6 healthy donors of diverse age, sex, and ethnicity was separately processed for total RNA preparation and RT-PCR amplification of human V_K , V_λ , and V_H encoding sequences. To include all human germlines, we used a total of 61 newly designed primers in 186 different and separate combinations for each of the 6 healthy donors. The V_K , V_λ , and V_H pool was subsequently used to assemble separate V_K - C_K - V_H and V_λ - C_λ - V_H cassettes in a single PCR amplification step. Cloning into pC3C by asymmetric *Sfi*I ligation resulted in 2 libraries consisting of approximately 1.0×10^9 (κ) and 0.5×10^9 (λ) independently transformed human Fab clones, respectively (Figure 1). The integrity and diversity of the Fab libraries was confirmed by ELISA and DNA fingerprinting. For selection by phage display, the 2 Fab libraries were combined and tested with tetanus toxoid (TT) as antigen. Three rounds of panning on immobilized TT resulted in the selection of a diverse panel of human anti-TT

Fab clones. All sequenced clones had a κ light chain. One of these clones, human Fab TT11, served as negative control throughout this study.

With a total of 1.5×10^9 independent clones, our naïve human Fab library provides a mid range size between the generally smaller immune antibody libraries (10^7 – 10^8 independent clones) and large naïve and synthetic antibody libraries (10^{10} – 10^{11} independent clones).^{13,14} Based on the documented correlation of library size and probability of finding high affinity antibodies^{20,21}, our library was anticipated to yield naïve human Fab with affinities in the range of 10 to 100 nM, thus providing a suitable starting point for affinity maturation.

Selection and affinity maturation of human anti-human NKG2D Fab

Next, the naïve human Fab library was selected by 10hage display through 4 rounds of panning on an immobilized recombinant fusion protein consisting of human Fc and the extracellular domain (amino acids 78–216) of human NKG2D (human Fc-NKG2D). A number of selected clones were subjected to an initial characterization by ELISA, DNA fingerprinting, and DNA sequencing, revealing the selection of a single human Fab clone that bound to human Fc-NKG2D but not to human Fc. This clone had a λ light chain and was designated KYK-1.0. An independent selection of the naïve human Fab library against soluble human Fc-NKG2D, using a mouse anti-human IgG1 Fc-specific mAb coated onto magnetic beads for capturing, again yielded KYK-1.0 and no additional clones.

These results suggested that KYK-1.0 provided the only selectable solution for human NKG2D binding in our naïve human Fab library. Since KYK-1.0 was generated by random combination of its V_L and V_H encoding sequences and the at best 1.5×10^9 different combinations in the library only represented a very small fraction of all possible combinations (e.g., 10^{14} for $10^7 V_L$ and $10^7 V_H$), we sought to search for additional selectable solutions by first replacing the light chain of KYK-1.0 with a naïve human light chain library generated from the same source as our naïve human Fab library. Subsequently, the same procedure could be applied to the heavy chain fragment of KYK-1.0. Using this sequential naïve chain shuffling procedure (Figure 2), a much larger fraction of all possible combinations can provide selectable solutions, likely resulting in an affinity maturation of KYK-1.0. Unlike other directed evolution strategies for affinity maturation that are based on dispersed or focused synthetic mutagenesis^{13,14}, sequential naïve chain shuffling does not further deviate from natural human antibody sequences, thereby preserving low immunogenicity.

For naïve human light chain shuffling, we generated a κ and a λ library using a modified pC3C phagemid in which the ampicillin resistance gene was replaced by a chloramphenicol resistance gene in order to preclude the selection of contaminating KYK-1.0 clones. The 2 libraries were selected separately by 3 rounds of panning on immobilized human Fc-NKG2D. A number of different clones that bound to human Fc-NKG2D with apparently higher affinity than KYK-1.0 as judged by ELISA were identified by DNA fingerprinting and DNA sequencing. Notably, all clones had a λ light chain. For subsequent naïve human heavy chain fragment shuffling, the selected panel of λ light chains was combined with a heavy chain fragment library using the original pC3C phagemid with ampicillin resistance. The library was selected by 4 rounds of panning on immobilized human Fc-NKG2D and selected clones were again subjected to an initial characterization by ELISA, DNA fingerprinting, and DNA sequencing. One Fab that dominated in terms of number of independently selected clones also revealed superior binding properties as judged by ELISA. This Fab was designated KYK-2.0.

Shown in Figure 3 are the amino acid sequences of the variable domains of V_L and V_H of KYK-1.0 and KYK-2.0 aligned with their corresponding human germlines based on IgBLAST analysis (www.ncbi.nlm.nih.gov/igblast/). The amino acid sequences harbor several interesting features. First, the FR1–FR3 regions of V_H of KYK-1.0 and KYK-2.0 are

remarkably conserved with respect to their shared V gene VH 3–30 and among each other. By contrast, the FR1–FR3 regions of V $_{\lambda}$ of KYK-1.0 and KYK-2.0 are highly divergent, derived from different V gene classes (V $_{\lambda}$ 3–21 and V $_{\lambda}$ 1–36, respectively), and contain more somatic hypermutations. Second, the CDR3 regions from both V $_{\lambda}$ (LCDR3) and V $_{\text{H}}$ (HCDR3) of KYK-1.0 and KYK-2.0 are highly divergent. Of particular interest is the finding that the HCDR3 region of KYK-2.0 is 3 amino acids longer than the HCDR3 region of KYK-1.0, suggesting a different HCDR3 conformation. Third, V $_{\lambda}$ of KYK-1.0 contains 3 clusters with negatively charged amino acids in CDR1 (GGDDIETKSVH), CDR2 (DDDDRPS), and CDR3 (QVWDDNNDEWV). It is conceivable that these negatively charged clusters promote binding to the highly positively charged NKG2D dimer interface that mediates ligand binding.²² Interestingly, the affinity maturation from KYK-1.0 to KYK-2.0 diminished these negatively charged clusters. Taken together, our sequential naïve chain shuffling procedure yielded a related, yet substantially divergent solution for human NKG2D binding that (i) was not present in the original naïve human Fab library, (ii) would have been missed by focused affinity maturation strategies such as CDR walking¹⁷, and (iii) did not accumulate more mutations deviating from the human germlines compared to the original solution.

Affinity and specificity of KYK-1.0 and KYK-2.0 Fab

KYK-1.0, KYK-2.0, and TT11 Fab were recombinantly equipped and expressed with a C-terminal (His)₆ tag using *Escherichia coli* expression vector pC3C-His²³ and purified by immobilized metal ion affinity chromatography (IMAC). ELISA on immobilized proteins was then used as a first assessment of the specificity of purified KYK-1.0 and KYK-2.0 Fab. As shown in Figure 4, both KYK-1.0 and KYK-2.0 Fab bound human NKG2D but neither mouse NKG2D nor a panel of other proteins that were tested in parallel. The lack of species cross-reactivity of both KYK-1.0 and KYK-2.0 was expected as human and mouse NKG2D only share 60% amino acid sequence identity, reflecting the pace of divergent evolution of immune systems in general and NK cells and NKG2D in particular. The ELISA also suggested a substantially higher affinity of the evolved KYK-2.0 Fab compared to the original KYK-1.0 Fab. The control Fab, TT11, revealed specific binding to tetanus toxoid (Figure 4).

For quantitative analysis of the thermodynamic and kinetic binding properties, the interaction of KYK-1.0 and KYK-2.0 Fab, respectively, with human NKG2D was analyzed by surface plasmon resonance using a BIAcore 2000 instrument (Table 1). The affinities (K_d) were measured as 27 nM for KYK-1.0 Fab and 5.8 nM for KYK-2.0 Fab, demonstrating a ~4.5 fold overall improvement following affinity maturation. The affinity of KYK-1.0 Fab is well within the range of affinities obtained for naïve human and synthetic human Fab libraries that are more than 10 times larger in terms of number of independent Fab clones^{20,24,25}. The higher affinity of KYK-2.0 Fab was solely mediated by a ~8.5 fold slower dissociation compared to KYK-1.0 Fab, an expected result for the k_{off} -driven selection methodology we applied. Interestingly, KYK-1.0 Fab revealed an extraordinarily fast association with a k_{on} of $1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). This is the fastest association we have measured for a Fab selected by phage display, including chimeric rabbit/human Fab from immune libraries^{19,26} and human Fab evolved by CDR walking.²⁷ In general, the selection of a k_{on} that exceeds $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ has been confined to Fab that were derived from synthetic human libraries and further improved by affinity maturation.²⁸ It is conceivable that the extraordinarily fast association of KYK-1.0 Fab is driven by electrostatic attraction²⁹ between the negatively charged clusters of KYK-1.0 Fab and the positively charged interface of the NKG2D dimer. This is supported by the observation that the affinity maturation from KYK-1.0 to KYK-2.0 Fab not only diminished the negatively charged clusters but also reduced the k_{on} despite a gain in affinity (Table 1). It may thus be possible to preserve the k_{on} of KYK-1.0 Fab by a focused (rather than dispersed) mutagenesis strategy in which amino acid residues outside the negatively charged clusters are targeted for randomization and selection. Alternatively, k_{on} -driven selection or

screening methodologies³⁰ or the introduction of negatively charged amino acids outside the antigen binding site²⁹ could be applied to preserve or reinstate the k_{on} of KYK-1.0 during or following affinity maturation, respectively. As an independent confirmation, we also analyzed the interaction of KYK-2.0 Fab and human NKG2D by quartz crystal microbalance using an Attana A100 instrument. Under various conditions, KYK-2.0 Fab revealed a k_{on} of $4.5\text{--}8.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and a k_{off} of $1.2\text{--}1.8 \times 10^{-3} \text{ s}^{-1}$, resulting in an affinity of 1.9–3.0 nM (data not shown). Thus, surface plasmon resonance and quartz crystal microbalance measurements gave fairly consistent thermodynamic and kinetic binding data for the interaction of KYK-2.0 Fab and human NKG2D.

The suspected interaction of KYK-1.0 and KYK-2.0 Fab with the positively charged interface of the NKG2D dimer, which is highly conserved between mouse and human NKG2D and implicated in NKG2D ligand binding^{22,31,32}, suggested that the selected antibodies may interfere with NKG2D receptor/ligand interactions. To confirm this, we first generated HEK 293F cells stably expressing human NKG2D by co-transfecting human NKG2D-IRES-EGFP and human DAP10 mammalian expression vectors and subsequently selecting transfectants that expressed EGFP by FACS. Similarly, as negative control, we generated HEK 293F cells stably expressing human ROR1³³ following transfection with a human ROR1-IRES-EGFP mammalian expression vector. Performing ELISA on whole cells, HEK 293F/human NKG2D and HEK 293F/human ROR1 cells were incubated with recombinant human MICA-Fc, MICB-Fc, or ULBP2-Fc in the presence or absence of KYK-1.0, KYK-2.0, and TT11 Fab and detected with biotinylated goat anti-human Fc polyclonal antibodies followed by streptavidin conjugated to horseradish peroxidase. As shown in Figure 5, KYK-2.0 Fab blocked the binding of all three human NKG2D ligands as potently as the commercially available mouse anti-human NKG2D mAb 149810 in IgG format. By contrast, KYK-1.0 Fab was less potent, and TT11 Fab did not reveal any blocking activity.

Affinity and specificity of KYK-1.0 and KYK-2.0 IgG1

For functional studies, KYK-1.0, KYK-2.0, and TT11 Fab were cloned, expressed, and purified as fully human IgG1 using mammalian expression vector PIGG, transiently transfected HEK 293F cells, and Protein A or Protein G affinity chromatography as described^{19,27}. As expected from their gain in avidity and despite slower association kinetics, KYK-1.0 and KYK-2.0 IgG1 revealed a strong improvement in virtual affinity as measured by surface plasmon resonance (Table 1). KYK-2.0 IgG1 and mouse anti-human NKG2D mAbs 149810 and 1D11 revealed similar virtual affinities in the subnanomolar range (Table 1). Additional studies based on surface plasmon resonance suggested that KYK-2.0, 149810, and 1D11 recognize three distinct but partially overlapping epitopes displayed by the extracellular domain of human NKG2D (data not shown).

To confirm and further assess the specificity of KYK-2.0 IgG1, its binding to human peripheral blood mononuclear cells (PBMC) subpopulations was analyzed by flow cytometry and compared to mouse anti-human NKG2D mAb 149810 (positive control) and TT11 IgG1 (negative control). Revealing essentially identical specificities for human T cells and NK cells, KYK-2.0 IgG1 and 149810 bound to the majority of human CD8+, CD16+, and CD56+ cells as well as to a small fraction of human CD4+ cells (Figure 6). Human B cells (CD19+) were not bound by either antibody and TT11 IgG1 was negative for all human PBMC subpopulations. Thus, KYK-2.0 IgG1 was found to selectively recognize human lymphocytes known to express NKG2D.¹

Dual antagonistic and agonistic activity of KYK-2.0 IgG1

The ability of KYK-2.0 Fab to potently interfere with human NKG2D receptor/ligand interactions suggested an antagonizing activity of KYK-2.0 IgG1 in solution. To test this, we

first used a novel *ex vivo* expansion protocol based on IL-15, IL-15R α , and 4-1BBL that was formulated to increase the cytolytic activity of human NK cells (H. Z. and C. L. M., manuscript in preparation). When compared for their cytolytic activity toward human chronic myelogenous leukemia (CML) cell line K562, a classical NK cell target expressing NKG2D ligands and not expressing MHC class I ligands, *ex vivo* expanded human NK cells revealed twice the activity measured for fresh human NK cells (Figure 7). In contrast to TT11 IgG1, both KYK 2.0 IgG1 and mouse anti-human NKG2D mAb 149810 significantly blocked this increase in cytolytic activity. The remaining cytolytic activity is likely mediated by other activating receptors, such as the natural cytotoxicity receptors NKp30/NKp44/NKp46/NKp80 which are not blocked by KYK 2.0 IgG1 and mouse anti-human NKG2D mAb 149810. Remarkably, the *ex vivo* expanded human NK cells also exhibited substantial cytolytic activity toward human Burkitt's lymphoma cell line Daudi (Figure 7). Like K562 cells, Daudi cells express NKG2D ligands and do not express MHC class I ligands. Unlike K562 cells, however, Daudi cells are known to be resistant to fresh human NK cells which we confirmed (Figure 7). Again, KYK 2.0 IgG1 and mouse anti-human NKG2D mAb 149810, but not TT11 IgG1, were found to significantly block the acquired cytolytic activity of *ex vivo* expanded human NK cells.

These findings suggested that *soluble* KYK-2.0 IgG1 exhibits antagonistic activity through interfering with effector cell to target cell recognition mediated by NKG2D receptor/ligand interactions. We next investigated whether the agonizing activity of target cell surface NKG2D ligands can be mimicked by *immobilized* KYK-2.0 IgG1. For this, we used the degranulation markers CD107a and CD107b which correlate with NK cell cytotoxicity.^{34,35} KYK-2.0 IgG1, in parallel to TT11 IgG1, mouse anti-human NKG2D mAb 149810, and nonspecific polyclonal mouse IgG, was coated on a 24-well tissue culture plate and incubated with IL-2 stimulated human PBMC from 4 different healthy donors. Subsequently, the percentage of degranulated NK cells (CD56+ CD107a/CD107b+) among total NK cells (CD56+) was quantified by flow cytometry (Figure 8 and Table 2). Whereas the percentage of degranulated NK cells did not increase following incubation with immobilized TT11 IgG1, KYK-2.0 IgG1 potently induced NK cell degranulation in PBMC from all 4 different healthy donors. By contrast, we did not observe NK cell degranulation in the presence of soluble KYK-2.0 IgG1 (data not shown). Mouse anti-human NKG2D mAb 149810 had been previously shown to exhibit agonistic activity in a redirected cross-species lysis assay with cell line P815 as target cells (mouse Fc γ R+) and human cell line NKL as effector cells (human NKG2D+).¹¹ We confirmed the agonistic activity of mouse anti-human NKG2D mAb 149810 in our degranulation assay by comparison with nonspecific polyclonal mouse IgG. Notably, despite matching avidities (Table 1) and indistinguishable antagonistic activities (Figure 7), KYK-2.0 IgG1 was found to exhibit substantially stronger agonistic activity than mouse anti-human NKG2D mAb 149810 (Figure 8 and Table 2).

Conclusions

Using phage display technology, we generated and evolved the fully human anti-human NKG2D mAb KYK-2.0 IgG1. To our knowledge, this is the first report of a mAb suitable for clinically investigating the therapeutic potential of NKG2D targeting. Through modulating NKG2D, KYK-2.0 IgG1 displays dual agonistic and antagonistic activity toward human NK cells. This activity profile in concert with designed minimized immunogenicity suggests broad and potent therapeutic utility of KYK-2.0 IgG1 and its derivatives in autoimmune diseases, infectious diseases, and cancer. Antagonizing NKG2D with non-depleting KYK-2.0 antibody formats (such as Fab, IgG4, or non-glycosylated IgG1) promises therapeutic applications in type I diabetes⁹, multiple sclerosis³⁶, colitis³⁷, rejection of solid organ transplants³⁸, and hepatitis³⁹. Due to diminished or absent Fc γ receptor binding, these non-depleting KYK-2.0 antibody formats are also less likely to display the agonistic activity we observed for

immobilized KYK-2.0 IgG1 and may thus be a preferred choice for initial clinical investigations. On the other hand, we are particularly interested in exploiting the agonistic activity of immobilized KYK-2.0 IgG1 for cancer therapy. Bispecific antibodies consisting of a tumor cell targeting moiety linked to a KYK-2.0 moiety for NK cell recruitment and activation are envisioned to mediate potent antitumor activity as has already been shown for analogous bispecific proteins based on NKG2D ligands.^{40,41} While the therapeutic ramifications of the observed antagonistic and agonistic activities remain to be shown, KYK-2.0 IgG1 and its derivatives belong to a new generation of mAbs designed to engage the host immune system by manipulating immune checkpoints on NK cells and T cells.⁴²

Materials and Methods

Generation of naïve human Fab library

Total RNA was prepared from bone marrow that was freshly harvested from 6 healthy donors of diverse age, sex, and ethnicity (Poietics Human Bone Marrow; Cambrex). Using established protocols⁴³, 10 mL bone marrow from each donor was homogenized with PowerGen 125 homogenizer (Thermo Fisher Scientific) and total RNA was extracted with TRI Reagent (Molecular Research Center) and further purified by LiCl precipitation. First-strand cDNA synthesis from total RNA using an oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) were carried out according to the manufacturer's protocol. V_K , V_λ , and V_H encoding sequences were separately amplified from each donor's first-strand cDNA by PCR using recombinant Taq DNA polymerase (Fermentas) and combinations of 12 sense/1 antisense primers for V_K , 20 sense/3 antisense primers for V_λ , and 19 sense/6 antisense primers for V_H , for a total of 186 different combinations, encompassing all human germlines. (Note: The antisense primers for V_λ and V_H align to J_λ and J_H germlines, respectively, whereas the antisense primer for V_K aligns to the C_K encoding sequence). Three pools combining V_K , V_λ , and V_H , respectively, from all donors were generated to increase the complexity of the libraries. Human C_K -pelB and C_λ -pelB encoding sequences required for the V_K - C_K - V_H and V_λ - C_λ - V_H cassette assembly, respectively, were amplified from pC_K¹⁹ and pC_λ. For the latter, a sequence encoding human C_λ (IGLC2; GenBank accession number J00253) was amplified by PCR from human bone marrow and cloned and confirmed analogous to pC_K.¹⁹ V_K - C_K - V_H and V_λ - C_λ - V_H cassettes were assembled in one fusion step based on 3-fragment overlap extension PCR, digested with *Sfi*I, and cloned into pC3C as described.¹⁹ Transformation of *E. coli* strain ER2738 (New England Biolabs) by electroporation yielded approximately 1.0×10^9 and 0.5×10^9 independent transformants for the κ and λ phagemid libraries, respectively. Randomly picked independent transformants from each library were analyzed for Fab expression by ELISA and for sequence diversity by DNA fingerprinting as described.²⁶ Using VCSM13 helper phage (Stratagene), the phagemid libraries were converted to phage libraries as described⁴⁴ and stored at 4°C after adding 0.01 volume 2% (w/v) sodium azide.

Selection of KYK-1.0 by phage display

Based on established protocols⁴⁴, the re-amplified and combined naïve human Fab libraries were selected by 4 rounds of panning against immobilized human Fc-NKG2D (R&D Systems) or 3 rounds of panning against immobilized tetanus toxoid (TT; prepared from Sanofi Pasteur vaccine formulation by dialysis against PBS). During the panning against immobilized human Fc-NKG2D, polyclonal human IgG (Pierce) were added as decoy at a final concentration of 2.5 µg/µL. Both selections yielded a number of clones that were positive when tested for binding to human Fc-NKG2D or TT by ELISA. Further analyses of these clones by DNA fingerprinting with *Alu*I as well as by DNA sequencing revealed a single repeated λ clone (KYK-1.0) from the selection against Fc-NKG2D. By contrast, the selection against TT gave a number of different repeated κ clones of which TT11 was pursued to serve as negative control for all subsequent studies. The re-amplified and combined naïve human Fab libraries were also

selected by 4 rounds of panning against human Fc-NKG2D in solution, using mouse anti-human IgG1 Fc-specific mAb 10G/2C11 (Meridian Life Science) that was coated onto surface activated magnetic beads (MyOne Tosylactivated Dynabeads; Invitrogen) according to the manufacturer's protocol and used for capturing as described.⁴⁴ Again, KYK-1.0 was selected as single repeated clone.

Affinity maturation of KYK-1.0 by phage display

Affinity maturation of KYK-1.0 was performed sequentially for light chain and heavy chain fragment by naïve chain shuffling. For the first step, a modified pC3C phagemid, pC3C-Cam, was used in which the ampicillin resistance gene was replaced by the chloramphenicol resistance gene from plasmid pPCR-Script Cam SK(+) (Stratagene). The previously amplified V_K and V_λ encoding sequences from all 6 donors were combined with the V_H encoding sequence of KYK-1.0 through V_K - C_K - V_H and V_λ - C_λ - V_H cassette assembly as described for the generation of the naïve human Fab library, digested with *Sfi*I, and cloned into pC3C-Cam. Transformation of *E. coli* strain ER2738 by electroporation yielded approximately 1.5×10^7 independent transformants for each κ and λ phagemid libraries. The corresponding phage libraries were selected separately by 3 rounds of panning on immobilized human Fc-NKG2D, yielding a number of repeated λ clones, but no κ clones, that were positive when tested for binding to human Fc-NKG2D by ELISA and revealed sequence diversity when analyzed by DNA fingerprinting with *Alu*I as well as by DNA sequencing. (These clones were designated KYK-1.N for KYK-1.1, KYK-1.2, KYK-1.3 etc.). For the second step, the V_λ encoding sequences of approximately 100 KYK-1.N clones were amplified by PCR and combined with the previously amplified V_H encoding sequences from all 6 donors using V_λ - C_λ - V_H cassette assembly and *Sfi*I cloning into the original pC3C phagemid with the ampicillin resistance gene. Transformation of *E. coli* strain ER2738 by electroporation yielded approximately 5×10^8 independent transformants. The corresponding phage library was selected by 4 rounds of panning on immobilized human Fc-NKG2D, yielding several repeated clones of which one, designated KYK-2.0, was dominating as revealed by DNA fingerprinting with *Alu*I and DNA sequencing. KYK-2.0 also gave the strongest signal when tested for binding to human Fc-NKG2D by ELISA.

Expression and purification of KYK-1.0 and KYK-2.0 Fab

To remove the gene III fragment of pC3C (Figure 1) and add a C-terminal (His)₆ tag, the expression cassettes encoding KYK-1.0, KYK-2.0, and TT11 Fab were transferred by *Sfi*I cloning into pC3C-His.²³ Following transformation into *E. coli* strain XL1-Blue (Stratagene) and expression through IPTG induction, KYK-1.0, KYK-2.0, and TT11 Fab were purified from culture supernatants by IMAC as described.²³ The quality and quantity of purified Fab was determined by SDS-PAGE and A₂₈₀ absorbance.

ELISA on immobilized proteins

The following proteins were used for coating: Human Fc-NKG2D, mouse Fc-NKG2D, human CD22-Fc, human ULBP2-Fc (all from R&D Systems), human CD23 (Lab Vision Corporation), tetanus toxoid (Sanofi-Pasteur), and human Fc-ROR1.³³ Using 100 ng of protein for coating, ELISA was carried out as described.¹⁹

Surface plasmon resonance

Surface plasmon resonance for the measurement of the affinity of KYK-1.0 and KYK-2.0 Fab and the virtual affinity (avidity) of KYK-1.0 and KYK-2.0 IgG1 as well as mouse anti-human NKG2D mAbs 149810 (R&D Systems) and 1D11 (BD Biosciences) to human Fc-NKG2D (R&D Systems) was performed on a BIAcore 2000 instrument (GE Healthcare) as described¹⁹ except for using 20 mM NaOH (instead of 25 mM HCl) for regeneration.

Stable transfection of HEK 293F cells

Full-length human NKG2D cDNA, kindly provided by Dr. Charles L. Sentman⁴⁵, and full-length human ROR1 cDNA (OriGene) were cloned into mammalian expression vector pIRES2-EGFP (Clontech; with neomycin resistance gene) downstream of CMV promoter and upstream of IRES. The resulting plasmids were transfected into HEK 293F cells with 293fectin (Invitrogen) using conditions recommended in the manufacturer's protocol. Mammalian expression vector pCMV6-XL5 containing the full-length cDNA of human DAP10 under the control of a CMV promoter (OriGene; without neomycin resistance gene) was co-transfected (1:1) to permit cell surface expression of human NKG2D. The transfected cells were maintained in 25-cm²-flasks in plain FreeStyle serum-free medium (Invitrogen) supplemented with 200 µg/mL G418 (Invitrogen). Subsequently, attached cells were transferred to fresh flasks and expanded in plain FreeStyle serum-free medium. Flow cytometry revealed that >90% of the cells expressed EGFP. Fluorescent cells were further enriched with a FACSVantage SE DiVa instrument (BD Biosciences), expanded in plain FreeStyle serum-free medium, and transferred in Recovery Cell Culture Freezing Medium (Invitrogen) for cryopreservation in liquid nitrogen. Freshly thawed HEK 293F/human NKG2D and HEK 293F/human ROR1 cells were recovered and expanded in plain FreeStyle serum-free medium prior to subsequent experiments.

ELISA on whole cells

In a 96-well tissue culture plate (Corning), 4×10^5 stably transfected HEK 293F/human NKG2D or HEK 293F/human ROR1 cells were incubated with 2 µg KYK-1.0 Fab, KYK-2.0 Fab, TT11 Fab, mouse anti-human NKG2D mAb 149810, or no antibody in 2% (v/v) nonimmune goat serum (Jackson ImmunoResearch Laboratories) in PBS for 1 h on ice. Subsequently, 100 ng of human MICA-Fc, human MICB-Fc, and human ULBP2-Fc (all from R&D Systems) were added to the cells and incubated for 1 h on ice. After washing twice with PBS through centrifugation at 500 g for 5 min at 4°C, the cells were incubated with a 1:3000 dilution of biotinylated goat anti-human Fc polyclonal antibodies (Jackson ImmunoResearch Laboratories) in 2% (v/v) nonimmune goat serum in PBS for 1 h on ice. Subsequently, after washing twice with PBS as before, the cells were incubated with a 1:3000 dilution of HRP-coupled streptavidin (BD Biosciences) in 2% (v/v) nonimmune goat serum in PBS for 30 min on ice. After washing twice with PBS as before, HRP substrate 2,2'-Azino-bis(3-ethyl-benzthiazoline)-6-sulfonic acid (Roche) was prepared and added according to the manufacturer's directions and incubated at room temperature until a green color developed (5–10 min). The cells were spun down as before and the supernatants were transferred to a 96-well ELISA plate to measure the absorbance at 405 nm in a VersaMax microplate reader (Molecular Devices).

Expression and purification of KYK-1.0 and KYK-2.0 IgG1

For the expression of fully human KYK-1.0 IgG1 λ , KYK-2.0 IgG1 λ , and TT11 IgG1 κ , the V_H and light chain encoding sequences were PCR amplified using appropriately designed primers and cloned into mammalian expression vector PIGG as described.¹⁹ Using 293fectin, 300 µg of PIGG-KYK-1.0, PIGG-KYK-2.0, or PIGG-TT11 plasmids were transiently transfected into 3×10^8 HEK 293F cells and kept in 300 mL FreeStyle serum-free medium in a 500-mL spinner flask on a stirring platform at 75 rpm (CELLSPIN System; Integra) in a humidified atmosphere containing 8 % CO₂ at 37°C. After 4 days, the medium was collected after centrifugation, replaced for additional 3–4 days, and collected again. Pooled supernatants were then processed and IgG1 purified using 1-mL recombinant Protein A or Protein G HiTrap columns (GE Healthcare) as described.¹⁹ The quality and quantity of purified IgG1 was determined by SDS-PAGE and A₂₈₀ absorbance.

Flow cytometry

Purified KYK-2.0 and TT11 IgG1 were biotinylated using the BiotinTag Micro-Biotinylation Kit (Sigma-Aldrich). Human PBMC were prepared from freshly drawn whole blood of healthy donors obtained from the Department of Transfusion Medicine at the NIH by density gradient separation on lymphocyte separation medium (ICN Biochemicals) and kept on ice in undiluted human AB serum (Invitrogen) for 15 min to block Fcγ receptors. Blocked PBMC were diluted to 5×10^5 cells in 10% (v/v) human AB serum in PBS and incubated with 10 μg/mL biotinylated KYK-2.0 or TT11 IgG1 for 1 h on ice in a total volume of 50 μL. After washing twice with 2% (v/v) human AB serum in PBS, the cells were incubated with 2 μg/mL PE-coupled streptavidin (BD Biosciences) and APC-coupled co-staining mAbs (see below) for 30 min on ice, washed twice as before, and resuspended in 400 μL 2% (v/v) human AB serum in PBS. PBMC subpopulations were gated by co-staining with APC-coupled mouse anti-human CD4, CD8, CD16, CD19, and CD56 mAbs (all from BD Biosciences); 7-aminoactinomycin D (7-AAD; Invitrogen) was added to exclude dead cells from the analysis. PE-coupled mouse anti-human NKG2D mAb 149810 (R&D Systems) was used as positive control. Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). A total of 20,000 gated events were collected for each sample in a list mode file and data were analyzed using CellQuest software (BD Biosciences).

Human NK cell cytotoxicity assay

Human PBMC were prepared from whole blood as described above. Human NK cells (CD16 + CD56+) were negatively selected and purified from human PBMC by magnetic activated cell sorting (MACS) using the NK Cell Isolation Kit (Miltenyi Biotec). The purity of the selection was greater than 95%. Expansion was carried out for 1 week in the presence of 10 ng/mL recombinant human IL-15 (PeproTech) and artificial antigen presenting cells (aAPCs)⁴⁶ expressing human 4-1BBL and human IL-15Rα at a ratio of 1–2 to 1 (cell line 2D11; H. Z. and C. L. M., manuscript in preparation). The cytolytic activity of purified human NK cells as effector cells before or after expansion was tested in a conventional ⁵¹Cr release assay using human cell lines K562 and Daudi (American Type Culture Collection) as target cells. Briefly, target cells (T) were radiolabeled with Na⁵¹CrO₄ (PerkinElmer) for 1 h at 37°C and 5% CO₂, then washed and co-incubated with effector cells (E) in 96-well U-bottomed plates at an E/T ratio of 40:1 in triplicates of 5,000 target cells/well. To test the blockade of cytolytic activity, KYK-2.0 IgG1, TT11 IgG1 (negative control), and mouse anti-human NKG2D mAb 149810 (positive control) were added to a final concentration of 20 μg/mL. After 4 h at 37°C and 5% CO₂, supernatants were collected and counted in a gamma counter (PerkinElmer). The percent of specific lysis was calculated as (experimental release minus spontaneous release) times 100 divided by (maximum release minus spontaneous release). Maximum release was determined through lysis in the presence of 0.1 N HCl.

Human NK cell degranulation assay

Human PBMC from 4 different healthy donors were prepared from whole blood as described above or from leukocytes collected by apheresis and were cultured in IMDM medium (Invitrogen) supplemented with 10% (v/v) human AB serum (Invitrogen), penicillin/streptomycin, and 100 U/mL IL-2 (PeproTech) at a density of 2×10^6 cells/mL for 4–10 days before the experiment. Every 3–4 days, half of the culture medium was replaced with fresh medium. One day before the experiment, a 24-well tissue culture plate was coated with 500 μL/well of 5 μg/mL KYK-2.0 IgG1, TT11 IgG1, mouse anti-human NKG2D mAb 149810, or nonspecific polyclonal mouse IgG (Jackson ImmunoResearch Laboratories) in PBS at 4°C overnight. After washing 3 times with PBS, 1×10^6 cells of the non-adherent fraction of the prepared PBMC diluted in 1 mL of the same medium plus 0.67 μL GolgiStop (BD Biosciences; a protein transport inhibitor containing monensin) were added to each well and incubated for

3 h at 37°C and 5% CO₂. Subsequently, the cells were stained with a mixture of FITC-coupled mouse anti-human CD107a and mouse anti-human CD107b mAbs (BD Biosciences) to measure degranulation. NK cells were gated by co-staining with APC-coupled mouse anti-human CD56 mAb and dead cells were gated out by 7-AAD co-staining. Flow cytometry was performed using a FACSCalibur instrument and analyzed using CellQuest software as described above.

Abbreviations

CDR, complementarity determining region; FR, framework region; HA, hemagglutinin; IMAC, immobilized metal ion affinity chromatography; PBMC, peripheral blood mononuclear cells.

Acknowledgements

This research was supported by the Intramural Research Program of the Center for Cancer Research, NCI, NIH. We thank Dr. Charles L. Sentman (Dartmouth Medical School, Lebanon, NH) for full-length human NKG2D cDNA; Dr. David H. Margulies (NIAID, NIH, Bethesda, MD) for providing access to his BIAcore 2000 instrument; Johan Lindberg and Dr. Alexander Kovacs (Attana AB, Stockholm, Sweden) for facilitating and carrying out quartz crystal microbalance measurements, respectively; Veena Kapoor (NCI, NIH, Bethesda, MD) for sorting stable transfectants by FACS; and Drs. Steven J. Burgess and Francisco Borrego (NIAID, NIH, Rockville, MD) for technical advice on the degranulation assay.

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Figure 1.

Generation of a naïve human Fab library in phage display vector pC3C. Using a total of 186 different and separate combinations, human V_κ, V_λ, and V_H encoding sequences were amplified by RT-PCR from bone marrow of 6 healthy donors, assembled into V_κ-C_κ-V_H and V_λ-C_λ-V_H cassettes by 3-fragment overlap extension PCR, and cloned into phagemid pC3C by asymmetric *Sfi*I ligation. The resulting naïve human Fab library was subsequently converted from phagemid to phage by transformation of *E. coli* and addition of helper phage, resulting in a library size of approximately 1.5×10^9 independent Fab clones.

Figure 2. Selection and affinity maturation of a human anti-human NKG2D mAb. (A) Using phage display, a human Fab, termed KYK-1.0 Fab, with high specificity for human NKG2D was selected from a naïve human Fab library consisting of 1.5 billion independent human Fab with both kappa and lambda light chains (1). For affinity maturation *in vitro*, KYK-1.0 Fab was

subsequently subjected to a tandem chain shuffling process. In the first chain shuffling step (2), the human lambda light chain of KYK-1.0 Fab was replaced with a naïve human lambda and kappa light chain library which was then re-selected by phage display against human NKG2D. In the second chain shuffling step (3), the human heavy chain fragment, also termed Fd fragment, of KYK-1.0 Fab was replaced with a naïve human Fd fragment library which was then also re-selected against human NKG2D. The evolved human Fab was termed KYK-2.0 Fab. Note that both KYK-1.0 and KYK-2.0 Fab have human lambda light chains. KYK-1.0 Fab and KYK-2.0 Fab were expressed in *E. coli* using *E. coli* expression vector pC3C-His6. **(B)** KYK-2.0 Fab was converted (4) to IgG1 using mammalian expression vector PIGG and expressed in human embryonic kidney (HEK) 293F cells.

V_{λ}		FR1	CDR1	FR2	CDR2	FR3
V_{λ} 3-21	SY-----P-----K---T-	--NN-GS----	-----V-	--S----	-----S-----T-----	
KYK-1.0	QPVLTQPSVSVAPGETARIPC	GGDDIETKSVH	WYQQKPGQAPVLVIY	DDDDRPS	GIPERFFGSNSGNTATLSISRVEAGDEADYYC	
	** * * * *	* * * * *	* * * *	* *	* * * * *	
KYK-2.0	QSALTQPASVSGSPGQSITISC	SGSSSNIGNNAVN	WYQQLPGKAPKLLIY	YDDLPS	GVSDRFSGSKSGTSAFLAISGLQSEADYYC	
V_{λ} 1-36	--V---P---EA-R-RV----	-----	-----	-----	-----S-----	
		CDR3	FR4			
KYK-1.0	QVWDDNNDEWV	FGGGTQLTVL				
	** * * *	*				
KYK-2.0	AAWDDSLNGPV	FGGGTKLTVL				
V_H		FR1	CDR1	FR2	CDR2	FR3
V_H 3-30	Q-----	-----	-----	-----	-----	-----
KYK-1.0	EVQLVESGGGVVQPGGSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	FIRYDGSNKYYADSVKG	RFTISRDN SKNTLYLQMNSLRAEDTAVYYCAK	
	* * *					
KYK-2.0	QVQLVESGGGLVKPGGSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	FIRYDGSNKYYADSVKG	RFTISRDN SKNTLYLQMNSLRAEDTAVYYCAK	
V_H 3-30	-----V-Q-----	-----	-----	-----	-----	-----
		CDR3	FR4			
KYK-1.0	DR...FGYYLDY	WGQGTITVTVSS				
	* * *	*				
KYK-2.0	DRGLGDGTYFDY	WGQGTITVTVSS				
		CDR3	FR4			

Figure 3.

Amino acid sequence alignment of the variable domains V_{λ} and V_H of KYK-1.0 and KYK-2.0 with their corresponding human germlines (only V genes). Shown are the 4 framework regions (FR) and 3 complementarity determining regions (CDR). Dashes indicate amino acids that are identical in the human germlines. Differences between KYK-1.0 and KYK-2.0 are indicated by asterisks.

Figure 4.

Analysis of KYK-1.0 and KYK-2.0 Fab specificity by ELISA. Shown is the binding of KYK-1.0 and KYK-2.0 Fab to a panel of proteins immobilized on an ELISA 96-well plate at 100 ng/well. TT11 Fab, which was selected from the same naïve human Fab library against tetanus toxoid, served as control. Goat-anti-human Fab polyclonal antibodies conjugated to horseradish peroxidase were used for detection. The absorbance at 405 nm was read before the signal obtained for KYK-2.0 Fab on immobilized human NKG2D reached saturation. The signal obtained for KYK-1.0 Fab on immobilized human NKG2D increased further relative to background after longer exposure (data not shown). Error bars indicate mean $\pm$ SEM ($n = 3$).

Figure 5. Analysis of KYK-1.0 and KYK-2.0 Fab for interfering with NKG2D receptor/ligand interactions. In an ELISA 96-well plate, HEK 293F transfectants that stably express cell surface human NKG2D (black bars) or human ROR1 (white bars) were incubated with recombinant human MICA-Fc, MICB-Fc, or ULBP2-Fc in the presence or absence of KYK-1.0 Fab, KYK-2.0 Fab, TT11 Fab (negative control), and mouse anti-human NKG2D mAb 149810 (positive control). Biotinylated goat anti-human Fc polyclonal antibodies followed by streptavidin conjugated to horseradish peroxidase were used for detecting ligand binding. Error bars indicate mean $\pm$ SEM (n = 4).

Figure 6.

Analysis of the binding of KYK-2.0 IgG1 to human PBMC subpopulations by flow cytometry. Freshly isolated human PBMC were stained with APC-coupled mouse mAb to CD4, CD8, CD16, CD19, or CD56 (x axes) and with biotinylated KYK-2.0 IgG1 or TT11-IgG1 (negative control) followed by streptavidin-PE (y axes). PE-coupled mouse anti-human NKG2D mAb 149810 was used as positive control.

Figure 7. Blockade of the cytolytic activity of *ex vivo* expanded human NK cells by KYK-2.0 IgG1. Human K562 (top) or Daudi cells (bottom) were labeled with ^{51}Cr and incubated with fresh human NK cells or *ex vivo* expanded human NK cells at an E:T ratio of 40:1 in the absence or presence of KYK-2.0 IgG1, TT11 IgG1 (negative control), and mouse anti-human NKG2D mAb 149810 (positive control). The cytolytic activity was measured as specific lysis based on ^{51}Cr release. Error bars indicate mean $\pm$ SD ($n = 3$); the probability (p) is based on a paired one-tailed t-test.

Figure 8.

Degranulation of human NK cells by immobilized KYK-2.0 IgG1. Freshly isolated human PBMC were stimulated with IL-2 and then incubated with immobilized mAbs. Activation was detected with a mixture of FITC-coupled mouse anti-human CD107a and mouse anti-human CD107b mAbs. NK cells were detected with an APC-coupled mouse anti-human CD56 mAb. CD56⁺ CD107a/CD107b⁺ cells are gated (R3). Typical results for one healthy donor are shown. The percentage of degranulated NK cells (CD56⁺ CD107a/CD107b⁺) among total NK cells (CD56⁺) is shown in Table 2 for 4 different healthy donors.

Table 1

Analysis of KYK-1.0 and KYK-2.0 Fab and IgG1 binding to human NKG2D by surface plasmon resonance using a BIAcore 2000 instrument. Mouse anti-human mAbs 149810 and 1D11 in IgG format were included for comparison.

	k_{on} ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_{off} ($\times 10^{-2} \text{ s}^{-1}$)	K_d (nM)
KYK-1.0 Fab	14.0	3.7	27
KYK-1.0 IgG1	3.1	0.056	"1.8"
KYK-2.0 Fab	7.4	0.43	5.8
KYK-2.0 IgG1	3.2	0.012	"0.38"
149810	1.4	0.0051	"0.36"
1D11	0.4	0.0029	"0.73"

Table 2

Degranulation of human NK cells by immobilized KYK-2.0 IgG1 and controls.

	Donor 1	Donor 2	Donor 3	Donor 4
PBS	2.6 %	2.9 %	16.8 %	6.0 %
KYK-2.0 IgG1	24.5 %	27.9 %	48.0 %	25.5 %
TT11 IgG1	2.4 %	5.1 %	16.8 %	5.4 %
149810	13.6 %	16.3 %	24.3 %	8.4 %
Mouse IgG	2.2 %	5.2 %	13.4 %	4.5 %