

# CD16A Activation of NK Cells Promotes NK Cell Proliferation and Memory-Like Cytotoxicity against Cancer Cells

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## Abstract

CD16A is a potent cytotoxicity receptor on human natural killer (NK) cells, which can be exploited by therapeutic bispecific antibodies. So far, the effects of CD16A-mediated activation on NK cell effector functions beyond classical antibody-dependent cytotoxicity have remained poorly elucidated. Here, we investigated NK cell responses after exposure to therapeutic antibodies such as the tetravalent bispecific antibody AFM13 (CD30/CD16A), designed for the treatment of Hodgkin lymphoma and other CD30<sup>+</sup> lymphomas. Our results reveal that CD16A engagement enhanced subsequent IL2- and IL15-driven NK cell proliferation and expansion. This effect involved the upregulation of CD25 (IL2R $\alpha$ ) and CD132 ( $\gamma_c$ ) on NK cells, resulting in increased sensitivity to low-dose IL2 or to IL15. CD16A engagement initially induced NK cell cytotoxicity. The lower NK cell

reactivity observed 1 day after CD16A engagement could be recovered by reculture in IL2 or IL15. After reculture in IL2 or IL15, these CD16A-experienced NK cells exerted more vigorous IFN $\gamma$  production upon restimulation with tumor cells or cytokines. Importantly, after reculture, CD16A-experienced NK cells also exerted increased cytotoxicity toward different tumor targets, mainly through the activating NK cell receptor NKG2D. Our findings uncover a role for CD16A engagement in priming NK cell responses to restimulation by cytokines and tumor cells, indicative of a memory-like functionality. Our study suggests that combination of AFM13 with IL2 or IL15 may boost NK cell antitumor activity in patients by expanding tumor-reactive NK cells and enhancing NK cell reactivity, even upon repeated tumor encounters. *Cancer Immunol Res*; 6(5): 517–27. ©2018 AACR.

## Introduction

Natural killer (NK) cells are cytotoxic innate lymphoid cells that are in the first line of defense against virally infected cells and cancer cells (1). In contrast to CD8<sup>+</sup> T cells, NK cells recognize abnormal cells through a defined set of germline-encoded receptors, such as the inhibitory KIR and NKG2A receptors and the activating NKG2D, DNAM-1, and NKp30 receptors (2, 3). NK cells respond to cells that display an incompatible repertoire or reduced levels of MHC class I molecules, enabling recognition of certain cancer cells which may evade CD8<sup>+</sup> T cell responses. Low expression of inhibitory ligands in combination with high levels of activating ligands on target cells result in NK cell activation and the release of perforin and granzyme B, mediating target cell death (4). The

responsiveness of NK cells to target cells can be augmented by prior activation with cytokines, such as IL2 or IL15.

In addition to their direct anti-tumor activity, NK cells contribute to the induction of adaptive anti-cancer responses and can fulfill immune regulatory functions (5). NK cells can acquire immunological memory and other properties associated with adaptive immunity, such as specific subset expansion and antigen-specific responses (6). In this context, preactivation of NK cells with IL12/15/18 has been shown to amplify and prolong NK cell responsiveness to tumor cells or cytokines, which was associated with epigenetic remodeling of the IFN $\gamma$  locus (7–11). Because these NK cells remember the previous exposure to IL12/15/18, resulting in more vigorous IFN $\gamma$  production upon restimulation, they are referred to as memory-like NK cells (6, 8). Without further activation, human naïve NK cells isolated from peripheral blood are reactive to only a limited number of tumor cell lines, such as the prototypical target cell line K562 (4). In particular, NK cells from cancer patients show low reactivity toward autologous tumor cells (12–14).

Therapeutic antibodies can significantly improve the cytotoxicity of naïve NK cells toward tumor cells, even in the presence of self-MHC class I (12–14). This antibody-dependent cellular cytotoxicity (ADCC) is conferred by the binding of the Fc portion of therapeutic human IgG1 antibodies to the NK cell-activating receptor CD16A (Fc $\gamma$ RIIIA), triggering NK cell activation through ITAM-containing adapters (15–17). A role for NK cells and ADCC in the clinical response to therapeutic antibodies has been inferred from the observation that patients carrying high-affinity CD16A allotypes (158V vs. 158F gene polymorphisms) have a more favorable prognosis after treatment (18, 19).

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However, the disparity in CD16A affinities in cancer patients, as well as the potential competition for CD16A binding of human serum IgG with conventional therapeutic antibodies, may limit the potential of ADCC by NK cells *in vivo* (20, 21). To improve CD16A engagement, antibody formats have been developed that bind CD16A in an Fc-independent manner with high affinity (22–24). As such, AFM13, a tetravalent bispecific tandem diabody (TandAb) without Fc domains, is the first bispecific antibody with high affinity and high specificity, bivalent binding to human CD16A on NK cells and to CD30 expressed on Hodgkin Reed–Sternberg cells (25). After completion of a phase I study in relapsed/refractory Hodgkin lymphoma patients (26), AFM13 is currently being tested (i) in a phase 2 monotherapy trial; (ii) in combination with anti-PD-1 (pembrolizumab) in a phase Ib clinical trial in patients with relapsed or refractory classic Hodgkin lymphoma; and (iii) in a translational phase Ib/IIa trial in patients with CD30<sup>+</sup> lymphoma with cutaneous manifestation.

In our study, we have investigated how CD16A engagement by therapeutic antibodies, such as AFM13, modulates the phenotype and proliferation of primary NK cells and their subsequent responsiveness to cytokines and cytotoxicity toward tumor cells. Our results reveal a thus far unappreciated role for CD16A in amplifying NK cell proliferation and expansion and in priming enhanced NK cell cytotoxicity and cytokine production when restimulated with cancer cells.

Taken together, our results suggest that AFM13-based immunotherapy in combination with NK cell-activating cytokines may improve NK cell cytotoxicity against tumor cells upon repeated exposure.

## Materials and Methods

### Cell lines

The erythroleukemic cell line K562 (ATCC), the Burkitt lymphoma cell line Daudi (ATCC), the classic Hodgkin lymphoma cell lines L428, HDLM-2, and L1236 (from P. Lichter, DKFZ, Heidelberg), the anaplastic large cell lymphoma cell line Karpas-299 (2016, Sigma-Aldrich), the acute T cell lymphoma line Jurkat (from R. Arnold, DKFZ), the cutaneous T cell lymphoma line HuT-78 (from P. Krammer, DKFZ), and murine Ba/F3 cell transfectants were maintained in RPMI 1640 supplemented with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Sigma-Aldrich). Cell line identities were confirmed by the MCA assay (Multiplexion GmbH) in 2016 (HuT-78 and L428) and 2017 (HDLM-2, K562, and Karpas-299). All cell lines were cultured for up to 4 months and were confirmed to be negative for *mycoplasma* infection (Multiplexion GmbH). Ba/F3 cells were generated by retroviral transduction using pmx-pie retroviral vectors and amphotropic Phoenix packaging cells to overexpress B7-H6 (provided by C. Watzl, Technical University Dortmund, Germany) or MICA, as previously described (27). To overexpress CD155, full-length unspliced CD155 cDNA was amplified by PCR from SiHa cervical carcinoma cells (forward primer: AGCTGCTAGCAGCAACTGGCAT containing a *NheI* restriction site, reverse primer: CAGCTTTCCTGAGTCTAGAGATCCTT containing an *XbaI* restriction site) and cloned it into the pcDNA3.1<sup>+</sup> vector (Invitrogen). Ba/F3 cells ( $4 \times 10^6$ ) were transfected with 20 to 40  $\mu$ g of plasmid DNA in OptiMEM medium (Invitrogen) by electroporation. Stable transfectants were selected using 1 mg/mL G418 (Invitrogen).

### NK cell isolation and culture

Buffy coats were collected from healthy adult blood bank donors (DRK-Blutspendedienst), according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all donors prior to blood donation, and ethical approval 87/04 was granted by the Ethik Kommission II of the Medical Faculty Mannheim. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient (density 1.077 g/mL; Biochrom, VWR) centrifugation. NK cells were purified from PBMCs by negative selection using the Human NK cell Isolation Kit (Miltenyi Biotec) or the MojoSort Human NK cell Isolation Kit (Biolegend), using LS separation columns (Miltenyi Biotec), according to the manufacturer's protocols. The purity of CD56<sup>+</sup>CD3<sup>+</sup>CD19<sup>−</sup>CD14<sup>−</sup> NK cells was 85% to 90% as measured by flow cytometry. Freshly isolated NK cells, hereafter referred to as "naive", were maintained overnight in SCGM medium (CellGenix) containing 10% human serum (Invitrogen; complete SCGM medium). PBMCs were maintained in RPMI 1640 medium containing 10% FCS (complete RPMI medium).

### NK cell activation by therapeutic antibodies

NK cells were cocultured with Karpas-299 or L428 cells at 1:1 ratio (each  $1 \times 10^6$  cells) in 24-well plates in the presence of AFM13 (CD30/CD16A) or AFM12 (CD19/CD16A; both provided by Affimed GmbH) at 0.1 to 1  $\mu$ g/mL for 20 hours in complete RPMI medium. Alternatively, NK cells were cultured in non-tissue culture–treated 24-well plates that had been coated with 10  $\mu$ g/mL of AFM13, rituximab (MabThera; Roche) or murine IgG1 (coated ctrl; does not engage human CD16A; Biolegend) in 0.5 mL PBS for 20 hours. When indicated, NK cells were treated with IL2 (12.5–400 U/mL; NIH), IL15 (0.6–10 ng/mL; Peprotech), or a combination of IL12 (10 ng/mL; Peprotech), IL15 (20 ng/mL), and IL18 (100 ng/mL; MBL), hereafter referred to as IL12/15/18, and cultured in complete SCGM medium for 2 to 5 days.

### NK cell proliferation and expansion

NK cell cultures were loaded with 2  $\mu$ mol/L CFSE (Sigma-Aldrich), incubated at room temperature for 15 minutes in the dark, and afterward washed in 5 mL pure FCS and 5 mL complete RPMI medium. NK cells were then cultured with IL2 (12.5–400 U/mL) or IL15 (0.6–10 ng/mL) at low cell densities ( $0.5 \times 10^6$ /mL) in 24-well plates in complete SCGM medium for 3 to 7 days. CFSE fluorescence was measured by flow cytometry. CFSE dilution was quantified by calculating the percentage of NK cells that underwent at least four divisions, as indicated by CFSE dilution peaks. Absolute NK cell numbers to evaluate NK cell expansion were obtained by counting trypan blue–negative and live-gated cells by microscopy and by flow cytometry (relative to counting beads), respectively.

### <sup>51</sup>Cr release assay, degranulation, and IFN $\gamma$

In <sup>51</sup>Cr release assays, NK cells were cocultured for 4 hours with  $2.5 \times 10^3$  Na-<sup>51</sup>chromate (<sup>51</sup>Cr)–labeled tumor target cells at escalating effector:target ratios (E:T; starting at 25:1 or 5:1 as depicted) in the presence of AFM13, AFM12, anti-EGFR cetuximab, or without therapeutic antibodies. The release of radioactive <sup>51</sup>Cr into the cell supernatant was measured on a  $\gamma$ -counter (Perkin Elmer) and indicated target cell lysis. When indicated,

10  $\mu\text{g}/\text{mL}$  of a combination of purified neutralizing antibodies against NKG2D (149810, R&D Systems; 1D11 LEAF, BioLegend) or mouse IgG1 (mIgG1) isotype control (BioLegend) was included at final concentration throughout the 4-hour incubation. Spontaneous and total release was obtained by incubation in medium and 10% Triton X-100 (Sigma-Fluka), respectively. The specific lysis was calculated by the following formula: percentage of specific lysis =  $100 \times (\text{experimental release} - \text{spontaneous release} / \text{total release} - \text{spontaneous release})$ .

To assess degranulation and intracellular IFN $\gamma$  expression, NK cells were cocultured with and without target cells at 1:1 ratio (each  $5 \times 10^4$  cells), AFM13, IL12/15/18 (10/20/100 ng/mL), or PMA (50 ng/mL) with ionomycin (1 mmol/L) in round-bottom 96-well plates for 4 hours in the presence of anti-CD107a-PE (H4A3; BioLegend) and GolgiPlug (1/100 v/v; BD Bioscience). Extracellular CD107a, a marker for degranulation, and intracellular IFN $\gamma$  expression were measured by flow cytometry.

Secretion of IFN $\gamma$  into cell supernatants (hereafter referred to as "production") was analyzed using the human IFN $\gamma$  ELISA MAX kit (BioLegend) after 24-hour coculture of NK cells with tumor cells at 1:1 ratio (each  $5 \times 10^4$  cells) in the presence of IL12/15 or PMA/ionomycin.

#### Flow cytometry

Extracellular staining was performed using  $5 \times 10^4$  NK cells in ice-cold PBS with 2% FCS and 0.05%  $\text{NaN}_3$  (Sigma-Aldrich) for 30 minutes in the dark. Intracellular staining was performed using the FoxP3 Staining Buffer Set (eBioscience) for IFN $\gamma$ , Fc $\epsilon$ R1 $\gamma$  and CD3 $\zeta$  and the Cytofix/Cytoperm kit (BD Bioscience) for perforin and granzyme B, according to the manufacturer's protocols. The following fluorescent monoclonal antibodies were applied: CD25-APC (BC96), CD132-PE (TUGh4), CD122-PE-Cy7 (TU27), CD69-APC (FN50), CD137-APC (4B4-1), ICAM-1-PE (HCD54), CD96-PE (NK92.39), CD16-FITC (3G8), NKG2D-APC (1D11), DNAM-1-PE (DX11), NKp46-Alexa 647 (9E2), NKp30-PE (p30-15), CD2-FITC (TS1/8), 2B4-APC (C1.7), NKp44-APC (p44-8), CD56-APC (HCD56), CD56-PE-Cy7 (HCD56), perforin-PE (BD48), granzyme B-Pacific Blue (GB11), (CD3 $\zeta$ -PE 6B10.2), IFN- $\gamma$ -APC (4S.B3), CD107a-FITC (H4A3) (all from BioLegend), NKG2A-APC (REA110; Miltenyi Biotec), and Fc $\epsilon$ R1 $\gamma$ -FITC (FCABS400F; Millipore). Samples were acquired on a FACSCalibur or FACSCanto II (BD Bioscience) and analyzed with FlowJo v10 software (FlowJo LLC). Y-axes in overlay histograms show counts (modal).

#### Statistical analysis

In GraphPad Prism, means of two groups were compared by paired two-tailed Student *t* tests. Means of multiple groups were assessed by one-way ANOVA to compare one parameter or by two-way ANOVA to compare two parameters, followed by Bonferroni multiple comparison *post hoc* test, as indicated. Error bars represent the SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

## Results

### AFM13 induces NK cell effector functions and expression of NK cell-activating receptors

To investigate NK cell functionality upon CD16A stimulation by AFM13, we first determined the effect of AFM13 on NK cells cocultured with different types of lymphoma cells. The presence of tetravalent bispecific AFM13 (CD30/CD16A) improved the

cytotoxic activity of naïve NK cells toward CD30<sup>+</sup> cancer cell lines of classic Hodgkin lymphoma (L428, HDLM-2), anaplastic large cell lymphoma (Karpas-299), and non-Hodgkin lymphoma (Jurkat, HuT-78) in 4-hour <sup>51</sup>Cr release assays (Fig. 1A; Supplementary Fig. S1A and S1B). Lysis of CD19<sup>+</sup>/CD30<sup>-</sup> Daudi cells could be increased by AFM12, a tetravalent bispecific CD19/CD16A antibody used as a control (Fig. 1A). Prior incubation of NK cells with IL2, IL15, or IL12/15/18 for 2 days potentiated AFM13-mediated NK cell cytotoxicity, especially against tumor cells weakly susceptible to cytokine-activated NK cell lysis (Supplementary Fig. S1C).

Next, we tested whether the presence of AFM13-opsonized tumor cells that were not labeled with <sup>51</sup>Cr (cold targets) could influence NK cell-mediated lysis of non-opsonized <sup>51</sup>Cr-labeled tumor cells (hot targets). Indeed, during the interaction of NK cells with AFM13-opsonized targets, lysis of non-opsonized bystander CD30<sup>+</sup> tumor cells was induced (Fig. 1B). Lysis of CD30<sup>-</sup> A431 tumor cells was not observed (Supplementary Fig. S1D), indicating that bystander cell lysis was specific for CD30<sup>+</sup> tumor cells and only observed in the presence of AFM13. Thus, NK cells were able to lyse bystander CD30<sup>+</sup> tumor cells when AFM13 had been presented on neighboring AFM13-opsonized target cells. In addition to NK cell cytotoxicity, intracellular IFN $\gamma$  expression and IFN $\gamma$  production was enhanced in response to AFM13-opsonized CD30<sup>+</sup> tumor cells (Supplementary Fig. S1E).

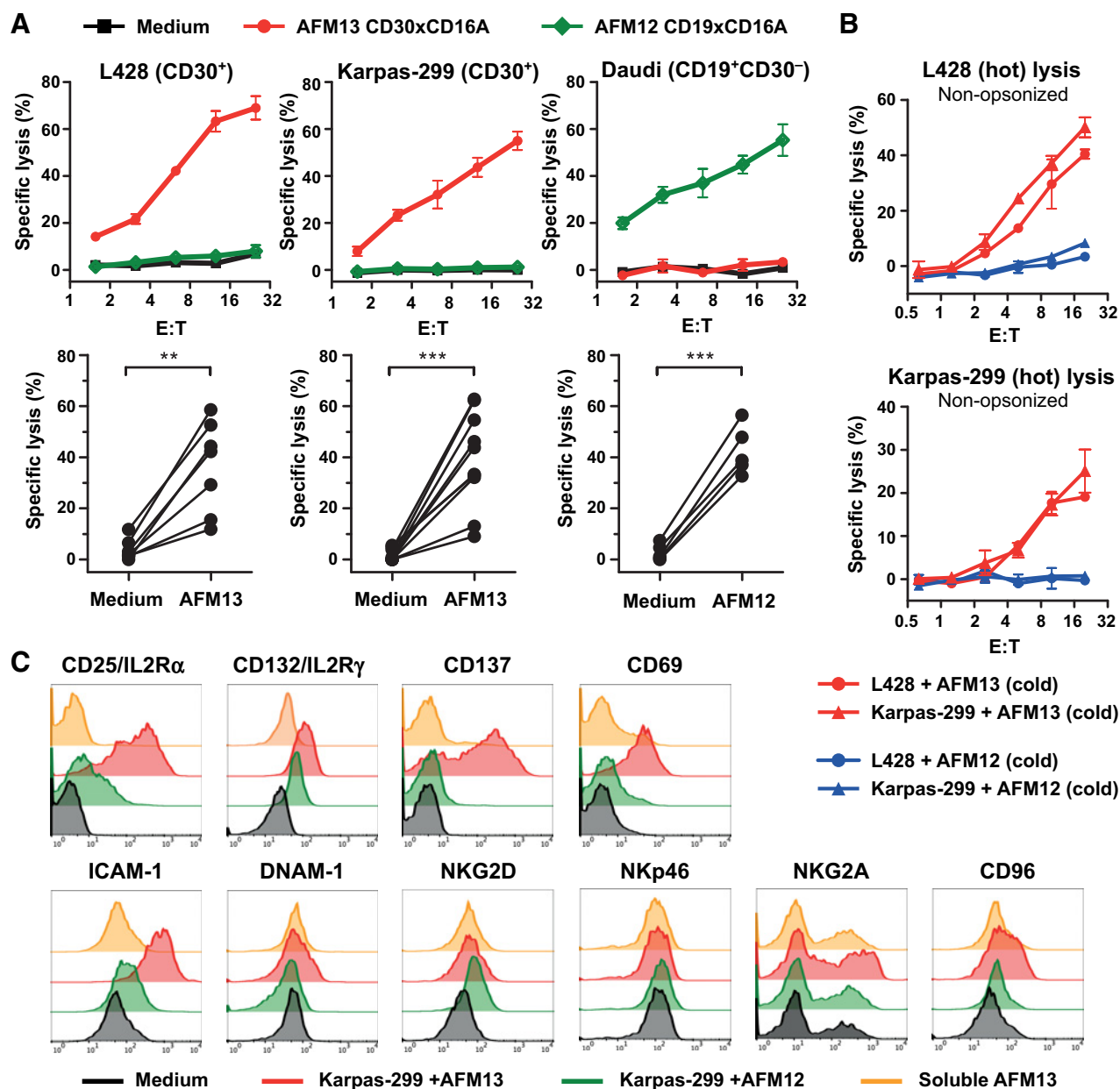
After coculture with AFM13-opsonized Karpas-299 or L428 cells, the expression of activating receptors, such as CD25 (IL2R $\alpha$ ), CD137, and CD69, was strongly induced on CD56<sup>dim</sup> CD16<sup>+</sup> NK cells, whereas AFM13 alone or tumor cells in the presence of AFM12 had little effect (Fig. 1C; Supplementary Fig. S1F). CD132 (common  $\gamma$ -chain ( $\gamma_c$ ) and part of the IL2R and IL15R), ICAM-1, and DNAM-1 were increased (Fig. 1C), whereas NKG2D and NKp46 remained unchanged. The expression of the inhibitory receptors NKG2A and CD96 was slightly increased.

### Preactivation of NK cells via CD16A amplifies cytokine-driven NK cell proliferation

Because CD25 (IL2R $\alpha$ ) and CD132 ( $\gamma_c$ ) were upregulated after CD16A engagement, we investigated whether this preactivation by AFM13 enhanced IL2-dependent NK cell proliferation. CFSE-labeled NK cells were incubated in a tumor-free system on plates with immobilized AFM13 (hereafter referred to as "coated") for 20 hours (Fig. 2A). Analogous to AFM13-opsonized tumor cells, coated AFM13 resulted in the upregulation of CD25 and CD132 on NK cells, whereas the expression of CD122 (IL2R $\beta$ ) remained unchanged (Fig. 2B). Thus, the phenotypic changes on NK cells in response to AFM13-opsonized tumor cells were a direct effect of CD16A engagement. After replating and reculture in IL2 for an additional 3- to 7-day period (Fig. 2A), cytokines alone were a weak stimulus for NK cell proliferation at the low cell densities applied here (Fig. 2C). In contrast, AFM13-preactivated NK cells displayed a robust dilution of CFSE after reculture in IL2, which became most evident on day 6 and increased further to day 8 (Fig. 2C). In contrast, NK cells, which were previously only exposed to medium, showed considerably less IL2-driven CFSE dilution. The percentage of NK cells that underwent at least four divisions was significantly higher in AFM13-preactivated NK cells, indicating enhanced IL2-mediated proliferation after AFM13 exposure (Fig. 2D).

Next, we assessed whether preactivation by AFM13 altered the sensitivity to low doses of IL2. AFM13-preactivated NK cells

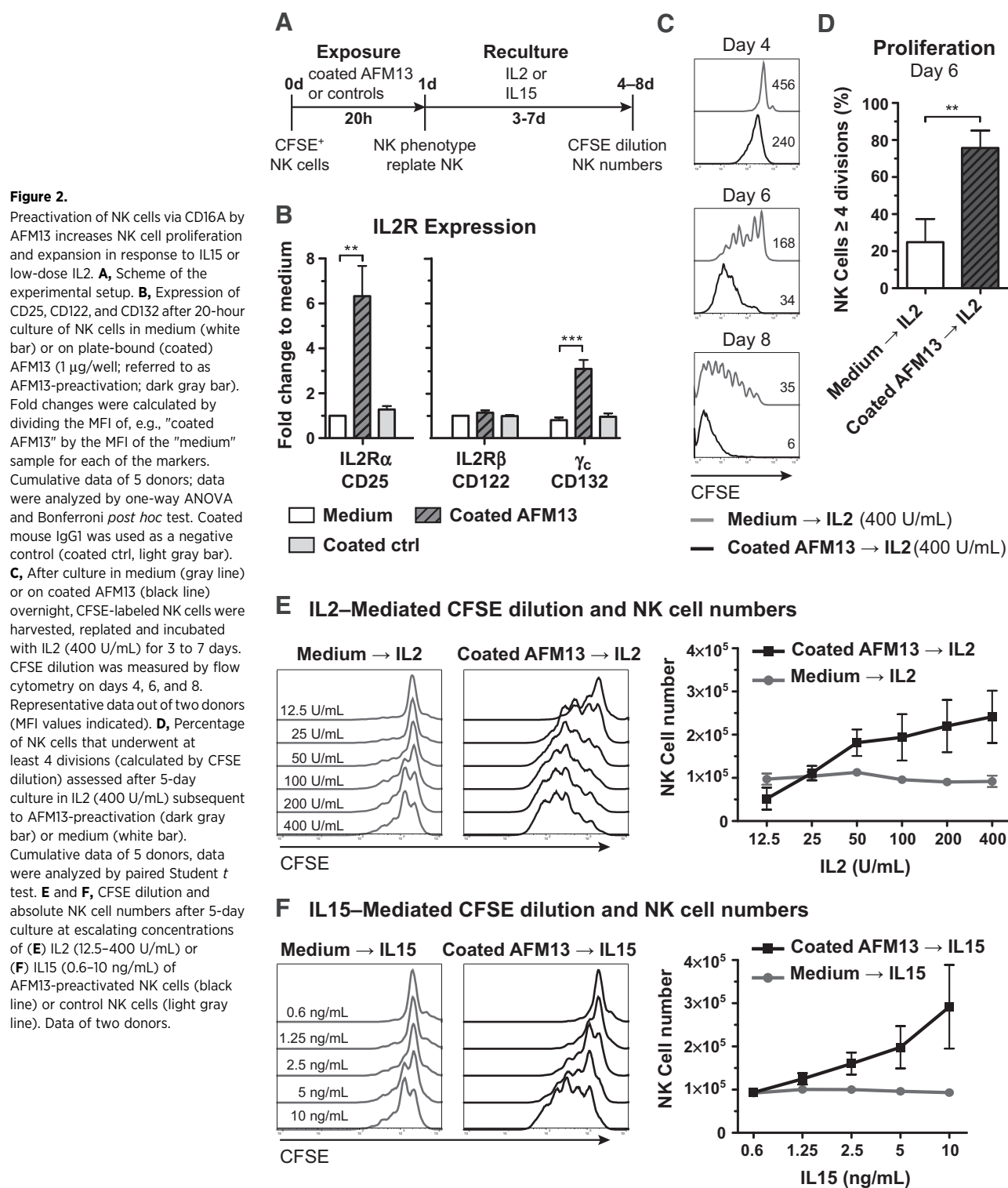
Pahl et al.

**Figure 1.**

AFM13-opsonized tumor cells induce NK cell cytotoxicity and an increase in activating receptors. **A**, Specific lysis of CD30<sup>+</sup>/CD19<sup>-</sup> Karpas-299 and L428 cells, and CD30<sup>-</sup>/CD19<sup>+</sup> Daudi cells by freshly isolated primary peripheral blood NK cells was measured in 4-hour <sup>51</sup>Cr release assays in the presence of CD30/CD16A-bispecific AFM13 (red circles), CD19/CD16A-bispecific AFM12 (green diamonds; all 10  $\mu$ g/mL), or no antibody (black squares) at escalating effector–target (E:T) ratios; representative data (top) and cumulative data of 7 to 10 donors (bottom; E:T 6:1) are depicted. Data were analyzed by paired Student *t* tests. **B**, Specific lysis of <sup>51</sup>Cr-labeled (“hot”) nonopsonized L428 or Karpas-299 cells after 4-hour culture with NK cells in the presence of unlabeled (“cold”) L428 (circles) or Karpas-299 (triangles) cells. Prior to this assay, “cold” L428 or Karpas-299 cells had been opsonized with AFM13 (red lines) or AFM12 (blue lines), followed by multiple washing steps to exclude transfer of unbound AFM13 or AFM12 to the assay. Afterward, “hot” and “cold” targets were mixed at a 1:1 ratio before the addition of NK cells and 4-hour incubation. Representative data out of four donors. **C**, Expression of indicated NK cell surface markers was analyzed by flow cytometry after 20-hour coculture of NK cells with Karpas-299 cells (E:T 1:1) plus AFM13 (0.1  $\mu$ g/mL) (red line). Controls: NK cells incubated with AFM13 without tumor cells (orange line); coculture of NK cells with Karpas-299 cells in the presence of AFM12 (green line); and NK cells only (gray line). Representative histograms out of three donors.

showed a profound dilution of CFSE even at a low IL2 concentration of 50 U/mL, whereas without AFM13-pretreatment, no proliferation was observed at low IL2 concentrations (Fig. 2E). CFSE dilution of AFM13-pretreated NK cells at low IL2 con-

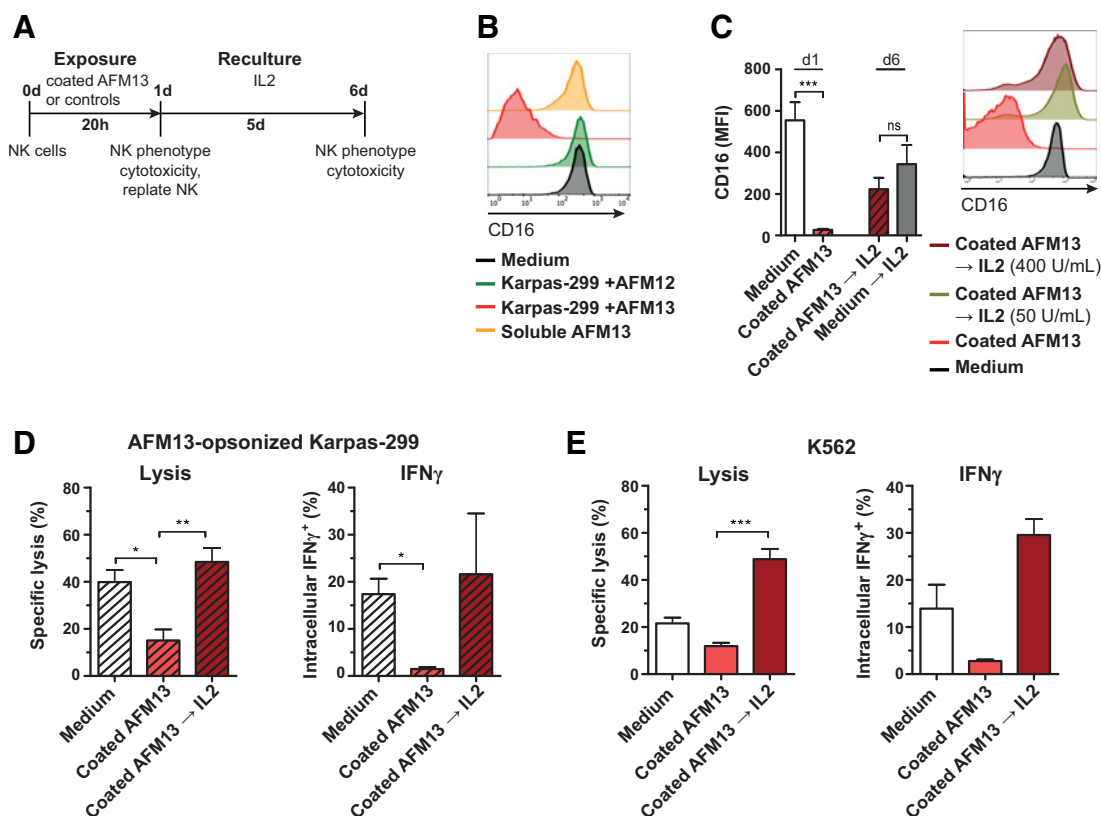
centrations was equally pronounced as the higher dose of 400 U/mL. Absolute NK cell numbers were substantially increased after reculture in low or high doses of IL2, resulting in an up to 4-fold expansion of NK cell numbers. Similarly, IL15-mediated NK cell



proliferation and absolute NK cell numbers were enhanced after AFM13 preactivation. However, this effect was observed mainly at the highest tested dose of IL15 (10 ng/mL) (Fig. 2F). Similar to the results obtained with AFM13, exposure of NK cells to the IgG1 anti-CD20 therapeutic antibody rituximab led to enhanced NK cell proliferation and increased NK cell numbers that associated

with increased expression of CD25 and CD132 (Supplementary Fig. S2). In summary, CD16A engagement of naïve NK cells by AFM13 or rituximab upregulated CD25 and CD132 expression and, thereby, improved responsiveness of NK cells to IL15 or low doses of IL2, leading to enhanced NK cell proliferation and expansion.

Pahl et al.

**Figure 3.**

NK cell function is recovered by IL2 after AFM13 exposure. **A**, Scheme of the experimental setup. **B**, CD16A expression was measured on NK cells after 20-hour coculture with Karpas-299 cells plus AFM13 (red line). Controls: NK cells incubated with AFM13 without tumor cells (orange line); coculture of NK cells with Karpas-299 cells in the presence of AFM12 (green line); and NK cells only (gray line). Representative histograms of 6 donors. **C**, CD16A expression on NK cells after exposure to medium (white bar) or coated AFM13 (light red bar) and on NK cells after reculture in IL2 (400 U/mL) for 5 days subsequent to exposure to coated AFM13 (dark red bar) or without pre-exposure (IL2 culture only, gray bars) as indicated. MFI data of nine donors; data were analyzed by one-way ANOVA and Bonferroni *post hoc* test. Representative histograms show CD16A expression after reculture in high-dose IL2 (400 U/mL; dark red line) and low-dose IL2 (50 U/mL; green line). **D**, Lysis of AFM13-opsonized Karpas-299 cells and intracellular IFN $\gamma$  expression toward AFM13-opsonized Karpas-299 cells by NK cells after exposure to medium (white bar) or coated AFM13 (light red bar) and by NK cells after reculture in IL2 (400 U/mL) for 5 days subsequent to exposure to coated AFM13 (dark red bar). Cumulative data (E:T 2.5:1) of 2 to 5 donors; data were analyzed by one-way ANOVA and Bonferroni *post hoc* test. **E**, Lysis of K562 cells and intracellular IFN $\gamma$  expression toward K562 cells by NK cells that had been cultured as described in **D**. Cumulative data (E:T 2.5:1) of 2 to 6 donors.

### NK cell functionality can be recovered after CD16A engagement

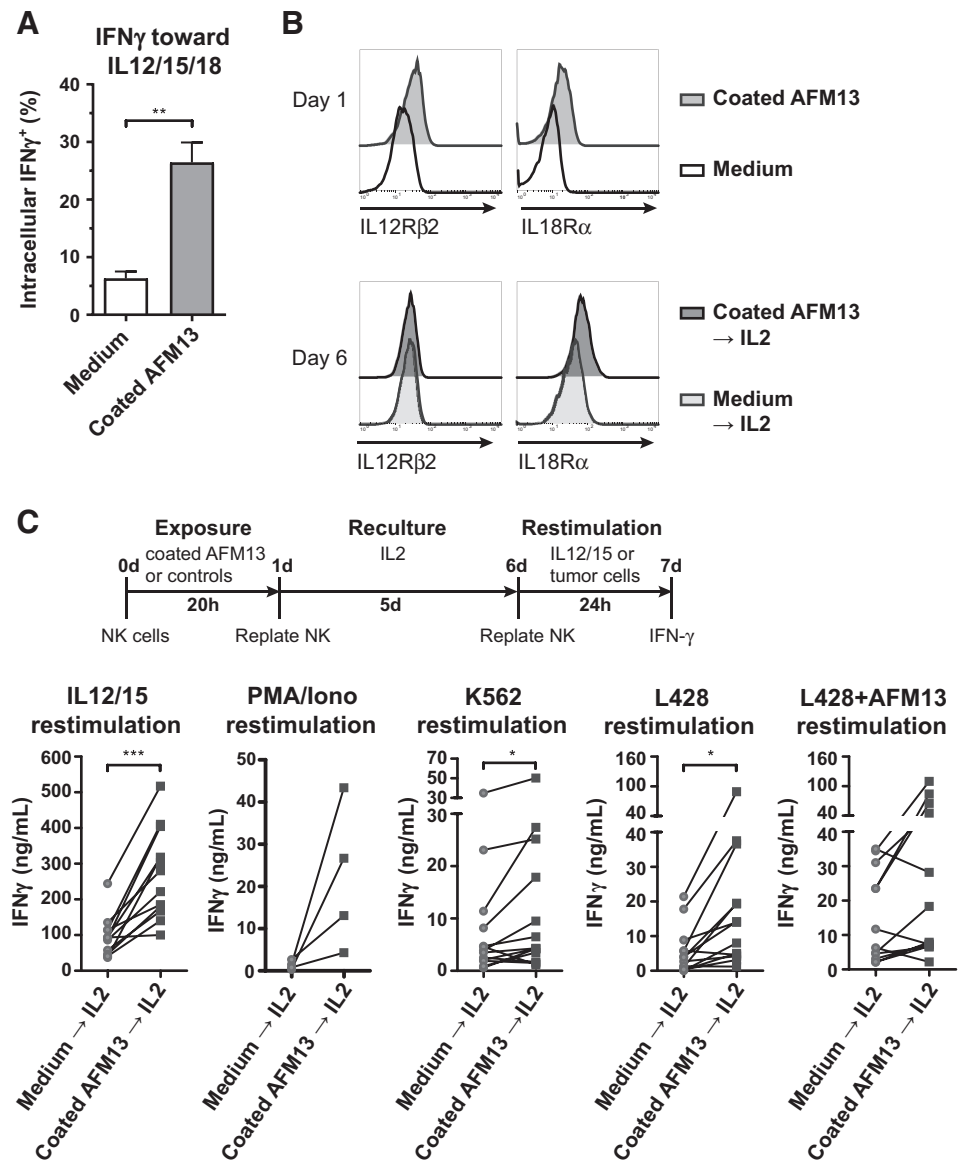
Concomitant with the induction of activation markers, we observed an almost complete loss of CD16A expression on NK cells after 20-hour culture with AFM13-opsonized target cells or coated AFM13 (Fig. 3A–C; Supplementary Fig. S3A). However, this effect was transient because CD16A expression could be restored when the NK cells were replated after AFM13 exposure and subsequently recultured in high or low doses of IL2 (Fig. 3C–D) or IL15 (Supplementary Fig. S3B) for 5 days. CD16A downregulation, as detected by flow cytometry, was not due to epitope masking because the detection of CD16A by anti-CD16 3G8 was not altered in the presence of AFM13 (Supplementary Fig. S3C). Instead, CD16A downregulation was, at least in part, attributable to metalloproteinase-mediated cleavage, as previously reported for CD16A downregulation by anti-CD16 3G8, rituximab, and bispecific killer engagers (BiKE; Supplementary Fig. S3D; refs. 24, 28, 29).

Next, we tested whether a reduction in CD16A expression compromised NK cell cytotoxicity. NK cells, which had previously

been cocultured with L428 cells in the presence of AFM13, showed impaired cytolytic activity toward fresh AFM13-opsonized target cells compared with previously non-cocultured NK cells (Supplementary Fig. S3E). NK cell cytotoxicity remained unaltered after coculture with L428 cells in the presence of AFM12. To dissect this impaired cytotoxic function in a tumor cell-free system, NK cell cytotoxicity was assessed after exposure to coated AFM13. After 20-hour culture on coated AFM13, NK cell cytotoxicity and intracellular IFN $\gamma$  expression in response to AFM13-opsonized Karpas-299 cells were significantly reduced (Fig. 3D). Similarly, lysis of AFM13-opsonized L428 as well as lysis induced by AFM12 toward Daudi cells was impaired (Supplementary Fig. S3F). Still, the residual AFM13-mediated lysis was higher than lysis of non-opsonized tumor cells. The reduced NK cell cytotoxicity and IFN $\gamma$  expression following AFM13 exposure could be fully restored after subsequent reculture in IL2 (Supplementary Fig. S3D and S3F) for 5 days. Similar to AFM13, coated rituximab impaired CD16A expression and NK cell cytotoxicity, and this effect could be restored after reculture in IL2 (Supplementary Fig. S3G–S3H).

**Figure 4.**

Preactivation of NK cells via CD16A by AFM13 enhances IFN $\gamma$  production upon restimulation with IL12/15 or lymphoma cells. **A**, NK cells were cultured in medium (white bar) or exposed to coated AFM13 (light gray bar). Intracellular IFN $\gamma$  expression of NK cells in response to IL12/15/18 was measured. Cumulative data of 4 donors; data were analyzed by paired Student *t* test. **B**, Expression of the high-affinity IL12R $\beta$ 2 and IL18R $\alpha$  on NK cells previously activated by coated AFM13 or cultured in medium as indicated (top pair of histograms); and by NK cells recultured in IL2 for 5 days subsequent to AFM13 exposure or in IL2 without preexposure as indicated (bottom pair of histograms). Representative data of 4 donors. **C**, Scheme of the experimental setup and IFN $\gamma$  production in response to 24-hour restimulation by IL12/15; PMA/ionomycin; or K562 cells, L428 cells, or AFM13-opsonized L428 cells by NK cells recultured in IL2 (100–400 U/mL) for 5 days subsequent to the exposure to coated AFM13 (dark gray squares), or cultured only in IL2 without pre-exposure (light gray circles). Cumulative data of 2 to 16 donors; data were analyzed by paired Student *t* tests.



Because the expression of perforin and granzyme B was unaltered after exposure to coated AFM13 or rituximab (Supplementary Fig. S3I), the impairment of NK cell cytotoxicity was not due to the absence of cytolytic mediators. However, expression of the CD16A and ITAM-signaling adaptor Fc $\epsilon$ R1 $\gamma$  (but not CD3 $\zeta$ ) was reduced after exposure to coated AFM13 or rituximab, which was restored after IL2 reculture (Supplementary Fig. S3J). We additionally tested NK cell responsiveness to PMA/ionomycin and to the prototypical target cell line K562. IFN $\gamma$  expression upon PMA/ionomycin stimulation (Supplementary Fig. S3K), as well as "natural" cytotoxicity and IFN $\gamma$  expression in response to K562 cells (Fig. 3E), was impaired but functional following IL2 reculture. Hence, although NK cell functionality was enhanced in direct response to AFM13-opsonized target cells, subsequently, CD16A engagement led to lower NK cell reactivity toward tumor cells, which could be rescued by IL2 or IL15 reculture.

#### CD16A preactivation promotes enhanced IFN $\gamma$ production upon restimulation

In a next step, we investigated whether preactivation of NK cells via CD16A influenced IFN $\gamma$  production in their subsequent response to cytokines or tumor cells. IFN $\gamma$  expression in response to IL12/15/18 was increased directly after AFM13 exposure (day 1; Fig. 4A). This coincided with higher expression of the high-affinity IL12R $\beta$ 2 and IL18R $\alpha$  (day 1; Fig. 4B). The higher expression of IL12R $\beta$ 2 was also observed after subsequent reculture in IL2 (day 6). When IL2-recultured NK cells had initially been preactivated by coated AFM13 or coated rituximab, IFN $\gamma$  production was significantly enhanced in response to restimulation with IL12/15 (Fig. 4C; Supplementary Fig. S4A). After IL2 reculture, IFN $\gamma$  production was enhanced after restimulation with PMA/ionomycin and in response to K562, L428, or AFM13-opsonized L428 cells (Fig. 4C). Thus, after IL2 reculture, NK cells preactivated

through CD16A displayed enhanced IFN $\gamma$  production upon restimulation with IL12/15 or different tumor cells.

#### CD16A preactivation enhances "natural" cytotoxicity to CD30<sup>+</sup> and CD30<sup>-</sup> tumor cells

Thus far, we have demonstrated that the reduced NK cell cytotoxicity following AFM13 exposure could be rescued through a 5-day reculture in IL2 (Fig. 3D; Supplementary Fig. S3E). In a next step, we explored whether preactivation of NK cells via CD16A could modulate the "natural" cytolytic activity of IL2-cultured NK cells when restimulated with non-opsonized tumor cells, expressing ligands to the NK cell-activating receptors NKG2D, DNAM-1, and NKp30. The "natural" lysis of CD30<sup>+</sup> Karpas-299 and HDLM-2 cells was enhanced, when IL2-cultured NK cells had initially been exposed to AFM13 (Fig. 5A). These lymphoma cells were highly resistant to lysis by IL2-cultured NK cells not preactivated by AFM13 and fully resistant to naïve NK cells. Even the lysis of the CD30<sup>-</sup> and CD30<sup>low</sup> lymphoma cell lines Daudi and L1236, respectively, and to some extent to K562 cells, was amplified by the initial AFM13 exposure (Fig. 5A; Supplementary Fig. S4B). Preactivation by AFM13 and reculture in IL2 enhanced not only the "natural" cytotoxicity but also AFM13- and AFM12-mediated target cell lysis involving CD16A engagement (Supplementary Fig. S4B). This enhanced cytotoxicity of NK cells after IL2 reculture could not only be induced by preactivation with coated CD16A-recruiting bispecific antibodies, such as AFM13, but also by classical IgG antibodies such as rituximab (Supplementary Fig. S4C). The enhanced cytolytic activity was associated with increased degranulation toward tumor cells. In parallel, perforin and granzyme B levels remained unchanged (Supplementary Fig. S4D). After IL2 reculture, NKG2D surface expression was increased on NK cells preactivated by AFM13 or rituximab, whereas the expression of NKp30 or DNAM-1 was not altered (Fig. 5B; Supplementary Fig. S4E). Therefore, to determine which NK cell receptors and pathways contributed to the enhanced cytolytic activity due to preactivation via CD16A, lysis of murine Ba/F3 cells stably transfected with the NKG2D ligand MICA, the DNAM-1 ligand CD155, or the NKp30 ligand B7H6 was examined. Lysis of Ba/F3-MICA was enhanced by AFM13-primed NK cells, whereas lysis of Ba/F3-CD155 or Ba/F3-B7H6 cells was not significantly altered (Fig. 5C). The lysis of Ba/F3-MICA cells by AFM13-primed NK cells was abrogated in the presence of neutralizing antibodies against NKG2D (Supplementary Fig. S4F). In summary, antibody-experienced NK cells (e.g., by AFM13) recultured in IL2 or IL15 exhibited amplified cytotoxicity toward CD30<sup>+</sup> and CD30<sup>-</sup> lymphoma cells, due to improved killing through the activating NK cell receptor NKG2D.

## Discussion

CD16A is the only activating receptor that can trigger the cytotoxic activity of naïve human NK cells, even in the absence of costimulatory signals (30, 31). In our study, we demonstrate that CD16A activation by tetravalent, bispecific AFM13 improved the functionality of NK cells beyond their classical antibody-dependent cytotoxicity and IFN $\gamma$  production elicited in response to CD30<sup>+</sup> lymphoma cells, which are otherwise poorly killed by NK cells. We show that CD16A engagement increased the sensitivity of NK cells to IL15 or low-dose IL2. This led to an amplification of IL15 and IL2-dependent NK cell

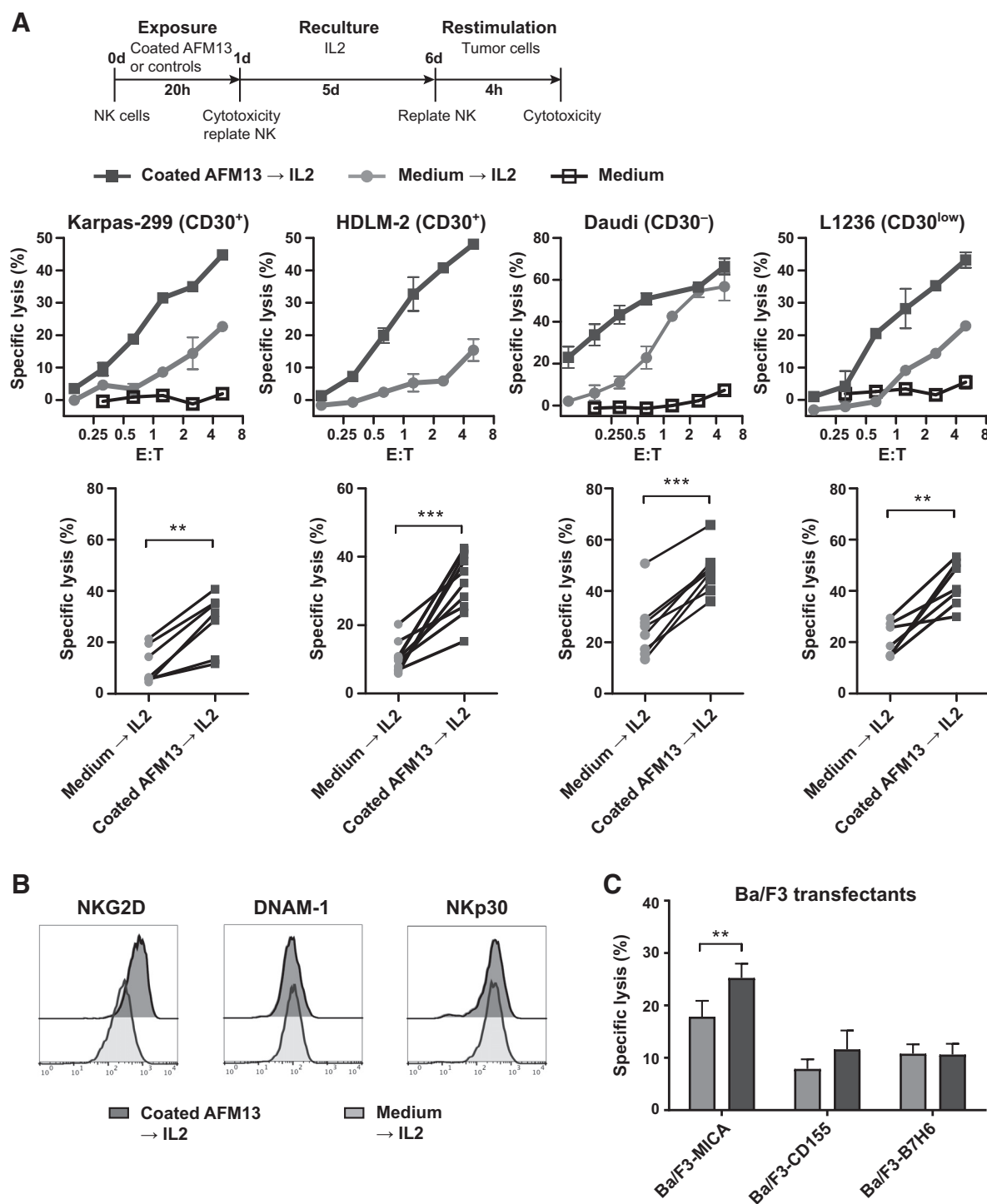
proliferation, resulting in increased numbers of functional NK cells. When preactivated via CD16A, CD16A-experienced NK cells displayed enhanced IFN $\gamma$  production and exerted enhanced cytotoxicity toward CD30<sup>+</sup> and CD30<sup>-</sup> lymphoma cells after IL2 reculture, which appeared to mainly involve the NK cell NKG2D pathway. The classic therapeutic antibody rituximab largely recapitulated the results of the tetravalent bispecific antibody AFM13, supporting these observations of CD16A-mediated priming of NK cells.

Our phenotypic analysis revealed that CD16A engagement by AFM13 leads to a defined signature of upregulated activating and inhibitory receptors on CD56<sup>dim</sup> CD16<sup>+</sup> NK cells. As expected, CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells remained largely unaffected. In our study with healthy donors, only low numbers of CD56<sup>dim</sup> CD16<sup>dim/-</sup> NK cells were detected, which has been previously described in leukemia patients (32). Increased frequencies of these subsets in cancer patients might be relevant for patient stratification before or during antibody therapy to predict optimal clinical responses. The signature of upregulated receptors on CD56<sup>dim</sup> CD16<sup>+</sup> NK cells may be further developed as biomarkers to monitor NK cell responses during AFM13 therapy and could be targeted in combinatorial treatments. For instance, cotargeting of activating receptors like CD137 with agonistic antibodies, as previously described (33), or inhibitory receptors, such as CD96, with antagonistic antibodies (34) may potentiate NK cell antitumor function after AFM13 exposure.

The improved sensitivity of CD16A-primed NK cells to IL15 and low-dose IL2 coincided with the induction of CD25 (IL2R $\alpha$ ), which, together with CD122 (IL2R $\beta$ ) and CD132 ( $\gamma_c$ ), assembles the trimeric high-affinity IL2 receptor, and the upregulation of CD132 together with CD122 assembles the dimeric intermediate-affinity IL2 and IL15 receptors (35). In our study, CD25 induction by AFM13-opsonized target cells was much stronger than previously shown upon CD16A cross-linking using anti-CD16 3G8 with a secondary antibody (36). Similarly, NK cell activation by IL12/15/18 has been shown to induce CD25 expression, boosting IL2-dependent proliferation *in vitro* and *in vivo* in tumor-bearing mice (9, 37). Hence, the induction of CD25 following AFM13 exposure may enable NK cells to compete for low amounts of IL2 with regulatory T cells, which strictly limit the availability of IL2 due to their constitutive CD25 expression, restraining NK cell expansion after adoptive cell transfer (38–40).

Following CD16A engagement, we observed a transient impaired function in CD16A-dependent as well as "natural" NK cell cytotoxicity and IFN $\gamma$  expression toward tumor cells. The impairment of CD16A-dependent activity may be explained by the nearly complete loss of CD16A expression upon CD16A engagement, which, at least in part, involved matrix metalloproteinase-mediated cleavage, consistent with previous studies, or receptor internalization, as additionally described (24, 28, 41–43). The transient lower responsiveness in "natural" NK cell antitumor reactivity toward K562 cells suggested a desensitization of other NK cell-activating receptors, such as NKp30 and NKG2D, that have previously been shown to be involved in K562 lysis (44, 45). The reduction in IFN $\gamma$  expression in response to PMA/ionomycin was indicative of a dysfunctional state that may also affect PKC activation and/or Ca<sup>2+</sup> mobilization, which are directly activated by PMA/ionomycin (46). Mobilization of Ca<sup>2+</sup> is critically involved in the signaling of CD16A and other activating receptors (31, 47). PKC activation can mediate IFN $\gamma$



**Figure 5.**

Preactivation of NK cells via CD16A by AFM13 enhances "natural" NK cell cytotoxicity upon restimulation with lymphoma cells. **A**, Lysis of nonopsonized CD30<sup>+</sup> Karpas-299 and HDLM-2 cells, CD30<sup>-</sup> Daudi and CD30<sup>low</sup> L1236 cells by naïve NK cells (open squares), by NK cells recultured in IL2 for 5 days subsequent to the exposure to coated AFM13 (dark gray squares), or by NK cells cultured only in IL2 without preexposure (light gray circles). Representative data (top) and cumulative data (Karpas-299, HDLM-2, and L1236 E:T 2.5:1; Daudi E:T 0.6:1; bottom) of 7 to 10 donors; data were analyzed by paired Student *t* tests. **B**, After exposure to coated AFM13 and IL2 reculture (dark gray line) or after IL2 reculture only (light gray line), surface expression of NKG2D, DNAM-1, and NKp30 was measured. Representative data of 6 donors. **C**, Lysis of murine Ba/F3 cells transfected with MICA, CD155, and B7H6 by NK cells recultured in IL2 subsequent to AFM13 exposure (dark gray bar) or by NK cells cultured in IL2 only (light gray bar). Lysis of Ba/F3 cells transfected with an empty vector was subtracted. Cumulative data (E:T 2.5:1) of 7 donors; data were analyzed by two-way ANOVA and Bonferroni *post hoc* test.

production and is important for K562 lysis but dispensable for ADCC, which, in turn, requires PI3K activation (48, 49). The observed impairment in our study after 20-hour exposure to AFM13 or rituximab may exceed the inhibitory effect of short-term (1.5-hour) CD16A engagement, which was reported to not affect IFN $\gamma$  production or responsiveness to PMA/ionomycin, while resulting in defective degranulation due to SHP-1 recruitment, inhibition of PLC $\gamma$ 2/Vav-1/SLP-76 phosphorylation, and both Fc $\epsilon$ R1 $\gamma$  and CD3 $\zeta$  degradation (42, 43, 50).

We found that the IFN $\gamma$  response of NK cells to IL12/15/18 after CD16A engagement was conserved or even further enhanced, indicating that the impaired IFN $\gamma$  expression after AFM13 exposure was selective for the response to tumor cells. This may be explained by the upregulation of the high-affinity IL12 and IL18 receptors, which potentially induce IFN $\gamma$ . Similarly, IFN $\gamma$  production in response to IL12 has recently been shown to be functional and enhanced after Fc-mediated antibody engagement of CD16A (43). It has been reported that CD16A and IL12 receptor activation can synergistically promote IFN $\gamma$  production (51). Because the signaling cascades for IFN $\gamma$  induction downstream of CD16A and IL12/18 receptors differ, it is tempting to speculate that the impaired IFN $\gamma$  production, as observed in our study, is selective for CD16A-ITAM-Syk-PI3K-ERK-AP-1-induced signaling, whereas IL12/18R-STAT4/AP-1-induced signaling remains competent (51–53).

The lower NK cell reactivity following AFM13 or rituximab exposure was reverted after reculture in IL2 or IL15 for 5 days. After IL2 reculture, these CD16A-experienced NK cells displayed, in particular, enhanced NKG2D-dependent cytotoxic activity, as well as enhanced IFN $\gamma$  production, when restimulated with otherwise weakly susceptible lymphoma cells or cytokines. Previously, IL12/15/18-preactivated, memory-like NK cells have been described to exert enhanced IFN $\gamma$  production and antitumor responses after reculture in IL2 or IL15 (8–11). It is possible that the novel functionality of "CD16A-induced memory-like NK cells" might have similarities to the enhanced antitumor potential of IL12/15/18-induced memory-like NK cells.

Taken together, our data imply that CD16A-mediated NK cell activation by AFM13 results in a distinctive phenotype that may

provide parameters to monitor AFM13-mediated NK cell activation in cancer patients. Our data support a strategy for rational treatment combinations of AFM13 with IL2 or IL15 that may boost NK cell functionality upon repeated tumor encounters and expand the quantity of tumor-reactive NK cells in lymphoma patients.

### Disclosure of Potential Conflicts of Interest

J.H.W. Pahl and A. Cerwenka report receiving a commercial research grant from Affimed. A. Cerwenka is a consultant/advisory board for SAB Dragonfly Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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# Cancer Immunology Research

## CD16A Activation of NK Cells Promotes NK Cell Proliferation and Memory-Like Cytotoxicity against Cancer Cells

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