Cytotoxicity of CD56-positive lymphocytes against autologous B-cell precursor acute lymphoblastic leukemia cells

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Abstract

Precursor B-lineage acute lymphoblastic leukemia (pre-B ALL) affects hematopoietic development and therefore is associated with immune deficiencies that can be further exacerbated by chemotherapy. It is unclear if and when monoclonal antibodies (mAbs) that stimulate antibody-mediated cellular cytotoxicity (ADCC) can be used for treatment because this depends on the presence of functional effector cells. Here, we used flow cytometry to determine that patient samples at diagnosis, post-induction and relapse contain detectable numbers of CD56+ cells. We were able to selectively expand CD56+ immune effector cells from bone marrow and peripheral blood samples at diagnosis and at various stages of treatment by co-culture with artificial antigen-presenting K562 clone 9.mbIL-21 cells. Amplified CD56+CD3- cells had spontaneous and anti-BAFF-R mAb-stimulated ADCC activity against autologous ALL cells, which could be further enhanced by IL15. Importantly, matched CD56+ effector cells also killed autologous ALL cells grown out from leukemia samples of the same patient, through both spontaneous as well as antibody-dependent cellular cytotoxicity. Since autologous cell therapy will not be complicated by graft-versus-host disease, our results show that expanded CD56+ cells could be applied for treatment of pre-B-ALL without transplantation, or for purging of bone marrow in the setting of autologous bone marrow transplants.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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Keywords

ADCC; NK; NKT; monoclonal antibodies; immune effector; pre-B ALL; BAFF-R; IL2; IL15

INTRODUCTION

There is increasing interest in the development and application of cell-based therapy in cancer. Such approaches are dependent on the presence of functional effector cells. These include NK cells that can spontaneously recognize and kill abnormal cells but can also be targeted to kill cells that are opsonized, in antibody-mediated cellular cytotoxicity (ADCC) reactions. Precursor B-lineage acute lymphoblastic leukemia (pre-B ALL) is a cancer that involves malignant transformation of blood-forming cells and because of this, patients typically present with a marked perturbation of normal hematopoiesis. As noted by Haining et al [1] and reviewed in [2] "ADCC requires functional immune effector mechanisms, which may be deficient in the setting of childhood leukemia." Deficiencies could be numerical, but also functional. Abnormalities of NK cell receptors and of their putative ligands are found in leukemia patients and this may result in defective NK cell function and loss of NK cell-mediated destruction of the leukemic cells [3]. It was also reported that in a haploidentical hematopoietic stem cell transplantation (HSCT) setting, NK cells are very effective in eliminating residual acute myeloid, but not acute lymphoid, leukemic cells. [4, 5]

It has become possible to expand NK cells from normal peripheral blood (PB) using co-culture with mitotically inactivated artificial antigen-presenting cells. [6, 7] Fujisaki et al [7] was able to expand NK cell numbers from normal peripheral blood (PB) samples and from 8 children with remission ALL undergoing chemotherapy upon co-culture with irradiated K562-mb15-41BBL cells. Denman et al [8] developed K562 cells expressing different co-stimulatory molecules including membrane-bound IL-21 to promote NK cell proliferation. Preclinical studies showed that such NK cells could be expanded from PB of patients with neuroblastoma. Expanded NK cells had significant cell killing effects against neuroblastoma alone or in combination with an ADCC-promoting monoclonal antibody (mAb) against GD2. [9]

A number of FDA-approved antibodies are in clinical use for mature B-lineage hematological malignancies, including rituximab (αCD20) for treatment of non-Hodgkin's lymphoma and Hodgkin's lymphoma, and alemtuzumab (αCD52) for treatment of chronic lymphocytic leukemia (CLL), and also increasingly being viewed for therapy of ALL as demonstrated for CD19 [10, 11] and reviewed in 2012. [12] We previously reported that the presence of the BAFF-R as tumor-specific antigen can be used as a therapeutic target in pre-B ALL and showed that an anti-BAFF-R antibody optimized for immunotherapy of mature B-lineage cancers in humans had significant ADCC- and antibody-dependent cellular phagocytosis-stimulating activity in NK and macrophage cell-mediated killing of ALL cells. [13] Thus, although cell-mediated antibody therapy for pre-B ALL may emerge as a promising alternative treatment, the possible lack of functional immune effector activity remains a troubling confounding factor. The current study was initiated to address this concern empirically, using direct investigation on human pre-B ALL clinical samples.

Interestingly, we show that CD56+ cells can be expanded *ex vivo* from pediatric ALL samples at diagnosis, remission and relapse, and have significant antibody-dependent and non-antibody dependent cytotoxicity in an autologous setting.

MATERIALS AND METHODS

Expression analysis and flow cytometry

The α-BAFF-R antibody used for ADCC assays was provided by Novartis and has been described. [13] To determine the percentage of NK cells in samples, cells were washed, treated with human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 minutes and then stained with CD56-PE and CD3-PerCP antibodies (Biolegend, San Diego, USA). For BAFF-receptor expression, cells were stained with CD19-FITC, BAFF-R-PE and CD10-APC (BD Biosciences, San Jose, CA). Cells were examined by flow cytometry on an Accuri flow cytometer (Ann Arbor, MI, USA). We analyzed effector cell numbers on a FACS Canto II (BD Biosciences) using CD45-PerCP, CD19-APC, CD10-FITC, BAFF-R-PE, CD56-FITC, CD16-PE, CD3-APC (BD Biosciences).

For expression of CD3, CD56, NKG2D, NKp46 and CD16, non-expanded PBMCs and corresponding *ex vivo* expanded NK cells were washed, treated with human Fc blocking reagent for 10 minutes and then stained with CD3-PerCP, CD56-FITC, NKG2D-APC, NKp46-PE-Cy7, (Biolegend) and CD16-BV510 (BD Bioscience, San Jose, CA). Cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences).

For analysis of CD107a and IFNγ, e*x vivo* expanded NK cells (1×10^6) from ALL patient samples were stimulated with nothing, or with US7 cells (2×10^5) in the presence or absence of 10 μg/ml human control IgG Ab or αBAFF-R mAb as indicated for 1 hr, with inclusion of CD107a-PE antibodies (BD Bioscience, San Jose, CA). Non-expanded PBMCs were stimulated with PMA (2.5 μg/ml) and ionomycin (1.0 μg/ml) as a positive control. Cells were then incubated for an additional 3 h at 37°C with brefeldin A (eBioscience, San Diego, CA) and monensin (Golgi-Stop, BD Biosciences). After washing and addition of Fc block (BD Biosciences), cells were stained with CD56-FITC, CD16 BV510 and CD3-PerCP for 30 min. After washing and fixing, cells were permeabilized with a BD Cytofix/Cytoperm[TM] kit, followed by intracellular staining for γ-interferon (γ-IFN)-APC (BD Bioscience, San Jose, CA) for an additional 30 min. Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences).

Cell culture

US7 cells have been previously described. [14] ALL patient samples were obtained on Children's Hospital Los Angeles IRB-approved protocols. Ficoll-Paque separated peripheral blood mononuclear cells (PBMCs) or bone marrow mononuclear cells (BMMCs) were tested freshly or stored in liquid nitrogen. OP9 mouse stromal cells (CRL-2749) were from the American Type Culture Collection (Manassas, VA). PBMCs or BMMCs from ALL patients were directly cultured with irradiated OP9 cells. Cell growth became evident after a variable lag period of up to 2 months. Co-culture of human ALL cells with OP9 cells was in MEM-α medium supplemented with 20% FBS, 1% L-glutamine and 1% penicillin/

streptomycin (Life Technologies, Grand Island, NY). We used lots of FBS that we had tested for ability to sustain optimal growth of previously described patient-derived pre-B ALL cells [14] for co-culture with primary human ALL cells. NK cells were expanded as previously described. [8, 9] Briefly, we started with 2×10^6 to 2×10^7 mononuclear, Ficoll-purified cells (PBMCs or BMMCs) for co-culture with irradiated K562 clone 9.mbIL-21 cells, as artificial antigen-presenting cells (aAPC). Co-cultures were grown in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin (Life Technologies, Grand Island, NY) and 50 ng/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ). Freshly irradiated K562 clone 9. mbIL-21 cells were added every week. Recombinant human IL-15 was purchased from PeproTech (Rocky Hill, NJ). Concentrations of secreted IFNγ from NK cells were determined with an enzyme-linked immunoassay (ELISA) kit (Biolegend, San Diego, CA).

ADCC assays

ALL cells (1×10^6/ml) were labeled with 5 μM calcein-AM (Lifescience Technologies, CA, USA) for 30 minutes at 37°C. ALL cells were washed with PBS, and added at 1×10^4 cells/ well to a 96-well plate. *Ex vivo* expanded NK cells (5×10^4/well) either from healthy donors or ALL patients were added with control human IgG Ab or α-BAFF-R mAb as indicated. After 1.5-3 hours of incubation, 50 μl of supernatant was transferred to a Black View Plate-96 well plate and arbitrary fluorescent units (AFU) were measured on a Tecan SPECTRAFLUOR (485 nm excitation/535 nm emission). The percentage of specific lysis from triplicate wells was determined using the following equation, in which "AFU mean spontaneous release" is calcein-AM release by target cells in the absence of antibody and NK cells and "AFU mean maximal release" is calcein-AM release by target cells upon lysis by detergent. % specific lysis = 100x (AFU mean experimental release – AFU mean spontaneous release)/(AFU mean maximal release – AFU mean spontaneous release)

Statistical analysis

Statistical analysis was performed with Prism software. Data are presented as mean ± SD of triplicate wells. For IFNγ ELISAs, values shown are averages of duplicate wells. Statistical significance of differences between groups was evaluated using one-way-ANOVA or two-way-ANOVA. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS

CD56+ cells can be expanded from residual effector cells in primary patient samples

To determine whether or not pre-B ALL patients retain NK cells that could mediate cell-based leukemia killing either alone or in the presence of antibodies, we performed flow cytometric analysis for NK cells on diagnosis, post-induction and relapse samples. Interestingly, CD56+CD3- NK cells were found in all samples (Supplementary Figure 1a), with the lowest percentage measured at 0.5%.

We isolated mononuclear cells from similar pre-B ALL samples to investigate if NK cells could be expanded from these by co-culture with mitotically inactivated K562 clone 9.mbIL-21 cells. At d0 new diagnosis and relapse samples contained significant numbers of

ALL blasts whereas two PB post-induction samples were blast-free. The lowest initial percentage of CD56+CD3- cells in the residual normal lymphocyte gate on d0 was 0.5% in ALL BM #6 (Table 1; original counts/μl of CD56+CD3- and CD3+CD+ before expansion Supplementary Figure 1b). However, although some of the samples exhibited no detectable CD56+ cell expansion for up to 14 days (Figure 1, #14) proliferating CD56+ cells eventually did start to emerge. Other samples such as BM #8/ PB #9 had more rapid expansion of CD56+ cells (Figure 1). We were able to expand CD56+ cells from BM and PB, including new diagnosis as well as relapse samples (Figure 1, Supplementary Figure 2, Table 1). We followed some of the samples for expansion of CD19+ cells to determine if such culture conditions would also support the proliferation of the original ALL blasts that had been present in the sample. However no proliferation of CD19+ cells was seen (Figure 5 and not shown).

CD56+ cells have allogeneic cytotoxic activity

We tested CD56+ cells expanded from two different ALL PB samples for cytolytic activity towards ALL cells, compared to NK cells expanded from normal PBMC from non-leukemia sources. Both ALL-derived CD56+ samples were able to lyse patient-derived US7 ALL cells in the absence of antibody (Figure 2b, d, 0 ng/ml antibody points). Moreover, as shown in Figure 2b, d, dose-response curves in the presence of an antibody that targets the BAFF-R showed that these cells had clear ADCC capability. The activity of such ALL-derived NK cells was comparable to that of normal PBMC-derived expanded NK cells (Figure 2e, f)

Comparison of CD56+CD3- and CD56+CD3+ populations

Depending on the initial sample, final CD56+ populations consisted of a varying percentage of CD3- and CD3+ cells, with CD56+CD3- populations predominating when PB was used as starting material. To characterize these, we prospectively analyzed CD56+CD3+ and CD56+CD3- populations from a relapse and three new diagnosis BM samples. As exemplified in Figure 3a (also see Figure 3b, Supplementary Figure 1), the percentage of CD56+CD3- cells in the initial samples was higher than that of CD56+CD3+ cells. Co-culture with K562 clone 9.mbIL-21 stimulated expansion of both over a 17-21 day period (Figure 3b). As illustrated for sample #25, in comparison to d0, the d17 CD56+CD3- NK population maintained high levels of expression of CD16, and increased expression of the activating NK cell receptors NKG2D and NKp46. CD56+CD3+ d17 cells also had increased NKG2D cell surface expression, but lacked NKp46, and had low levels of CD16 (Figure 3c; also Figure 3d). This expression pattern resembles that of CD56+CD3- / CD56+CD3+ cells initially present in normal PBMC from non-leukemia origin (Figure 3d) and grown out from them by co-culture with K562 clone 9.mbIL-21 (Supplementary Figure 3).

We also compared functional activity of ALL patient-derived CD56+CD3- and CD56+CD3+ populations. We used CD107a surface expression, which was reported, and has been subsequently widely used, as a validated readout for functional NK cell activity [15]. As illustrated in Figure 4a and c, patient-derived CD56+CD3- cells showed a marked increase in CD107a cell surface expression when used for ADCC assays with anti-BAFF-R mAb against allogeneic US7 pre-B ALL cells. CD56+CD3- cells in other samples also showed increased levels of activation in ADCC reactions, whereas CD56+CD3+ cells were

not stimulated by BAFF-R mAbs (Figure 4b, d), consistent with their lack of CD16 expression (Figure 3d). Intracellular IFNγ was also increased (Figure 4c). Analysis of CD107a and IFNγ-positive cells showed that around 50% of CD56+CD3- cells still retained CD16 after 4 hours but a significant percentage had lost expression (Supplementary Figure 4, #28), which may, in part, be caused by activation of the NK cells. [16] A similar effect was seen in non-amplified normal PBMC from non-leukemia origin after stimulation with PMA and ionomycin (Supplementary Figure 4, PBMC).

CD56+ cells have autologous cytotoxic activity

We also examined if CD56+ cells obtained from primary ALL patient samples have autologous activity. Different primary ALL cells do not reliably proliferate long-term in tissue culture with the exception of some samples from relapsed patients [17]. To obtain viable, actively growing ALL cells, we plated primary ALL cells directly on irradiated OP9 stromal cells using previously described culture conditions. [18, 19] Using this system, ALL cells of PB #12 as well as BM #5, #6, #10, and #14 grew out (Table 1). Flow cytometry showed that these cells were uniformly CD19+/CD10+ and also positive for expression of the BAFF-R (Figure 5a). We also expanded CD56+ cells from post-induction PB #5 and #10; from the same leukemic PB #12 and #14; and leukemic BM #6 to obtain matched autologous sets. The expanded CD56+ populations consisted of varying percentages of CD3- and CD3+ cells (Figure 5b).

Normal allogeneic (non-ALL) PBMC-expanded NK cells showed antibody-independent ALL cell killing activity against these ALL samples (Figure 6a-d, left panels 0 μg) and addition of the BAFF-R mAb stimulated significant specific ADCC activity (Figure 6a-e, left panels, anti-BAFF-R). Interestingly, the CD56+ cells expanded from the different ALL samples also showed non-antibody-dependent autologous cytotoxicity. Compared to normal PB-derived NK cells, all patient-derived CD56+ populations tested were stimulated to kill autologous ALL cells by the specific presence of BAFF-R antibodies (Figure 6, compare left and right panels).

Increasing cytotoxic effects of expanded NK cells

Cytotoxicity assays were terminated at a fixed time point. We therefore performed a time course to address whether or not such reactions reach a plateau. We found that lysis of target ALL cells continues for at least 5 hours (Figure 7a), and that both antibody-dependent and -independent reactions continue until complete lysis is reached after overnight incubation (not shown). We also investigated if the addition of IL2 or IL15 could further stimulate cytotoxicity of expanded NK cells. IL15 was reported to increase NK cell cytotoxicity. [20, 21] Figure 7b shows both cytokines had a varying extent of stimulatory effect on cytotoxicity of such NK cells, with ADCC most clearly enhanced by IL15. Cytotoxicity reactions continued for at least 3 hours (Supplementary Figure 5). The effect of longer-term exposure to IL15 was also examined. Compared to NK cells not incubated with IL15, those cultured overnight with added IL15 had significantly enhanced cytotoxicity (Figure 7c). We also determined the effect of cytokine addition on IFNγ secretion into the medium under these conditions. Both IL2 and IL15 enhanced IFNγ secretion by NK cells exposed to ALL cells although

these cytokines had no additive effect (Figure 7d). Long-term exposure to IL15 also enhanced IFNγ secretion (Figure 7e).

DISCUSSION

Although it is commonly accepted that patients with ALL are immune deficient, few studies have quantitated this in detail. Alanko et al [22] investigated recovery of NK cells after cessation of chemotherapy for leukemia. They found that NK cell numbers rapidly increased, but noted that NK cells from PB of two patients examined 5 months after therapy had low cytotoxic activity against the chronic myelogenous leukemia cell line K562. Haining et al [1] measured NK cell numbers in 19 patients at diagnosis and followed some of these over time, including three samples assayed up to 24 months after diagnosis. They concluded that pediatric patients have long-term immune deficiency with NK cell numbers below that of normal children. Based on flow cytometry, we detected the presence of NK cells in presentation, post-induction and relapse samples, raising the possibility that antibodies could be used for therapy. However, it is currently not known if there is a threshold for minimal effector cell numbers needed for ADCC, and thus lack of sufficient effector cell numbers remains a potential problem that will only be addressed if mAb therapy is tested on ALL patients in clinical trials.

Increasing NK cell numbers through infusion of *ex vivo* expanded NK cells has now become a realistic possibility. Surprisingly, although diagnosis bone marrow samples consisted mainly of ALL blasts, we were able to selectively expand CD56+ cells from these although the kinetics of expansion was less rapid than that of PB. Since some PB diagnosis samples containing blasts (#9 and #11) expanded as rapidly as PB #5, which was post-remission induction (Supplementary Figure 6), kinetics of expansion do not appear to correlate with the presence of ALL cells. Wang et al [23] reported that they expanded cytokine-induced killer (CIK) cells from AML patients but that especially in samples with high leukemia burden, the AML cells had to be removed before such cells could grow out. In our studies, starting material was not pre-purified by removal of the ALL cells but the presence of very large numbers of ALL cells did not appear to preclude outgrowth of CD56+ cells. Importantly, we have no evidence that these culture conditions also promoted expansion of the ALL cell population in the sample.

The overall pattern of expansion that we obtained over time by co-culture with K562 clone 9.mbIL-21, as measured by immunophenotyping, differed from that which we found in normal PBMC-derived samples. In the latter, between 85-95% of the end product was generally CD56+CD3- NK cells upon 14 days in culture, with 0.6 - 8.7% CD3+CD56- T cells and between 0 - 7.4% CD56+CD3+ cells. In contrast, a more heterogenous profile was obtained with ALL samples. Since these included samples from BM and PB (#1 and #3) there is no clear correlation with the origin of the sample, and the difference more likely relates to the shorter duration of co-culture, the different degree of activation or the differences in the composition of the starting material. Cells outgrowing from PB with a CD56+CD3+ phenotype have been called NKT cells or cytokine-activated killer (CIK) cells in different studies. Pievani et al [24] described CIK cells as "ex vivo activated lymphocytes that can be obtained in large numbers within 3 weeks of culture from human PB or BM" by

sequential stimulation with IFNγ, anti-CD3 and high dose [1000 ng/ml] IL-2. The resulting population was described as heterogenous to include a large majority of CD56+/CD3+ cells with a minor fraction of typical T cells (CD56-CD3+) and NK cells (CD56+CD3-). AML-derived CIK cells were reported to have high cytotoxicity against leukemia cell lines. [23]

Because NKT /CIK cells exhibit non-MHC restricted cytotoxicity, they are also viewed as immunotherapeutic cells with a high potential to treat cancer. [25, 26] In our initial patient pre-B ALL samples, as in normal PB, CD56+CD3- cells outnumbered CD56+CD3+ cells, and although both NK and NKT cells received a stimulus for amplification by co-culture with K562 clone9.mbIL21, final numbers of CD56+CD3- dominated those of CD56+CD3+ cells after amplification. Direct comparisons of the CD56+CD3- and CD56+CD3+ cells expanded from patient-derived samples clearly showed that BAFF-R mAb-stimulated ADCC activity was entirely attributable to the CD56+CD3- population, consistent with the high percentage of these cells expressing CD16. Interestingly, when such ADCC reactions were followed over time, difference in cell lysis with or without antibodies disappeared and eventually all leukemia cells were lysed. Since ADCC reactions may slow down because of loss of CD16 expression on the NK cells, or shaving of the antigenic epitope from the target cells by monocytes [16, 27] it may be advantageous to have a mixture of cells that are also capable of direct cytotoxicity, without dependency on ADCC, in a population of immunotherapy effector cells.

CD56+CD3- cells also produced the highest levels of IFNγ although we also detected some IFNγ production in one patient-derived CD56+CD3+ expanded cell sample when exposed to ALL cells. When we tested autologous cytotoxicity in five different sets of patient-derived CD56+ populations, with varying ratios of CD3- and CD3+ cells, all showed non-antibody stimulated as well as specific antibody-stimulated lysis of the ALL cells. Thus the presence of CD56+CD3+ cells in these preparations, if used in an autologous setting, is unlikely to have negative, and may have positive effects on ALL cell killing.

Our exciting results could have a relatively immediate application if CD56+ lymphocytes can be expanded to sufficiently useful numbers. At this point it is not clear how many cells would be required for clinical effectiveness if used as adoptive cell therapy in patients. Different clinical trials (reviewed in [28]) reported using up 6×10^9 NK cells per patient with leukemia. In our study we started with only 2×10^6 to 2×10^7 cells, of which a small percentage was actually CD56+ and therefore the absolute final yield of CD56+ cells typically did not exceed 10^7 cells. However, 3-week expansion rates of 50-300-fold with ALL PB samples (#9, #11, Supplementary Figure 6) and 10-230-fold for ALL BM (Figure 3b) indicate that significant cell numbers can be obtained from such pre-B ALL samples. Outgrowth to significant numbers could be achieved with larger amounts of starting materials [29], and our results show that activity of the NK cells can be further enhanced by IL15. Other applications, which could require lower cell numbers, include elimination of residual ALL cells in autologous bone marrow transplants, alone, or combined with a mAb such as the one used here.

Attack on autologous cells by NK cells is normally prevented by expression of inhibitory signals (self) and lack of activating signals on healthy cells. [30, 31] Hombach et al [32] reported

that *ex vivo* activated lymphocytes cells are not MHC-restricted. However, CD56+ cells that have been expanded on K562 clone 9.mbIL-21 were still able to discern healthy cells, since in mouse models no systemic damage was seen. [9] In addition, Fujisaki et al [7] found no significant cytotoxicity against non-transformed cells in tissue culture and in mice transplanted with AML cells and treated with NK cell infusions. Thus, therapy using *ex vivo* expanded autologous CD56+ cells could also be envisioned, since this would eliminate the potential complications of graft-versus-host disease that may affect adoptive cell therapy with allogeneic NK cell preparations containing residual T cells. We conclude that although the numerical and functional defects of NK cells reported in earlier studies in ALL could complicate treatment of this type of cancer using monoclonal antibodies to promote ADCC, the ability to generate autologous CD56+ cells may inspire new ideas of how to use cell-dependent therapy for ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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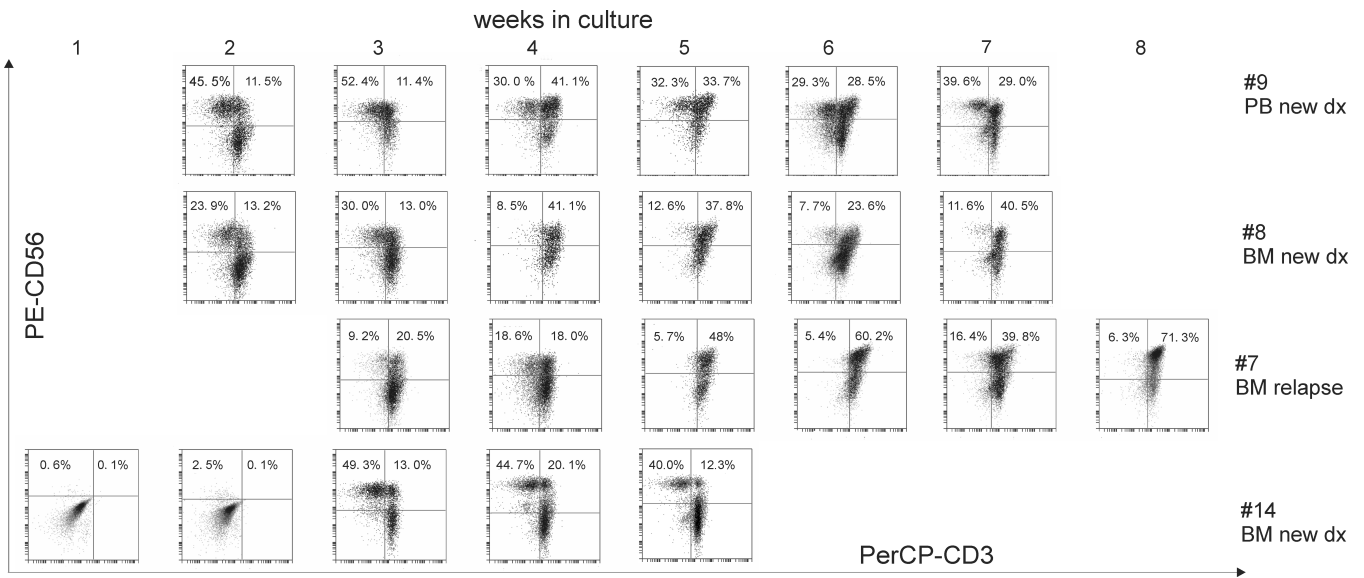
Figure 1.
Artificial antigen-presenting K562 clone 9.mbIL-21 cells stimulate selective outgrowth of CD56+ cells from pediatric pre-B ALL samples with high initial percentages of blasts. ALL samples as indicated containing 30, 80, 30 and 88% blasts on d0 (#9, #8, #7 and #14, respectively) were examined for the presence of CD56+ and CD3+ lymphocytes after co-culture with irradiated K562 clone 9.mbIL-21 cells for the number of weeks indicated above the panels. The percentage of CD56+, CD3- (UL) and CD56+, CD3+ (UR) cells is indicated in the panels.
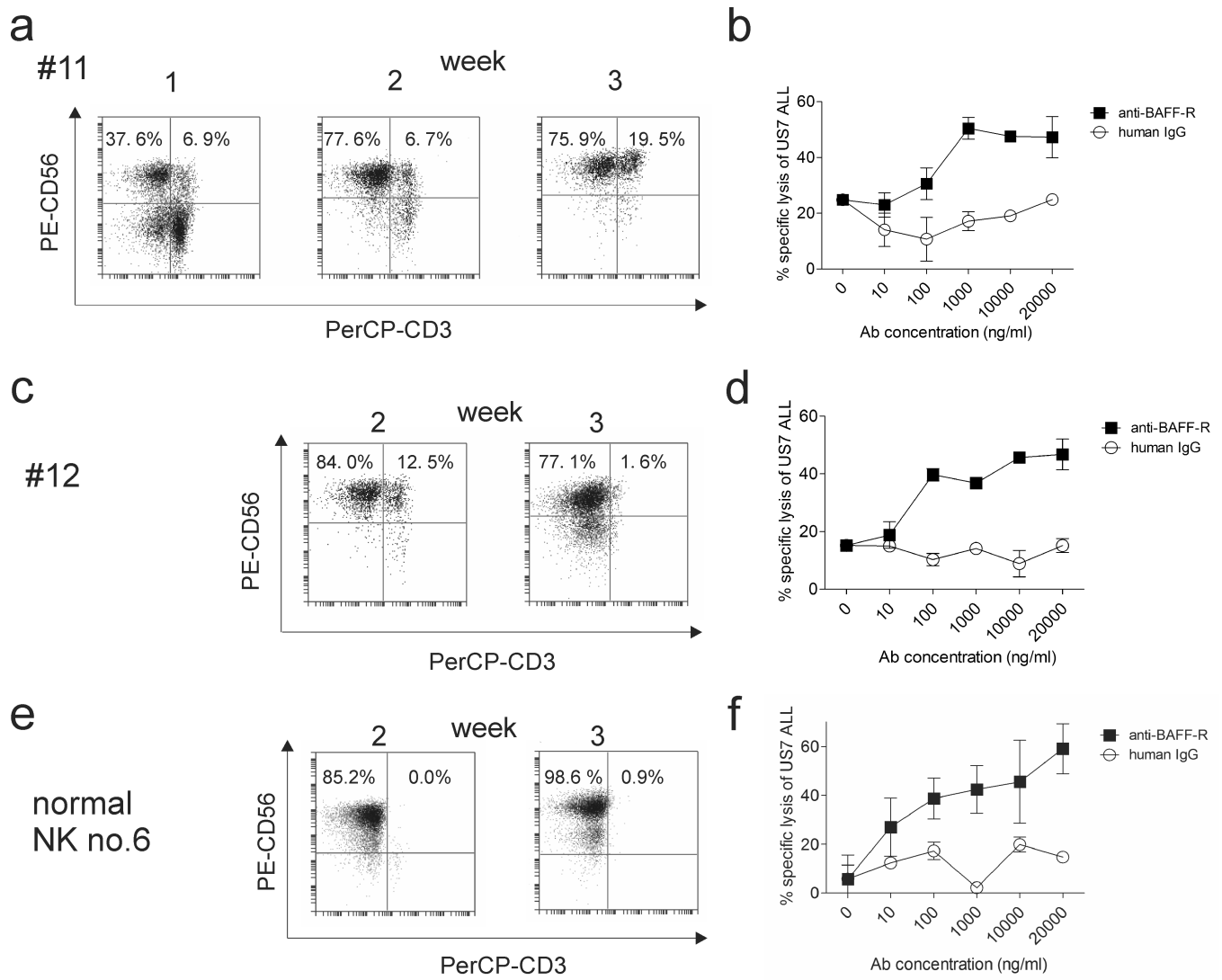
Figure 2.
Ex vivo expanded CD56+ effector cells from ALL PB are cytotoxic against allogeneic pre-B ALL cells. NK cells expanded *ex vivo* from the indicated primary patients and from a control normal PBMC sample by 3-week co-culture with K562 clone 9.mbIL-21 as evidenced by outgrowth of CD56+ cells (**a, c, e**) were tested for ADCC activity in a 1-hr reaction against human patient-derived US7 pre-B ALL cells in the presence of control IgG or anti-BAFF-R antibody (**b, d, f**).
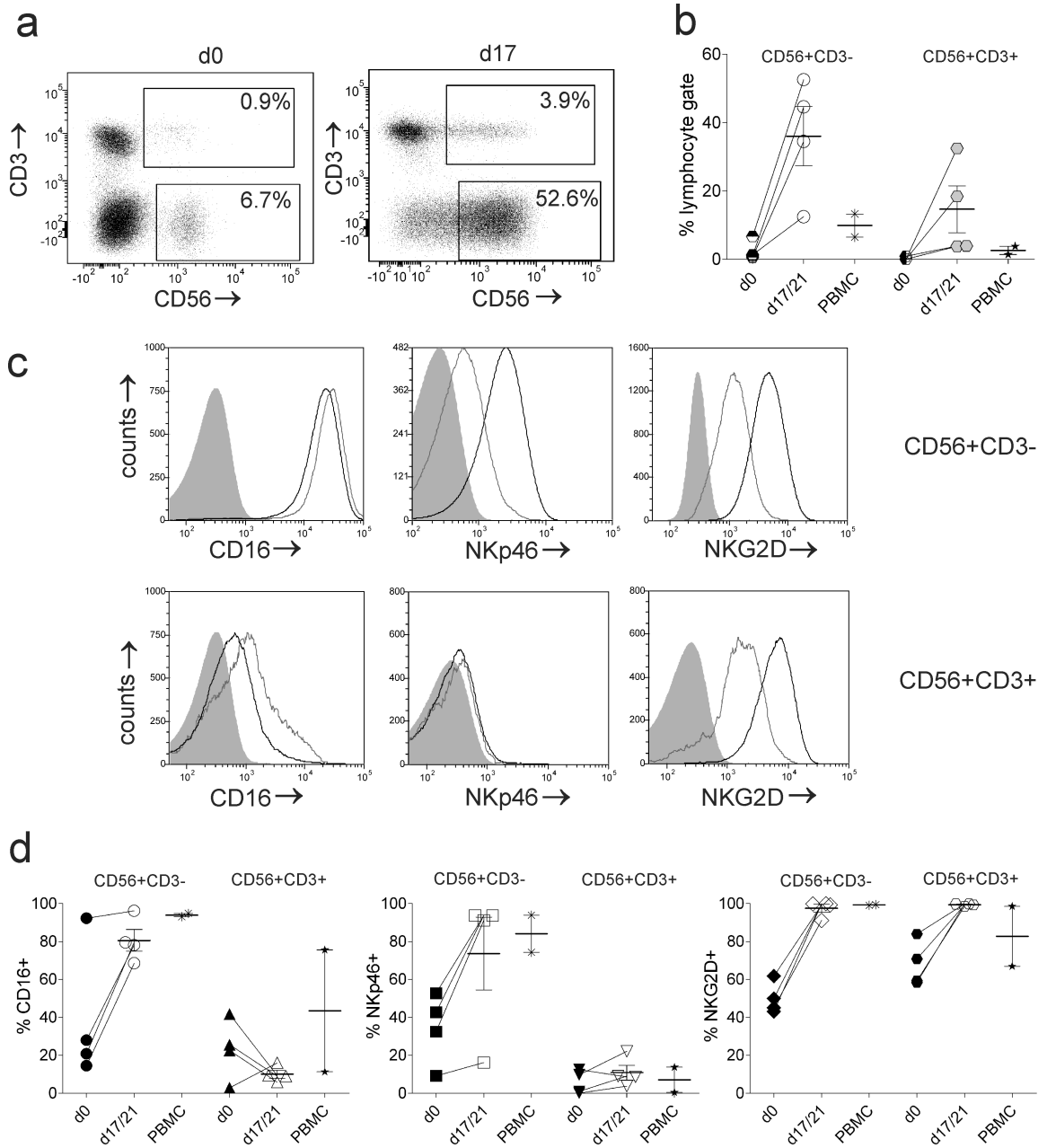
Figure 3.
Immunophenotype comparison of pre-B ALL bone marrow CD56+ populations expanded after co-culture with K562 clone 9.mbIL-21. (**a**) Percentage of live gated lymphocytes positive for CD56 or CD3 in pre-B ALL #28 BM on d0 and d17 of co-culture. (**b**) Summary of outgrowth of CD56+ cells from four different pre-B ALL BMs from d0 to d17/d21 of co-culture. PBMC, normal non-cultured PB mononuclear cells included as comparison. (**c**) Flow cytometry on #28 d0-d17 expanded CD56+ cells for expression of CD16, NKp46 and NKG2D. Isotype control, filled curve; d0, grey line; d17, black line. (**d**) Summary of differential expression of CD16, NKp46 and NKG2D on CD3- and CD3+ populations

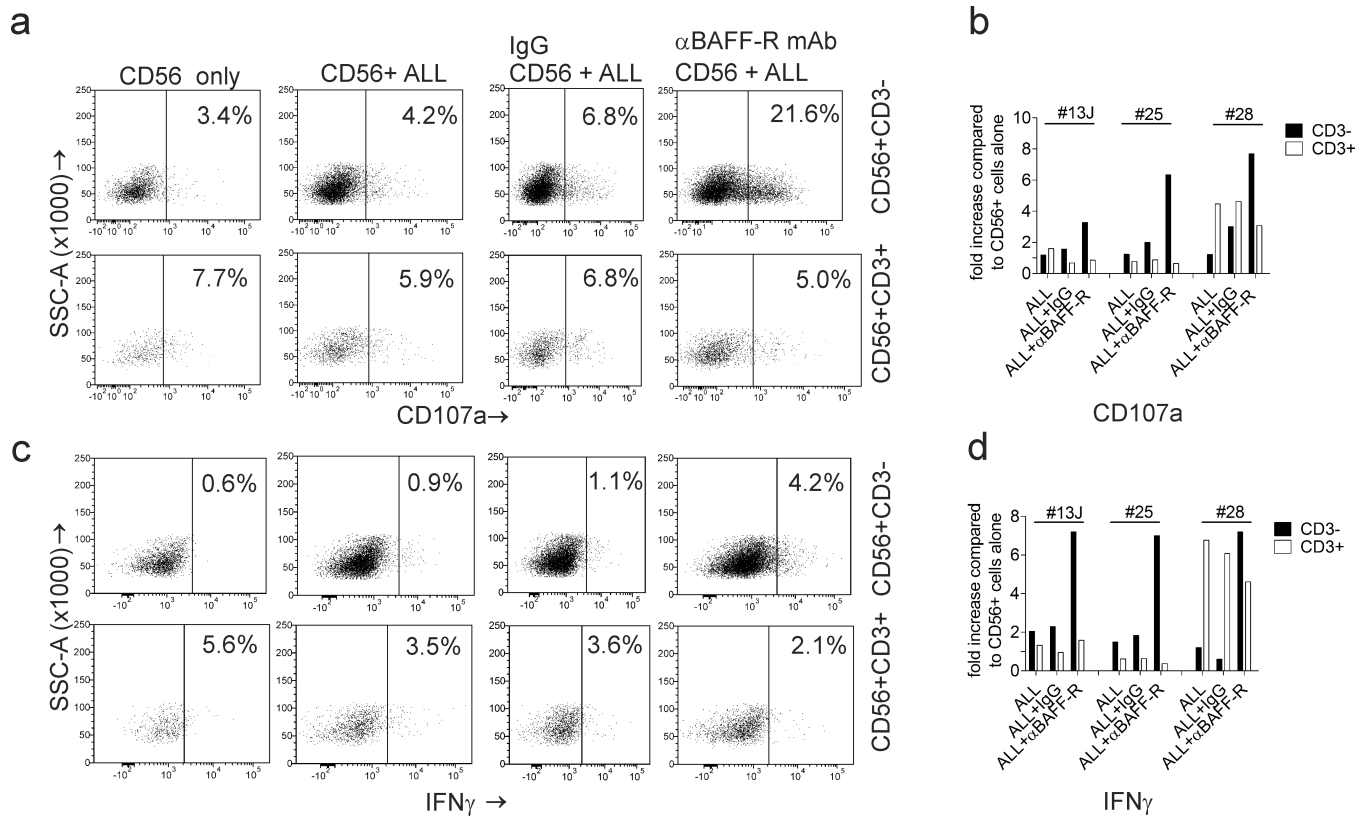within the CD56+ populations grown out from four pre-B ALL bone marrows from d0 to d17/21.

Figure 4.
Comparison of CD56+ cell activity from BM-expanded pre-B ALL samples. Flow cytometry for CD107a (**a**) and intracellular IFNγ (**c**) expression on sample #25. CD56+ populations are indicated to the right of the panels. The percentage of cells in the sections is indicated. NK, CD56+ cells alone; NK+ALL, cells + US7 pre-B ALL cells; NK+ALL+10 μg/ml human IgG; αBAFF-R mAb NK+ALL; 10 μg/ml mAb added. (**b, d**) Fold increase in CD107a (**b**) or IFNγ (**d**) positivity of cells incubated with ALL cells, ALL cells + human IgG or ALL cells + mAb, compared to cells alone. CD56+ cells were incubated with ALL cells, plus human IgG or αBAFF-R mAb for a total of 4 hrs.
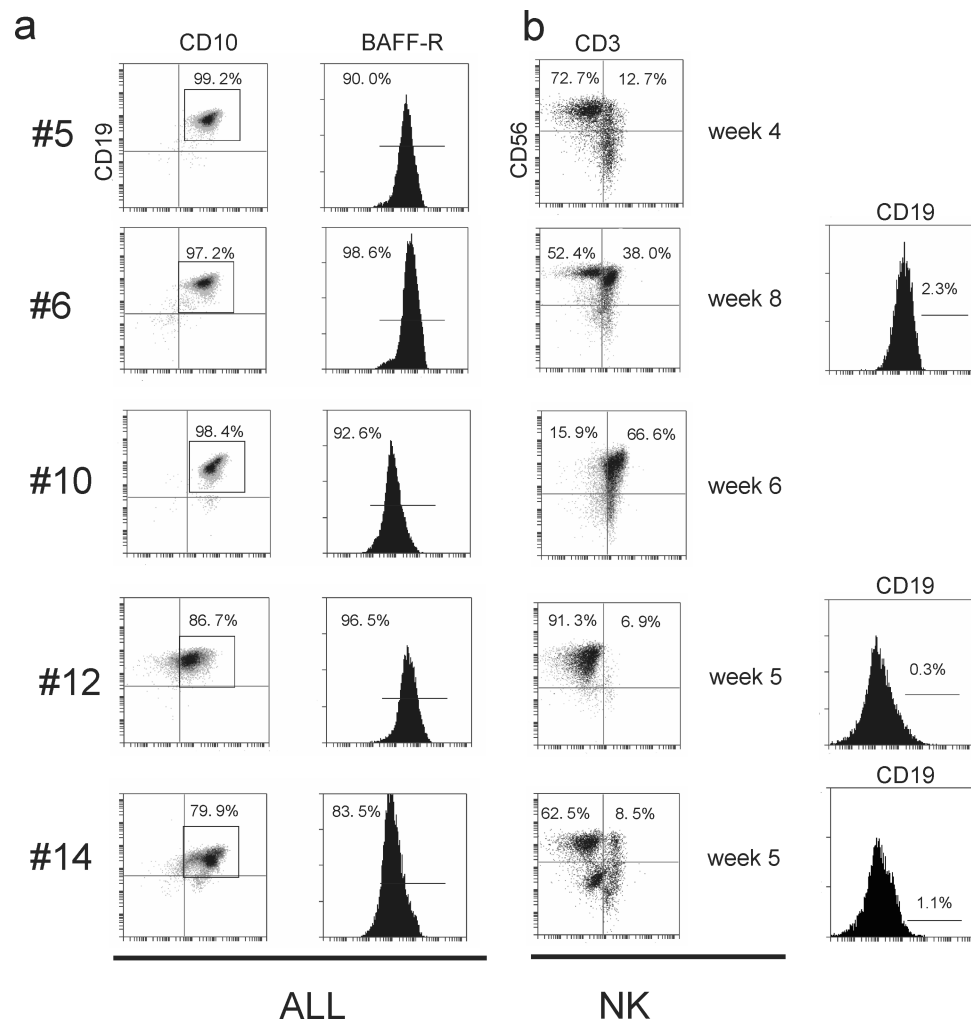
Figure 5.
Ex vivo growth of matched patient ALL cells and CD56+ activated effector cells. (**a**), Flow cytometry on primary ALL cells from BM (#5, #6, #10, #14) or PB (#12) expanded in co-culture with irradiated OP9 stromal cells for CD19 and CD10, and for expression of the BAFF-R. (**b**), CD56 and CD3 immunophenotype of mononuclear cells from PB (#5, #10, #12, #13) or BM (#6) co-cultured with irradiated aAPC-K562 cells at the indicated week numbers (#6, 10, 13 also see Supplementary Figure 2). Right panels, flow cytometry on CD56+ populations #6, #12 and #14 for the presence of CD19+ pre-B ALL cells.
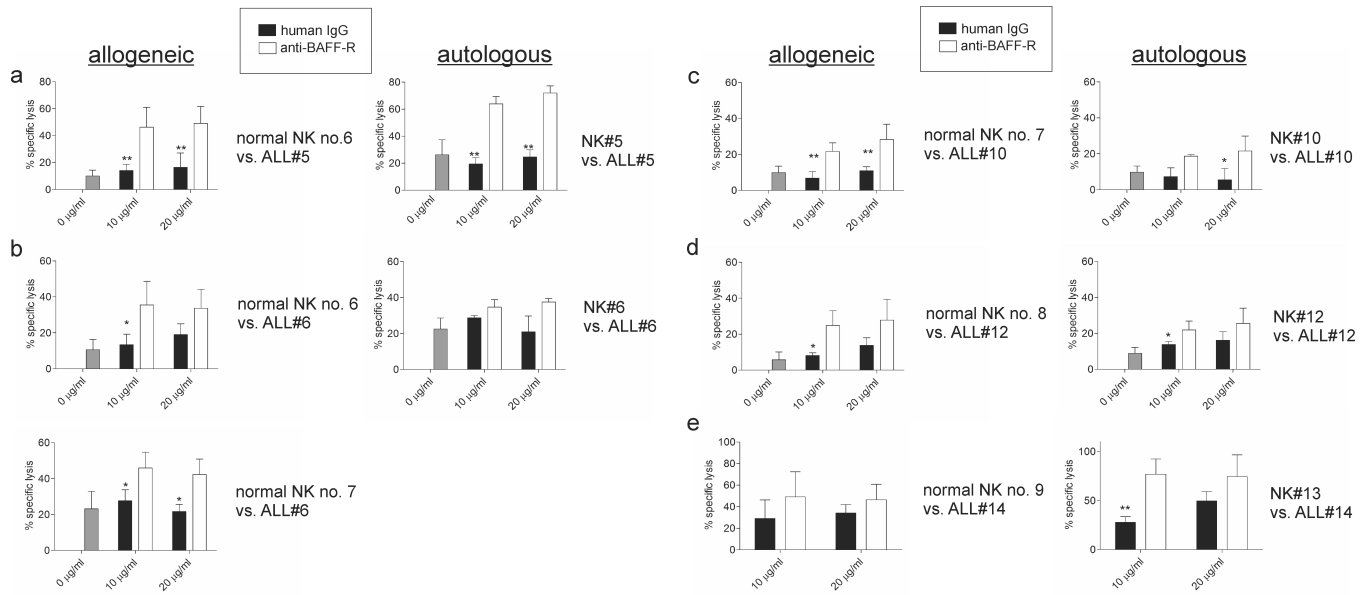
Figure 6.
Autologous patient-derived CD56+ effector cells exhibit spontaneous and antibody-stimulated ALL cell killing. ALL cells grown from patient samples #5, #6, #10 #12 and #14, and CD56+ cells generated by co-culture of normal PBMC or of patient samples with K562 clone 9.mbIL-21 (Figure 5 and Table 1) were tested for antibody-stimulated and basal cell-killing activity. 1-3.5 hrs incubation, E:T = 5:1 Experiments depicted in panels (**a, b**) (top 4) were done in one experiment. *p<0.05, **p<0.01 for human IgG compared to the same concentration of BAFF-R mAb (non-paired T-test).
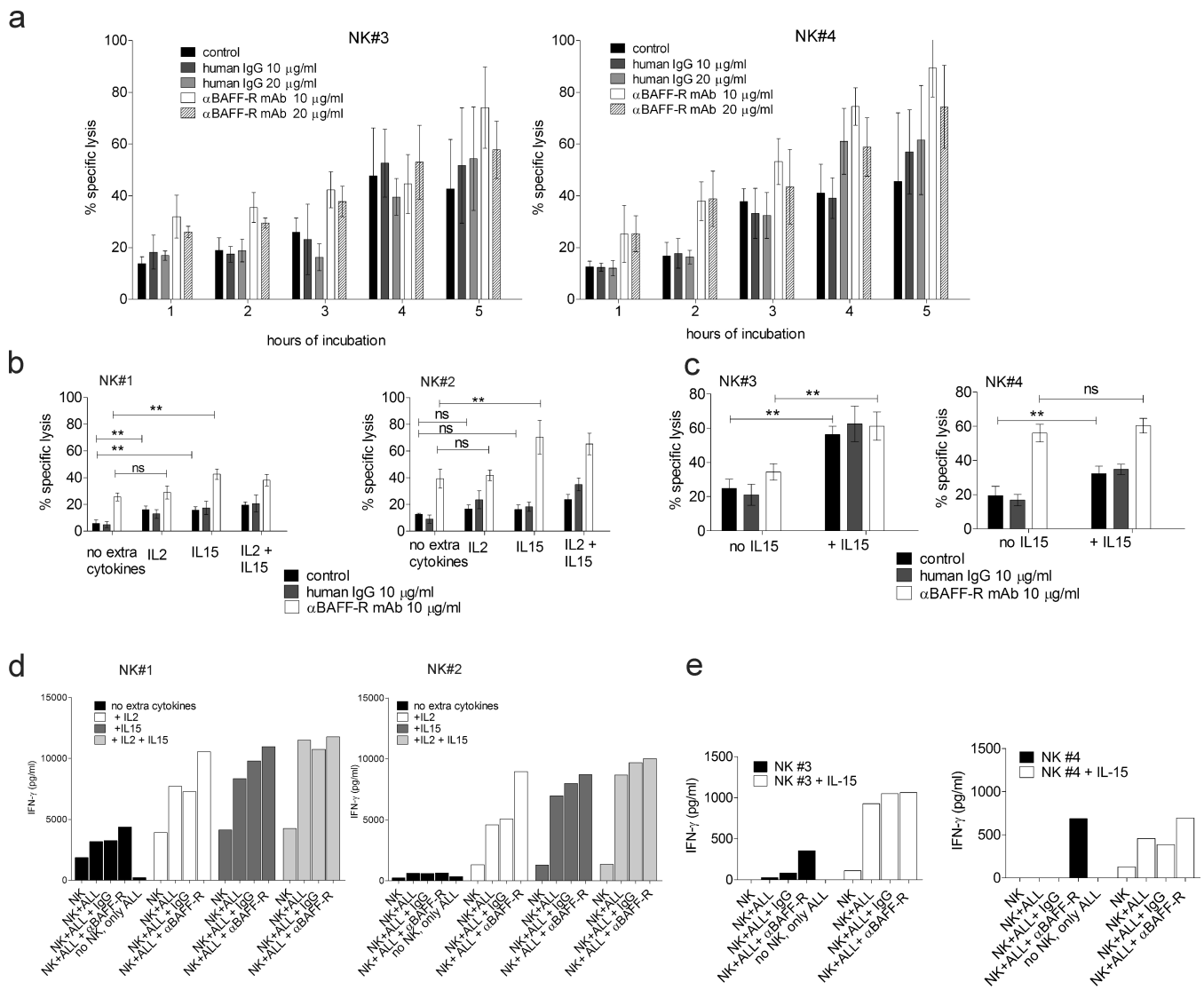
Figure 7.

Enhanced time- and cytokine-dependent cytotoxicity of *in vitro* expanded NK cells. NK cells expanded for 21-28 days from different non-leukemia PBMC samples were incubated in an E:T ratio of 5:1 with human pre-B US7 ALL cells. (**a**) ADCC over time of expanded NK cells in the presence of no antibodies (control), human IgG or anti-BAFF-R mAb as indicated. (**b**) Cytotoxicity of expanded NK cells in a 2-hr reaction with the inclusion of 50 ng/ml IL2, 50 ng/ml IL15 or both, in the reaction. Control, no added immunoglobulin. (**c**) NK cells co-cultured with aAPC-K562/IL2, with added 50 ng/ml hurIL15 overnight, tested in a 2 hr reaction for ADCC. (**d, e**) IFNγ secretion into the medium of samples from panel (**b**, **c**).

Table 1

Leukemia and NK specimen characteristics

Data from 20 samples of 18 different patients are listed. We analyzed new diagnosis PB and BM from the same patient (#9 PB/#8 BM; #13 PB/#14 BM) and two diagnosis (#5 BM and #10 BM) and post-induction (#5 PB and #10 PB) sets. The indicated percentages CD56+CD3− and CD56+CD3+ cells are residual cells in the normal lymphocyte gate.

Sample number	sample	% blasts in the sample	initial sample type	% BAFF-R expression	ALL expanded	CD56+ expanded	original % CD56+ CD3−	original % CD56+ CD3+
#1	BM relapse	58	ALL	73		Y	4.1	2.1
#2	BM relapse	24	AML	None on blasts		Y	7.8	4.2
#4	BM new Dx	90	ALL	97		Y	6.5	0.7
#5	BM new Dx	80	ALL	88	on OP9	Y	5.6	0.9
#6	BM new Dx	93	ALL	98	on OP9	Y	0.5	0.3
#7	BM relapse	30	ALL	93		Y	19.1	1.7
#8	BM new Dx	80	ALL	65		Y	2.2	1.6
#10	BM relapse	64	ALL	>90	on OP9	N	4.8	1.5
#14	BM new Dx	88	ALL	87	on OP9	Y	6.3	1.8
#13 J	BM relapse	92	ALL	89		Y	1.3	0.02
#25	BM new Dx	95	ALL	91		Y	1	0.04
#27	BM new Dx	97	ALL	94		Y	0.6	0.04
#28	BM new Dx	89	ALL	>90		Y	6.7	0.9
#3	PB Relapse	80	ALL	94		Y	12.1	0.01
#5	PB Post induction, in remission	0		na		Y	18	0.9
#9	PB new Dx	30	ALL	63		Y	8.9	2.4
#10	PB Post-induction, in remission	0		na		Y	14.1	1.3
#11	PB new dx	70	ALL	76		Y	4.1	2.3
#12	PB new Dx	70	ALL	99	on OP9	Y	6.3	1.3
#13	PB new Dx	63	ALL	84		Y	6	0.3