Zoledronic acid negatively affects the expansion of *in vitro* activated human NK cells and their cytolytic interactions with Ewing sarcoma cells

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Abstract. Disseminated Ewing sarcoma remains a fatal disease despite advanced multimodal treatment regimens. Immunotherapies as well as novel drugs and biologicals are currently being explored to eliminate minimal residual disease after conventional therapy thereby rescuing patients at a high risk for relapse. Insights into the interactions between novel therapies provide the basis for the development of effective combination strategies. We investigated the effects of the aminobisphosphonate zoledronic acid (ZA) on the *in vitro* expansion of human natural killer (NK) cells and their cytolytic activity against Ewing sarcoma cells. ZA significantly impaired the *in vitro* expansion of activated NK cells from both healthy donors and Ewing sarcoma patients in a dose-dependent manner. Expression of differentiation markers and activating receptors was unaffected by the drug. Activated NK cells from both healthy donors and patients had potent degranulation responses to Ewing sarcoma cells. In the presence of ZA at concentrations reflecting pharmaceutical serum levels, the *in vitro* antitumor activity of NK cells from Ewing sarcoma patients was significantly impaired. We conclude that ZA can impede *in vitro* NK cell expansion and cytolytic NK cell responses to Ewing sarcoma. These observations raise caution against the combination of adoptive NK cell transfer with ZA maintenance therapy in Ewing sarcoma. Future studies aim to identify potentiating interactions of novel drugs with cellular therapies.

Introduction

Ewing sarcoma is an aggressive cancer of bone. Successful therapy requires a multimodal strategy combining systemic multiagent chemotherapy with local treatment (1). Current 3-year event-free survival ranges between 70% in patients with localized disease and good response to chemotherapy and only 25-30% in patients with skeletal metastases. Novel therapies are needed to eliminate residual disease after conventional treatment and prevent disease recurrence. One example of a conceptually novel non-cytotoxic drug is the bisphosphonate compound zoledronic acid (ZA). Bisphosphonates are a class of drugs that inhibit osteoclast activity and bone resorption and are widely used in patients with osteolytic skeletal disorders (2). Based on the rationale that ZA may interfere with stimulatory interactions between the bone microenvironment and tumor cells and on observations of direct antiproliferative effects against tumor cells (3-5), the drug was also evaluated for the treatment of primary and metastatic bone tumors. As expected, ZA demonstrated clinical antitumor activity in multiple myeloma (6) and osteosarcoma (7) and against (micro)metastatic bone and/or bone marrow disease in solid tumors (8,9). In preclinical studies in Ewing sarcoma, ZA was found to inhibit *in vivo* tumor growth both alone and in synergism with chemotherapies (4,5), and initial clinical reports suggest that ZA combined with chemotherapy may be effective in refractory disease (10). The drug has now entered randomized clinical evaluation to improve overall survival as an add-on therapy in localized disease (4).

In a parallel development, cellular therapies have emerged as promising novel modalities for cancer treatment (11). Among pediatric solid tumors, Ewing sarcoma cells were found to be particularly sensitive to lysis by activated natural killer (NK) cells *in vitro* (12). NK cells are innate effector lymphocytes that provide a first-line defense against viral infection and tumor cells. Thus, NK cell therapies may have therapeutic benefit in Ewing sarcoma without additive toxicity. Modulatory or synergistic interactions between novel drugs and immunotherapies have only begun to be investigated. Antiapoptotic proteins, kinase inhibitors and epigenetic agents can sensitize cancer cells to antigen-specific immunotherapies and reverse immune escape (13-17). On the other hand, various non-cytotoxic anticancer drugs were found to negatively interfere with critical lymphocyte and antigen-presenting functions and thereby conflict with rational combinations of the two strategies (18,19).

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Apart from their antitumor and antiresorptive activities, ZA and other bisphosphonates have potent immunomodulating effects. At clinically relevant concentrations, these agents were found to induce specific activation and IL-2-dependent proliferation of a peripheral blood lymphocyte subset, γδ T cells (20). Moreover, ZA can inhibit the activation of monocyte-derived dendritic cells (21). The immune effects of ZA are further documented by the clinical inflammatory syndrome that occurs in many patients upon first doses and is associated with a transient decrease in the number of lymphocytes circulating in peripheral blood (22,23). Through indirect mechanisms, ZA was found to enhance NK cell effector functions (24), supporting the combination of the two strategies. In the present study, we explored the direct *in vitro* effects of ZA on expansion, phenotype and cytolytic activity of human NK cells in Ewing sarcoma.

Materials and methods

Cell lines. The identity of all cancer cell lines was confirmed by short tandem repeat (STR) profiling. The Ewing sarcoma cell lines TC-71 and CADO-ES1 were obtained from DSMZ (Braunschweig, Germany). VH-64 and WE-68 cells were gifts from Frans van Valen's Laboratory at the Institute of Experimental Orthopedics of the University of Muenster, Germany. These cell lines were characterized by the EuroBoNeT consortium (25). Tumor cells were cultured in collagen-coated 25-cm^2 tissue culture flasks (VH-64, WE-68, CADO-ES1) or in uncoated flasks (TC-71) in RPMI-1640 medium (Invitrogen, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Thermo Fisher, Bonn, Germany) and 2 mM L-glutamine and maintained at 37˚C in 5% CO_2. K562 (ATCC) is a human erythroleukemia cell line that is sensitive to lysis by NK cells. Generation of the K562-mb15-41BBL stimulator cells was previously described (26). The human myeloid ML-2 cell line (ATCC) was used as a control target.

In vitro expansion of human NK cells. Approval for using peripheral blood samples of both healthy donors and pediatric sarcoma patients was obtained from the University of Muenster Board of Ethics. Peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation and resuspended in RPMI-1640 medium, supplemented with 10% FCS and 2 mM L-glutamine (RPMI culture medium). The cells were seeded at $1x10^6$/well in a 24-well tissue-culture plate in the presence of 40 IU/ml recombinant human IL-2 (rhIL-2) (Proleukin; Chiron, Emeryville, CA, USA) in RPMI-1640 and 10% FCS and stimulated once with $0.75x10^6$ irradiated (120 Gy) K562-mb15-41BBL stimulator cells, as described by Imai *et al* (26). TCR γδ-expressing T cells were depleted by magnetic cell sorting using the anti-TCR γδ MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow cytometry. For immunophenotyping, lymphocytes were stained with fluorescence-labeled anti-human antibodies against the following surface molecules: CD3, CD4, CD56, CD16, CD137 (4-1BB), NKG2D, NKG2A, NKG2C, CD94, CD57, 2B4 (CD244), DNAM-1 (CD226), NKp30, NKp44, NKp46, TCRγδ for 30 min at 4˚C. Anti-NKG2A and anti-NKG2C antibodies were from R&D Systems (Minneapolis, MN, USA). All others were from BD Biosciences (Heidelberg, Germany). Cells were analyzed using a FACSCanto cytometer (BD Biosciences).

CD107a assay. Degranulation responses were assessed by flow cytometric analysis of CD107a expression after a 4-h co-incubation with target cells. Co-incubations were performed in the presence of PE-labeled anti-human CD107a antibody (BD Biosciences) and 2 μM monensin (Sigma, Munich, Germany). The NK cells were washed and stained with FITC-labeled anti-CD56 and PerCP-labeled anti-CD3 antibody, followed by analysis of cells within the $CD56^+CD3^-$ gate.

Statistics. The Student's t-test was used to test whether the means in each set of values differed significantly, assuming two possible tails as well as unequal variance. A P-value <0.05 was defined as indicative of statistical significance. Values depict the means ± standard deviation unless otherwise stated.

Results

Ewing sarcoma cells effectively induce NK cell degranulation responses. First, we assessed the capacity of individual Ewing sarcoma cell lines to functionally interact with *in vitro* activated and expanded human NK cells. CD107a upregulation corresponds to NK cell degranulation (27) and was used as a functional marker for cytolytic activity. PBMCs from three healthy donors were stimulated with irradiated K562 cells that had been gene-modified to express membrane-bound IL-15 and 41BB ligand, as described by Imai *et al* (26), and expanded in the presence of low-dose rhIL-2 for 12 days. This resulted in a mean 34.5±8.3-fold increase in $CD56^+CD3^-$ NK cells among the stimulated bulk populations on day 12 of culture (range, 27.8-43.7) and a mean purity of 66.8±11.9% (range, 53.1-74.2%). Expanded NK cells from all donors were highly responsive to the NK cell-sensitive target cell line K562, while the NK cell-resistant leukemia ML2 cell line failed to induce degranulation responses above the background of the medium alone (Fig. 1A). At a 1:1 stimulator-to-responder cell ratio, VH-64, WE-68 and TC-71 Ewing sarcoma cells effectively induced CD107a upregulation responses in all three donors, with 44.6±16.5% (range, 23.3-65.2%), 23.7±11.2% (range, 8.7-34.5%) and 34.7±17.9% (range, 16.2-53.9%) CD107a-expressing cells within the $CD56^+CD3^-$ NK cell gate, respectively. While degranulation responses to VH-64 cells were comparable to K562 cells in all donors, TC-71 and WE-68 cells induced variable responses among the three donors. Cado-ES-1 Ewing sarcoma cells failed to induce NK cell activation above the medium control in all donors (4.6±3.7%; range, 0.8-9.9%). Specific degranulation responses to VH-64 cells were maintained with reduced stimulator-to-responder cell ratios of 0.5:1 and even 0.1:1 (Fig. 1B). These results confirm that Ewing sarcoma cells can be highly efficient activators of allogeneic NK cells. Subsequent experiments were performed at the lowest ratio of 0.1:1 unless otherwise stated, and the NK cell-sensitive cell lines VH-64 and WE-68 were used as targets.

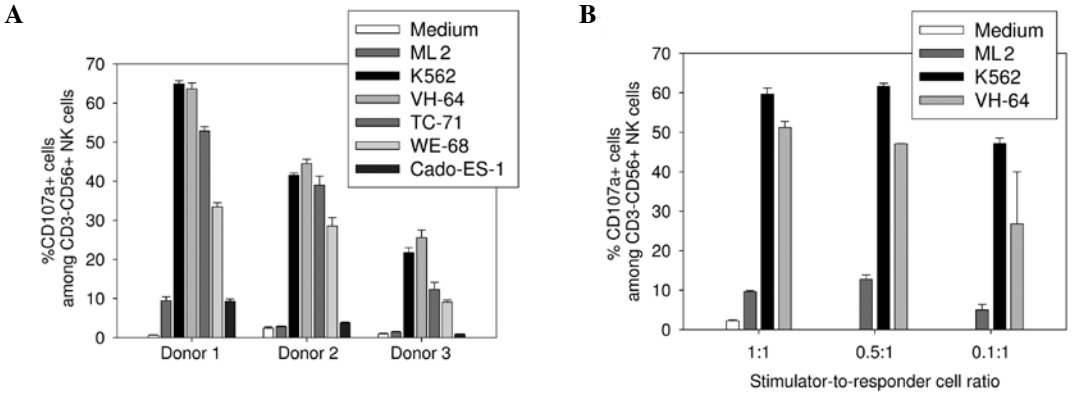

Figure 1. Ewing sarcoma cell lines induce potent NK cell degranulation responses. (A) The percentages of CD107a-expressing degranulating NK cells were determined by flow cytometry after a 4-h co-incubation of *in vitro* activated NK cells from three healthy donors with NK cell-sensitive (K562) and control (ML2) target cells, and with the Ewing sarcoma cell lines VH-64, TC-71, WE-68 and Cado-ES-1, respectively, at stimulator-to-responder cell ratio of 1:1 or in the presence of medium alone. To exclude residual non-NK lymphocytes within the cultures from analysis, the gate was set on $CD56^+/CD3^-$ cells. (B) Percentages of CD107a-expressing cells among $CD56^+$ NK cells in response to K562, ML2 and VH-64 cells and in the presence of medium alone at decreasing stimulator-to-responder cell ratios. Bars in the histograms represent means and standard deviations of results from triplicate wells.

Table I. Surface expression density of cell receptors on NK cells expanded in the presence or absence of aminobisphosphonate zoledronic acid.

	Without ZA		With 1 μM ZA		
Marker	MFI (means ± SD)	Range	MFI (means ± SD)	Range	P-value
CD56	3210±2133	1875-5670	2764±409	2292-3022	ns
CD57	160±47	130-214	138±24	113-161	ns
CD244 (2B4)	2007±546	1599-2627	1998±13	1568-2487	ns
NKG2D	5376±885	4378-6065	6187±2623	3213-8172	ns
CD137 (4-1BBL)	105±18	85-118	105±13	90-109	ns
CD226 (DNAM-1)	3841±461	3524-4370	3906±363	3641-4319	ns
NKp46	380±198	179-574	257±80	165-312	ns
NKp44	128±40	97-174	143±75	60-205	ns
NKp30	4025± 668	3510-4780	3923±1643	2049-5119	ns

The median fluorescence intensity (MFI) of expression was determined within the $CD56^+CD3^-$ lymphocyte gate following 10 days of NK cell expansion in the absence or presence of 1 μM ZA. Shown are the means and standard deviations (SD) of experiments with three donors. ns, statistically not significant. ZA, aminobisphosphonate zoledronic acid.

Zoledronic acid impairs the in vitro expansion of NK cells from healthy donors whereas the cytolytic responses to tumor cells are essentially maintained. To investigate the effects of ZA on the *in vitro* expansion and functionality of human NK cells, NK cells were *in vitro* activated and expanded from PBMCs of five healthy donors as described above, in the absence or presence of increasing concentrations of ZA. These concentrations correspond to plasma levels obtained in patients after ZA infusion (28). NK cell expansion was significantly diminished in the presence of ZA in a dose-dependent manner compared to the ZA-free medium control (Fig. 2A). Substantial inter-donor variability was observed; whereas addition of 10 μM ZA to the culture medium almost completely prevented NK cell expansion in three donors, expansion rates of 76.6 and 79.2% of controls, respectively, were obtained under the same conditions in the two other donors.

Next, we analyzed the phenotypes and NK cell receptor expression of NK cells expanded from three healthy donors in the presence or absence of ZA by gating on $CD56^+CD3^-$ lymphocytes and flow cytometryic analysis. Our *in vitro* stimulation method preferentially expanded NK cells with a $CD56^{bright}$ phenotype (Fig. 2B). Under the two culture conditions, equal proportions of NK cells expressed CD57, a marker of highly mature and differentiated NK cells (29). Over 95% of expanded NK cells co-expressed the activating receptors NKG2D and DNAM-1 (CD226), which have both been implicated in the recognition of target cells via specific ligands, as well as the (co)stimulatory receptor 2B4 (CD244) and the activating receptors NKp30 and NKp46, as well as NKp44 and CD94/NKG2C (data not shown), regardless of the presence or absence of ZA (Fig. 2B). CD94/NKG2A heterodimers, which have inhibitory function, were also equally expressed under all culture conditions. Median fluorescence intensities of expression of all of these receptors did not significantly vary between the two populations (Table I).

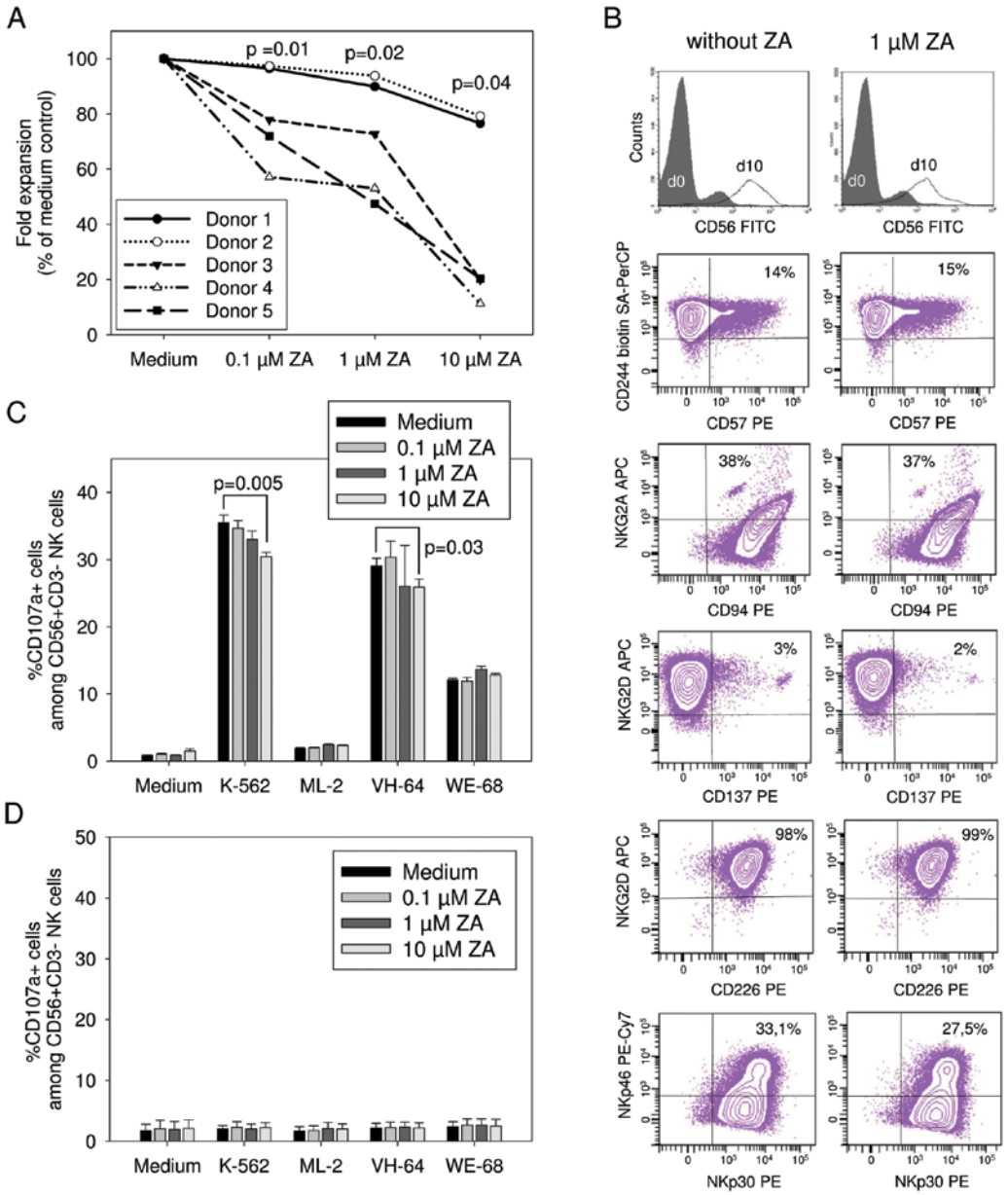

Figure 2. Phenotype and functionality of NK cell populations expanded in the presence or absence of ZA. (A) PBMCs from five healthy donors were co-cultured for 10 days with irradiated K562-mb15-41BBL cells and rhIL-2 in the presence of 0.1, 1 and 10 μM ZA or with medium alone, and the absolute numbers of NK cells were calculated after staining of viable cells with CD3- and CD56-specific antibodies. (B) Immunophenotyping of NK cells following 10 days of expansion in the absence (left panel) or presence (right panel) of 1 μM ZA. The initial gate was set on $CD56^+CD3^-$ cells. Shown is a representative experiment with one of three donors. (C) NK cells were *in vitro* expanded for 10 days in the absence or presence of 0.1, 1 and 10 μM ZA or with medium alone, then co-incubated with K562, ML2, VH-64 and WE-68 target cells or in medium alone for 4 h at a 0.1:1 stimulator-to-responder cell ratio. CD107a-expressing cells among the $CD56^+$ NK cells were determined by flow cytometry. Shown are triplicate values obtained for one of five representative healthy donors. (D) NK cells were positively selected from peripheral blood and co-incubated with target cells for 4 h at an 0.1:1 stimulator-to-responder cell ratio in the absence or presence of the indicated concentrations of ZA, followed by quantification of CD107a-expressing cells among the $CD56^+$ NK cells by flow cytometry. Shown are the combined means and standard deviations derived from triplicate wells from three healthy donors.

To address the effect of ZA on the *in vitro* functionality of NK cells, we compared CD107a upregulation by NK cells expanded either in the presence or absence of ZA in response to stimulation with K562 or tumor cells. Zoledronic acid is a strong activator of γδ T cells (20). Therefore, to avoid confounding effects by co-expanded γδ T cells, these cells were depleted prior to expansion by magnetic cell sorting. In the presence of 0.1 and 1 μM ZA, potent degranulation responses to K562 and VH-64 cells and intermediate responses to WE-68 cells were maintained (Fig. 2C). In two of the five donors, responses to K562 and VH-64 cells by NK cells were reduced when the highest concentration (10 μM) of ZA was added to the 4-h co-incubation.

We further investigated the effects of ZA on the functionality of native NK cells obtained by isolation from peripheral blood of three healthy donors without prior stimulation. NK cells were positively selected by magnetic cell selection, then co-incubated for 4 h with the various target cells at an 0.1:1 stimulator-to-responder cell ratio in the presence or absence of ZA. CD107a expression of non-activated NK cells from

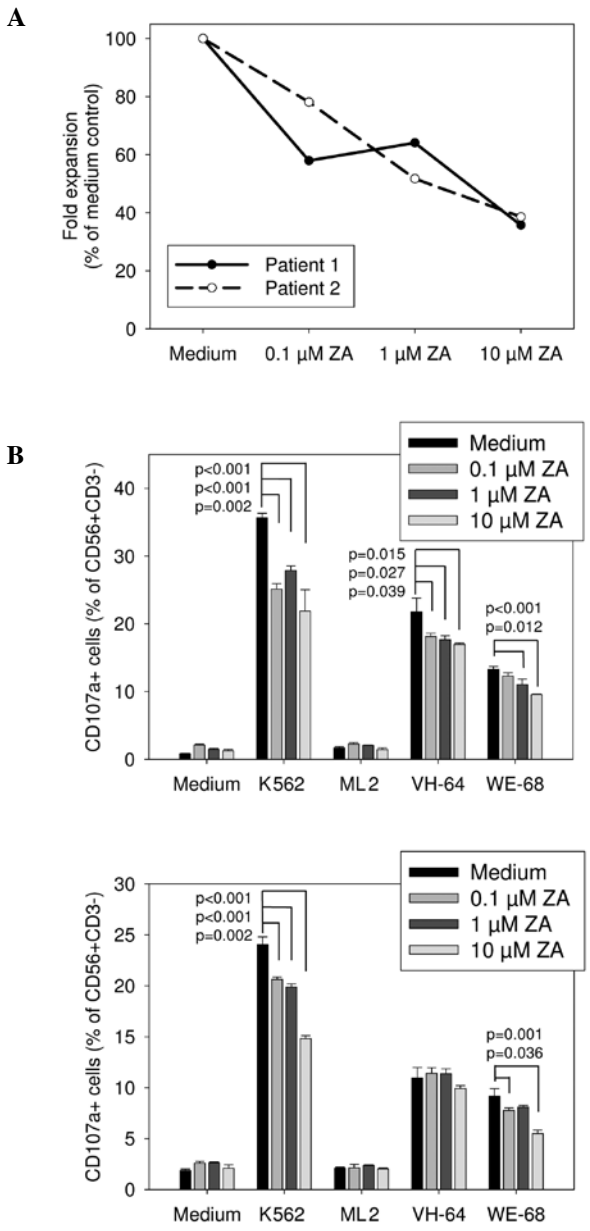

Figure 3. Expansion and functionality of NK cell populations in the presence or absence of ZA. (A) PBMCs from two Ewing sarcoma patients were co-cultured for 10 days with irradiated K562-mb15-41BBL cells and rhIL-2 in the presence of 0.1, 1 and 10 μM ZA or with medium alone, and the absolute numbers of NK cells were calculated after staining of viable cells with CD3- and CD56-specific antibodies. (B) On day 10 following *in vitro* expansion in the absence or presence of 0.1, 1 and 10 μM ZA, NK cells from two Ewing sarcoma patients were co-incubated with K562, ML2, VH-64 and WE-68 target cells or in medium alone for 4 h at an 0.1:1 stimulator-to-responder cell ratio. The proportions of CD107a-expressing cells among the $CD56^+$ NK cells were determined by flow cytometry. Shown are triplicate values for both donors.

all donors did not exceed background expression even upon co-incubation with K562 targets (Fig. 2D). These results were unaffected by the presence of ZA.

Thus, whereas *in vitro* expansion of NK cells from healthy donors was significantly impaired in the presence of ZA, their phenotype and activating receptor expression were unaffected by the drug, and degranulation responses of activated NK cells to tumor targets were maintained at ZA concentrations reflecting pharmaceutical serum levels.

Zoledronic acid impairs both in vitro expansion and degranulation responses of NK cells from patients with Ewing sarcoma. To determine the effects of ZA on the functionality of NK cells from Ewing sarcoma patients, PBMCs were obtained from two patients with relapsed refractory disease. NK cells from both patients were *in vitro* stimulated and expanded using the above-described technique, with 0.1 to 10 μM of ZA added during expansion. NK cells were effectively expanded after *in vitro* activation from both donors with a median 28.7-fold increase in $CD56^+CD3^-$ NK cells (range, 27.56-29.8) and a mean purity of 88.1% (range, 80.5-95.6%). Similar to the healthy donors, expansion of NK cells from both patients was significantly diminished in the presence of 10, 1 and even 0.1 μM ZA (Fig. 3A). Moreover, in contrast to healthy donors, NK cells expanded from Ewing sarcoma patients in the presence of even low concentrations of 1 and 0.1 μM ZA had significantly impaired degranulation responses to both K562 cells and several Ewing sarcoma targets (Fig. 3B). Thus, ZA negatively affects both *in vitro* expansion and cytolytic antitumor activity of activated NK cells from Ewing sarcoma patients.

Discussion

ZA has attracted recent interest as a non-cytotoxic anticancer drug and is under clinical investigation for use in various diseases, including primary bone tumors (3,4,7-9). Promising features of ZA include the observed anticancer synergies between bisphosphonates and cytotoxic chemotherapies (4,30), the favorable toxicity profile, and the proposed effect on the tumor-promoting bone/bone marrow microenvironment to prevent dissemination. However, drug therapy alone is unlikely to completely eliminate residual disease in patients with high-risk disseminated tumors. Instead, the combination of anticancer drugs and immune-based treatments is an attractive strategy to overcome drug resistance and clonal escape. Rational synergistic combinations may exploit the capacity of individual drugs to facilitate immune recognition of tumors by manipulation of immunogenicity and immunosuppressive effects of the microenvironment (13,14,16). Potential combination partners must also be explored for their effects on the antitumor effector functions of therapeutic immune cells.

In the present study, we demonstrated that ZA significantly impeded *in vitro* NK cell expansion. Moreover, cytolytic NK cell responses to Ewing sarcoma cells were substantially impaired in the presence of ZA. Both effects were even more apparent with NK cells from Ewing sarcoma patients in disease-refractory situations in which such alternative combination therapies may be considered.

One of the most studied effects of ZA is the selective activation of γδ T cells. ZA and other aminobisphosphonates stimulate γδ T cells through indirect mechanisms, including inhibition of a critical enzyme of the mevalonate metabolism. This results in accumulation of the upstream metabolite isopentenyl pyrophosphate that directly activates γδ T cells (31). By the same mechanism, pretreatment of solid cancer cells with ZA sensitizes cancer cells to γδ T cell-mediated killing (32). Apart from γδ T cells, ZA was reported to have profound effects on alternative immune cells, including dendritic cells (DCs) (21). Previous studies of ZA

and NK cells have shown activating interactions (24,33,34) which appear to contradict our own observations. A major conceptual difference was that these previous studies relied on the effects of ZA on accessory cells. ZA-activated γδ T cells and monocytes, respectively, were shown to induce NK cell cytolysis of tumor cells by providing CD137L-induced co-stimulation to upregulate NKG2D expression on NK cells (33) or by IFN-γ-mediated upregulation of TNF-related apoptosis-inducing ligand (TRAIL) (34). In another study, activation of NK cells in the presence of ZA was found to be a consequence of ZA-induced cytokine support by DC-like cells (24). In the present study, we investigated the effects of ZA on a population of *in vitro* preactivated NK cells under consideration for the cell therapy of cancer (12,26,35). Preactivation relies on stimulation with the human leukemia cell line K562 and co-stimulation by 4-1BBL along with cytokine support by IL-15 and subsequent low-dose IL-2. This method results in an enrichment of highly cytotoxic NK cells with strong potency against tumor cells, including Ewing sarcoma (12,35). Although we cannot exclude that residual non-NK cell lymphocyte populations contributed to our observations, interactions with γδ T cells were avoided by depleting this subset prior to expansion.

Whereas ZA significantly suppressed activation-induced expansion of NK cells in most donors and in both Ewing sarcoma patients, the phenotypes of the resulting cell populations were comparable and correspond to the *in vitro* activated phenotype that was previously reported for 4-1BBL-based stimulation methods (26,36). A practical consequence is that PBMCs or activated and expanded NK cell products should be cryopreserved prior to ZA treatment to maximize the rates of *ex vivo* NK cell expansion. More relevant from the translational perspective is the negative effect on degranulation responses particularly in Ewing sarcoma patients. Although potent responses were still found even at high concentrations of ZA, clinical efficacy of NK cell therapy may be more dependent on an optimal activity of therapeutic cells, and even moderate inhibitory effects may interfere with tumor cell clearance.

The mechanisms by which ZA affects NK cell expansion and responses to target cells remain to be resolved. Cytolytic responses of activated NK cells to Ewing sarcoma targets were shown to involve interaction of the NK cell activation receptors NKG2D and DNAM-1 with their respective ligands on tumor cells (12,37). Expression levels of these receptors were unaffected by ZA in our studies. Alternatively, at least the effect on degranulation responses may be explained by effects of ZA on the Ewing sarcoma target cells, e.g. by downregulation of activating receptor ligands. The variability observed among donors may be explained by the impact of allogeneic KIR (killer immunoglobulin-like receptor) mismatches on the outcome of the interaction.

Whether autologous NK cells in Ewing sarcoma patients have a role in the natural defense against this disease is unclear. In the present study, non-activated NK cells were not capable of functionally interacting with Ewing sarcoma targets, and the results were unaffected by ZA.

Collectively, our data suggest that ZA maintenance therapy in Ewing sarcoma is not an ideal platform for adoptive NK cell transfer. Drugs that potentiate the therapeutic immune effects of NK cells and the mechanisms underlying these interactions must be identified to develop effective combinations of novel drugs and cellular therapies.

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