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Chemotherapy resistant osteosarcoma is highly susceptible to IL-15 activated allogeneic and autologous NK cells

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ABSTRACT

INTRODUCTION: High-grade osteosarcoma occurs predominantly in adolescents and young adults and has an overall survival-rate of about 60%, despite chemotherapy and surgery. Therefore, novel treatment modalities are needed to prevent or treat recurrent disease. Natural killer (NK) cells are lymphocytes with cytotoxic activity towards virus infected or malignant cells. We explored the feasibility of autologous and allogeneic NK cell mediated therapies for chemotherapy resistant and sensitive high-grade osteosarcoma. METHODS: The expression by osteosarcoma cells of ligands for activating NK cell receptors was studied *in vitro* and *in vivo* and their contribution to NK cell mediated cytolysis was studied by specific antibody blockade. RESULTS: Chromium release cytotoxicity assays revealed chemotherapy sensitive and resistant osteosarcoma cell lines and osteosarcoma primary cultures to be sensitive to NK cell mediated cytolysis. Cytolytic activity was strongly enhanced by IL-15 activation and was dependent on DNAM-1 and NKG2D pathways. Autologous and allogeneic activated NK cells lysed osteosarcoma primary cultures equally well. Osteosarcoma patient derived NK cells were functionally and phenotypically unimpaired. CONCLUSION: In conclusion, osteosarcoma cells, including chemoresistant variants, are highly susceptible to lysis by IL-15-induced NK cells from both allogeneic and autologous origin. Our data support the exploitation of NK cells or NK cell activating agents in patients with high-grade osteosarcoma.

INTRODUCTION

High-grade osteosarcoma is the most common primary malignant bone sarcoma, occurring mainly in adolescents and young adults [125]. Despite multi-agent chemotherapy and surgery, overall survival is still poor at about sixty percent [24;32;141]. Therefore, novel treatment modalities are urgently needed to either prevent or treat chemotherapy refractory and recurrent disease. Immunomodulatory agents such as interferon (IFN)-α and muramyl-tripeptide (MTP) have been added to standard chemotherapy regimens in recent clinical trials [160;169;170]. Immunotherapy with stimulatory cytokines such as IL-2 and IL-15, or the adoptive transfer of *ex vivo* cytokine activated cytotoxic lymphocytes such as natural killer (NK) cells could be another adjunct to current therapy [148;174;255].

NK cells lack a clonally rearranged antigen-specific receptor. Instead, cytolytic activity towards virus infected or malignant cells is dependent on the balance between inhibitory and activating signals. NK cell activating signals are provided when the activating receptors Natural Killer Group 2, member D (NKG2D), DNAX accessory molecule-1 (DNAM-1) and the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 bind their respective ligands [178;186;272]. Although NK cell recognition of tumor cells has been reported to be partially mediated through NCRs, the responsible ligands are unknown [135]. The DNAM-1 ligands poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) are highly expressed by many tumors, including sarcomas [26;64;272]. Ligands for NKG2D are the stress-inducible major histocompatibility class I polypeptide-related sequence (MIC) A and B and the UL-16 binding proteins (ULBPs) 1 to 4. Inhibitory ligands are the classical and non-classical human leukocyte antigen (HLA) class I molecules expressed on all normal cells [118]. These ligands bind to inhibitory killer immunoglobulin-like receptors (KIRs) and the C-type lectin heterodimer CD94/NKG2A on NK cells, respectively. In addition to high expression of NKG2D and DNAM-1 ligands, many tumors show loss of HLA class I, possibly rendering them susceptible to NK cell mediated lysis [20;38].

In the current study, we demonstrated the sensitivity of chemotherapy resistant and sensitive osteosarcoma cells to lysis by IL-15 activated NK cells and identified the molecular mechanisms involved. NK cells of osteosarcoma patients were not functionally impaired and were able to lyse autologous tumor cells, supporting the use of NK cell activating agents in the treatment of osteosarcoma patients.

MATERIALS AND METHODS

Patient material

A tissue array was constructed from formalin fixed, paraffin embedded (FFPE) tissue retrospectively collected from 88 osteosarcoma patients treated at the LUMC as previously described [183] (Suppl. Table 5.1). Peripheral blood mononuclear cells (PBMCs) were collected from healthy controls and 22 pre-treatment osteosarcoma patients after written informed consent was obtained, as approved by the Institutional Review Board on Medical Ethics. Osteosarcoma tissue samples were used for research in accordance with national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of

Table 5.1 Expression of inhibitory and activating NK ligands by osteosarcoma. Eight osteosarcoma cell lines (HOS, 143B/HOS, SJSA-1, OHS, ZK-58, U2-OS and SAOS-2), six chemotherapy resistant variant cell lines (doxorubicin (DX), methotrexate (MTX) and cisplatinum (CDDP) resistant variants of U2-OS and SAOS-2) and five short-term cultures (L2808, L2599, L2792, L2635 and L2531; all no later than passage 3) were evaluated for the expression of NK cell ligands by flow cytometry. Expression of the inhibitory ligands CD48 and MHC class I and expression of ligands for the activating receptors NKG2D (MICA, MICB, ULBP-1, ULBP-2 and ULBP-3) and DNAM-1 (CD112 and CD155). Expression of the adhesion molecules CD54 and CD58 and of the death receptor CD95 (Fas). (−) mean fluorescence intensity (MFI) ratio of specific staining versus isotype control <2; (+/−) MFI ratio between 2 and 5; (+) MFI ratio between 5 and 10; (++) MFI ratio >10.

							U2-OS				SAOS-2								
	HOS	143B/ HOS	SJSA-1	IOR/ OS-14	OHS	ZK-58	U2-OS	DX 580	MTX 300	CDDP 4	SAOS-2	DX 580	MTX 1	CDDP 6	L2531	L2599	L2635	L2792	L2808
MHC class I	++	++	++	++	+/-	+	++	++	++	++	++	++	++	++	++	++	++	++	++
CD48 (2B4 ligand)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA	+/-	+	+/-	+/-	-	+/-	++	+	+	++	++	++	++	+	-	-	++	+/-	-
MICB	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ULBP-1	+/-	+	+/-	+/-	+/-	-	-	-	-	-	-	+/-	+/-	+/-	-	-	-	-	-
ULBP-2	+/-	++	+	+	+	+	++	+	++	+/-	++	++	++	++	-	+/-	+/-	+/-	+/-
ULBP-3	-	+/-	-	+/-	+	+/-	+	+/-	+	+/-	+/-	-	+/-	-	-	-	-	+/-	+/-
CD112 (Nectin-2)	+	+	++	+	+/-	+/-	++	++	++	++	+	+/-	+	+/-	+/-	+/-	+/-	+/-	++
CD155 (PVR)	+/-	++	+	+	+	+/-	++	++	++	+	+	+	+	+	+/-	+/-	++	+/-	+
CD54 (ICAM-1)	++	++	++	++	-	+/-	-	-	-	-	-	-	-	-	-	++	+/-	+/-	+
CD58 (LFA-3)	++	+	++	+	++	++	++	+	++	++	++	++	++	+	+	+/-	++	+/-	+
CD95 (Fas)	+/-	+/-	+/-	+/-	+/-	+/-	+	+/-	+	+	+/-	-	+/-	-	+/-	+	-	+/-	+

Medical Scientific Societies). All patient material was handled in a coded fashion. Clinical and pathological details of all patients can be found in Suppl. Table 5.2.

Cell lines and primary cultures

The osteosarcoma cell lines HOS, 143B/HOS, IOR-OS14, SJSA-1, OHS, ZK-58, U2-OS and SAOS-2 were characterized and maintained as described earlier [199]. The EBV B-LCL cell line 107 (EBV) and the erythroleukemic cell line K562 were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin/streptomycin (PS, Invitrogen). The chemotherapy resistant variant cell lines of U2-OS and SAOS-2 were established as described previously [204;235;236] and were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% FCS and PS. The doxorubicin (DX) resistant variants U2-OS-DX580 and SAOS-2-DX580 were cultured in the presence of 580 µg/mL DX. The methotrexate (MTX) resistant variants U2-OS-MTX300 and SAOS-2-MTX1µg were cultured in the presence of 300 and 1000 ng/mL MTX, respectively. The cisplatin (cis-diamminedichloroplatinum, CDDP) resistant variants U2-OS-CDDP4µg and SAOS-2CDDP6µg were cultured in the presence of 4 and 6 µg/mL CDDP respectively.

Fresh osteosarcoma samples L2808, L2599, L2792, L2635 and L2531 were cultured as described previously by our group for related tumors [252]. Clinical and histopathological details can be found in Suppl. Table 5.3. L2531, L2792 and L2599 were derived from patients with poor histological response to pre-operative chemotherapy in the primary tumor. L2808, a pulmonary metastatic sample, was derived from a patient with good histological response in the primary tumor but who relapsed nonetheless. L2635 originated from a patient with good histological response to pre-operative chemotherapy who is currently in persistent first complete remission (follow-up since diagnosis 20 months). Collected tissue pieces were dissociated mechanically and cultured in RPMI 1640 medium supplemented with 20% FCS and PS. When subconfluence was reached, cells were harvested using 0.05% Trypsin/EDTA (Invitrogen) and passaged. Chromium release assays and flow cytometric analyses were performed no later than at passage 3.

Isolation and culture of NK cells

PBMCs were isolated using a Ficoll density gradient separation followed by NK cell enrichment using the MACS NK enrichment kit and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purity of NK cells was assessed by flow cytometry and was typically around 95% (less than 1% T cells). NK cells were cultured in AIM-V medium (Invitrogen), supplemented with 10% pooled human AB serum (Sanquin, Rotterdam, Netherlands), PS and glutamine (Glutamax I, Invitrogen). Activated NK cells were cultured with 10 ng/mL recombinant human interleukin-15 (IL-15) for three days or two weeks (Peprotech, Rocky Hill, NJ).

Flow cytometry

Surface staining of cells for flow cytometry was performed as described elsewhere [20]. Intracellular flow cytometry staining was done using permeabilization and fixation kits as per manufacturer's instructions (00-5123; 00-5223; 00-8333, eBioscience, San Diego, CA). An overview of antibodies can be found in Suppl. Table 5.4. Flow cytometry of PBMC of osteosarcoma patients

and healthy controls was performed on a BD LSRII and analyzed using FACS Diva Software 5.0 (both from Becton Dickinson, San Diego, CA). Mean fluorescence intensity (MFI) of cell subsets was calculated by subtracting the MFI of a negative population from the MFI of the population of interest within one individual to correct for interindividual variability of background staining. Flow cytometry of cell lines and purified NK cells was performed on a FACScalibur, and analyzed using Cellquest software (both Becton Dickinson). MFI ratio was calculated by MFI of the specific staining relative to the MFI of the appropriate isotype control staining.

Immunohistochemistry

Immunohistochemistry was performed on FFPE tissue array sections as previously described [183]. Testis was used as a positive control for the activating NK ligands and tonsil for the inhibitory ligands. Sections were blocked using 10% swine or goat serum in PBS and subsequently incubated with primary antibody diluted in 0.5% bovine serum albumin (BSA) in PBS overnight. As a negative control 0.5% BSA/PBS without primary antibody was used. All primary antibodies are listed in Suppl. Table 5.4. Anti-rabbit/rat/mouse PowerVision Poly-HRP (Leica Biosystems, Newcastle Upon Tyne, United Kingdom) was used as a secondary antibody, except for the MICA staining, in which case the Universal LSAB+ Kit (DAKO, Glostrup, Denmark) was used. DAB+ (DAKO) was used as a chromogen. Sections were counterstained using Mayer's hematoxylin. Tissue array images were acquired using the MIRAX slide scanner (3DHISTECH, Budapest, Hungary) and analyzed using the MIRAX viewer version 1.14 (3DHISTECH). Slides were scored by two observers (EPB and PCWH) in a modified semi-quantitative scoring system as proposed by Ruiter *et al* [225]. The intensity of staining was scored as 0, 1, 2 or 3 indicating absent, weak, clear or strong expression, respectively. Percentages of positive cells were scored as 0 for 0%, 1 for 1-30%, 2 for 31-70% and 3 for 71-100%.

Chromium release assays

Cytotoxicity was determined in standard four hour Chromium release assays. For experiments using PBMCs of OS patients and controls, PBMCs were thawed from storage in liquid nitrogen and allowed to recover for 16 h in RPMI 1640 supplemented with 10% FCS and PS. The E:T ratios in these experiments were corrected for the percentage of NK cells of PBMCs as determined by flow cytometry. For all other experiments, purified unstimulated or IL-15 activated NK cells were used as effector cells. Target cells (cell lines or primary cultures) were incubated with 100 µCi sodium-51-chromate (PerkinElmer, Wellesley, MA) for 1 h. Effector cells (PBMCs, unstimulated purified NK cells or activated NK cells) were incubated for 4 h with 2500 target cells at eight effector:target (E:T) ratios in triplicate. Maximum and spontaneous release was determined by incubating targets in 2N HCl or medium, respectively. Supernatants were harvested and measured in a gamma-counter (Wallac, PerkinElmer). Specific lysis was determined as: (experimental release-spontaneous release)/(maximum release-spontaneous release) × 100%. In all NK cytotoxicity experiments, K562 and EBV were used as positive and negative controls, respectively. For blocking experiments, NK cells preincubated with blocking anti-NKG2D (R&D systems, clone 149810) and/or blocking anti-DNAM-1 (BD Pharmingen, clone DX11) at a concentration of 20 µg/mL. To disrupt perforin/granzyme mediated cytolysis, NK cells were preincubated for 2 h at 37°C with or without

1 μM Concanamycin A (Sigma-Aldrich, Zwijndrecht, the Netherlands) prior to adding the NK cells to the target cells. To block Fas-induced apoptosis, target cells were preincubated with 2μg/mL neutralizing anti-Fas antibody (Clone ZB4, Millipore, Temecula, CA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (LaJolla, CA). Data with non-normal distribution or small sample size were analyzed using non-parametric methods (Mann-Whitney U, Kruskal-Wallis, Friedman and Dunns tests) and data with normal distribution were analyzed using parametric methods (t-tests, one way analysis of variance (ANOVA) and Bonferroni's tests). Survival analyses were performed using Kaplan-Meier curves and compared using the logrank method.

RESULTS

Osteosarcoma cells are highly susceptible to IL-15 activated allogeneic NK cells

We tested eight osteosarcoma cell lines for susceptibility to cytolytic activity of freshly isolated ('unstimulated') and IL-15 cultured ('activated') healthy donor derived NK cells. All cell lines were lysed by unstimulated allogeneic NK cells at levels comparable to the positive control cell line K562 (Fig. 5.1a and b). Cytolysis of all osteosarcoma cell lines was strongly enhanced when IL-15 cultured allogeneic NK cells were used.

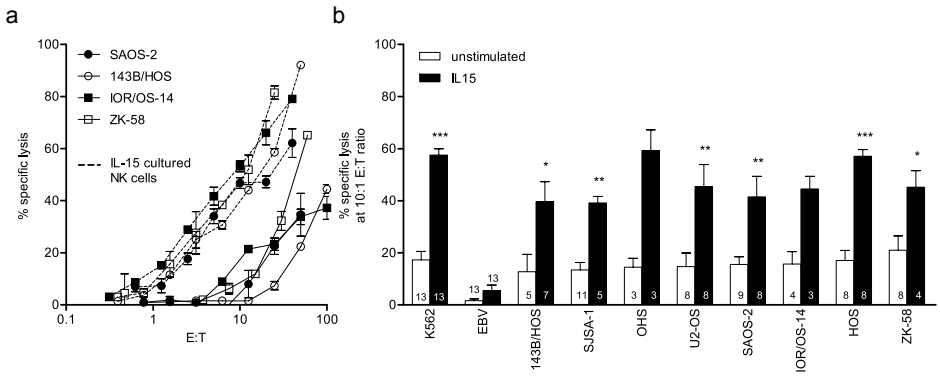

Fig. 5.1 Osteosarcoma cells were sensitive to lysis by freshly isolated NK cells (solid lines) and NK cells cultured in IL-15 for 2 weeks (dashed lines). **a**, examples of percentage of specific lysis are shown for the osteosarcoma cell lines SAOS-2 (●), 143B/HOS (○), IOR/OS-14 (■) and ZK-58 (□). Cell lines were incubated with increasing numbers of NK cells (E:T; effector to target ratio). Error bars represent standard error of the mean lysis of a representative experiment performed in triplicate. **b**, mean percentage of specific lysis by unstimulated (white bars) and IL-15 activated (black bars) NK cells of the osteosarcoma cell lines 143B/HOS, SJSA-1, OHS, U2-OS, SAOS-2, IOR/OS-14, HOS and ZK-58 at an effector to target ratio of 10:1. Error bars represent standard error of the mean of independent experiments using different healthy donor NK cells. Numbers in the bars represent number of experiments. K562 and an EBV transformed B-LCL ("EBV") were used as positive and negative controls, respectively. Mann-Whitney-U test was done comparing IL-15 activated NK cells with unstimulated NK cells for each cell line; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

Osteosarcoma cells express inhibitory and activating NK cell ligands

Osteosarcoma cells expressed activating NK cell ligands and HLA class I, both *in vivo* and *in vitro* (Table 5.1 and Fig. 5.2). All osteosarcoma cell lines expressed HLA class I, at least 3/5 NKG2D ligands and both DNAM-1 ligands. Expression of ligands *in vivo* was determined on the tissue array containing 144 samples of 88 patients. In chemotherapy-naive tumor material MICA, DNAM-1 ligands and HLA class I were also expressed, albeit at different levels (Fig. 5.2a). In tumor cells persisting after chemotherapy, levels of MICA, HLA class I and β-2 microglobulin expression were unaltered but the expression levels of the DNAM-1 ligands CD112 and CD155 were significantly decreased (Fig. 5.2b). There was a trend for patients with high (score > 4) vs. low (score </= 4) expression of MICA in pre-treatment diagnostic biopsies to have better overall survival (n=53, *P*-value logrank test = 0.07). Expression level of HLA class I in diagnostic biopsies as determined by staining with antibodies recognizing β-2 microglobulin, HLA-A and HLA-B/C did not correlate with tumor progression.

NK cells lyse osteosarcoma cells in a DNAM-1 and NKG2D dependent manner

NK cell-mediated cytolysis of osteosarcoma cells was dependent on NKG2D and DNAM-1 pathways, as was demonstrated by blocking DNAM-1, NKG2D or both receptors (Fig. 5.3). In resting NK cells the DNAM-1 pathway appeared to predominate the cytolytic potential, whereas the contribution of the NKG2D pathway was more prominent in the cytolytic activity of IL-15 cultured NK cells. In case of IL-15 cultured NK cells, blockade of both pathways was required for optimal inhibition of NK cytolytic activity. Levels of expression of HLA class I did not correlate with magnitude of lysis by unstimulated or IL-15 activated NK cells. Similarly, levels of expression of ligands for the activating receptors NKG2D or DNAM-1 did not correlate with degree of lysis by NK cells (data not shown).

Chemotherapy resistant osteosarcoma cells remain sensitive to lysis by IL-15 activated NK cells

To study whether chemotherapy resistant cell lines have become resistant to NK cell mediated lysis as well, the sensitivity of a panel of chemotherapy resistant variants of the osteosarcoma cell lines SAOS-2 and U2-OS (selected *in vitro* to be resistant to DX, CDDP or MTX) to lysis by NK cells was tested (Fig. 5.4a). Although some SAOS variants, *e.g.* CDDP, were less sensitive to lysis by resting NK cells, activation of NK cells with IL-15 greatly enhanced lysis of all U2-OS and SAOS-2 chemotherapy resistant variant cell lines (Fig. 5.4a). Expression levels of NKG2D and DNAM-1 ligands were similar in chemotherapy resistant variants and parental cell lines, as was the dependency on NKG2D and DNAM-1 signaling in cytotoxicity assays (Table 5.1 and Suppl. Fig. 5.1). Expression levels of HLA class I and of the adhesion molecules ICAM-1 and LFA-3 were unaltered in the chemotherapy resistant variants, but expression of CD95 (death receptor Fas) was lost in the SAOS-2 CDDP and DX-resistant variants (Table 5.1 and Fig. 5.4b). Since the loss of CD95 could provide an explanation for reduced susceptibility to NK cell induced lysis, we performed blocking experiments in which both CD95 and the granule exocytosis pathway were blocked with a blocking antibody and Concanamycin A, respectively. These experiments were performed using IL-15 activated NK cells at an effector to target ratio of 40 to 1. Blocking the

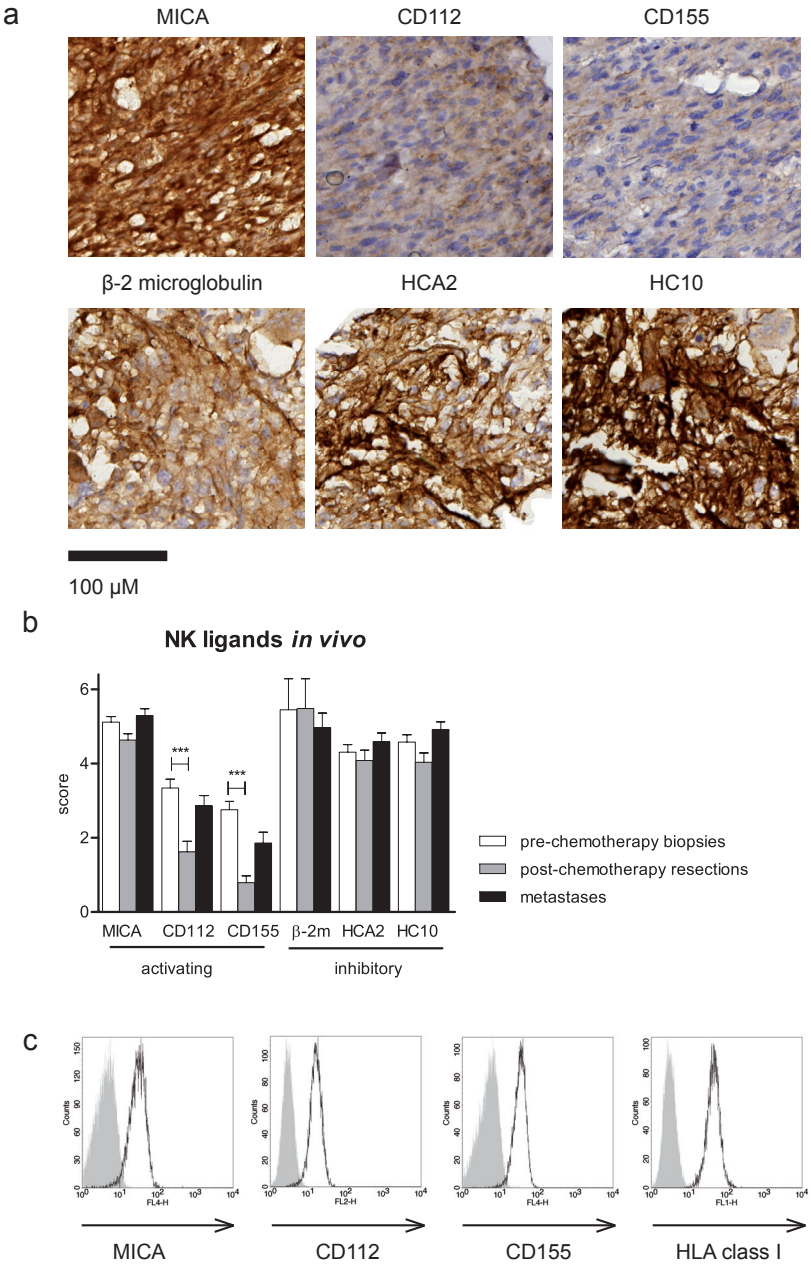

Fig. 5.2 **a**, representative example of immunohistochemical staining of ligands for the activating receptors NKG2D (MICA) and DNAM-1 (CD112 and CD155) and of β2-microglobulin, HLA-A (HCA2) and HLA-B/C (HC10) on an osteosarcoma sample. **b**, overview of the results of immunohistochemical stainings on pre-chemotherapy and post-chemotherapy samples of the primary tumor as well as metastatic osteosarcoma tissue. Expression levels of CD112 and CD155 but not the other ligands decreased significantly upon chemotherapy treatment (*P*-value Kruskal-Wallis test <0.001=***). **c**, example of flow cytometry plots for MICA, CD112, CD155 and HLA class I for the osteosarcoma cell line IOR/OS-14; isotype matched control staining is shown in grey

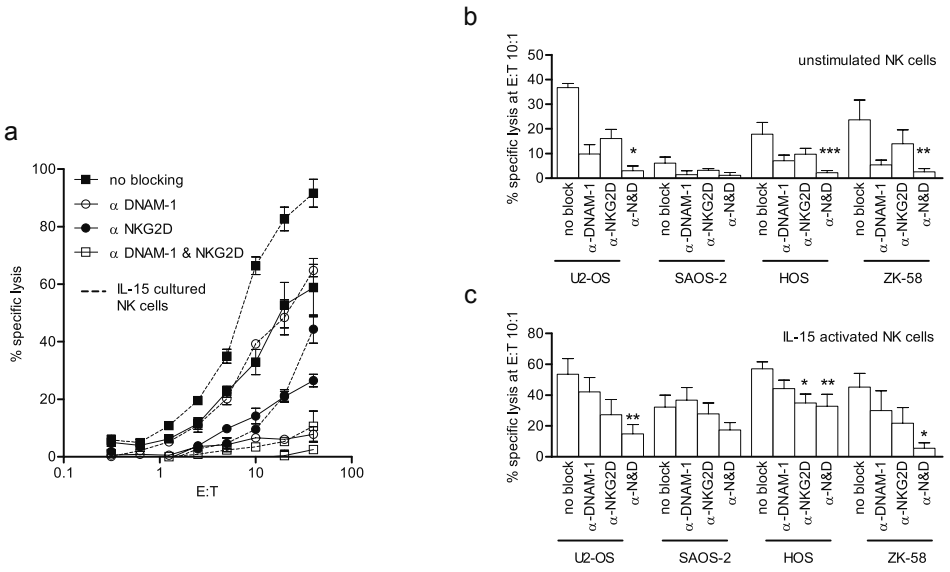

Fig. 5.3 **a**, cytolysis of U2-OS by unstimulated (solid lines) and IL-15 activated (dashed lines) NK cells was almost completely abrogated when the NK cells were pre-incubated with both anti (α)-DNAM-1 and α-NKG2D blocking antibodies (■ *vs.* □). Unstimulated NK cells were most dependent on DNAM-1 (○) signaling, whereas activated NK cells were most dependent on NKG2D (●). Error bars represent standard error of the mean lysis of experiment performed in triplicate. Similar results were obtained for SAOS-2, HOS and ZK-58 using unstimulated (**b**) and IL-15 activated NK cells (**c**). Bars represent mean lysis in at least three independent experiments using healthy donor NK cells; error bars represent standard error of the mean. Friedman test, Dunns post test compared to non-blocked; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

GrB but not the CD95 cytolytic pathway, almost completely abrogated NK cytolytic potential, demonstrating the predominance of the GranzymeB pathway in NK cell mediated lysis of parental as well as CDDP resistant variants of osteosarcoma cells (Fig. 5.4c). Similar results were obtained when the U2-OS parental cell line was used (data not shown).

Peripheral blood NK cell phenotype is unaltered and cytolytic potential is unimpaired in newly diagnosed osteosarcoma patients

Since peripheral NK cells in patients with other tumor types show altered phenotype and function, we analyzed PBMCs of 22 newly diagnosed osteosarcoma patients and 23 age-matched healthy controls by flow cytometry for NK cell number and phenotype (Suppl. Fig. 5.2a). NK cell number and phenotype were comparable between patients and controls (Fig. 5.5a and Suppl. Fig. 5.2b and c). Following three days of culture in IL-15, there was a larger increase in NKG2D and granzyme B expression levels on both CD56dim and bright NK cells of osteosarcoma patients than of healthy controls (Fig. 5.5a and Suppl. Fig. 5.2c). We assessed the functionality of NK cells of osteosarcoma patients at diagnosis in cytotoxicity assays using unstimulated and 3 days IL-15 activated PBMCs as effector cells. Resting NK cells from osteosarcoma patients and healthy donors lysed the allogeneic target HOS equally well (Fig. 5.5b), but IL-15 cultured NK cells of patients lysed HOS significantly

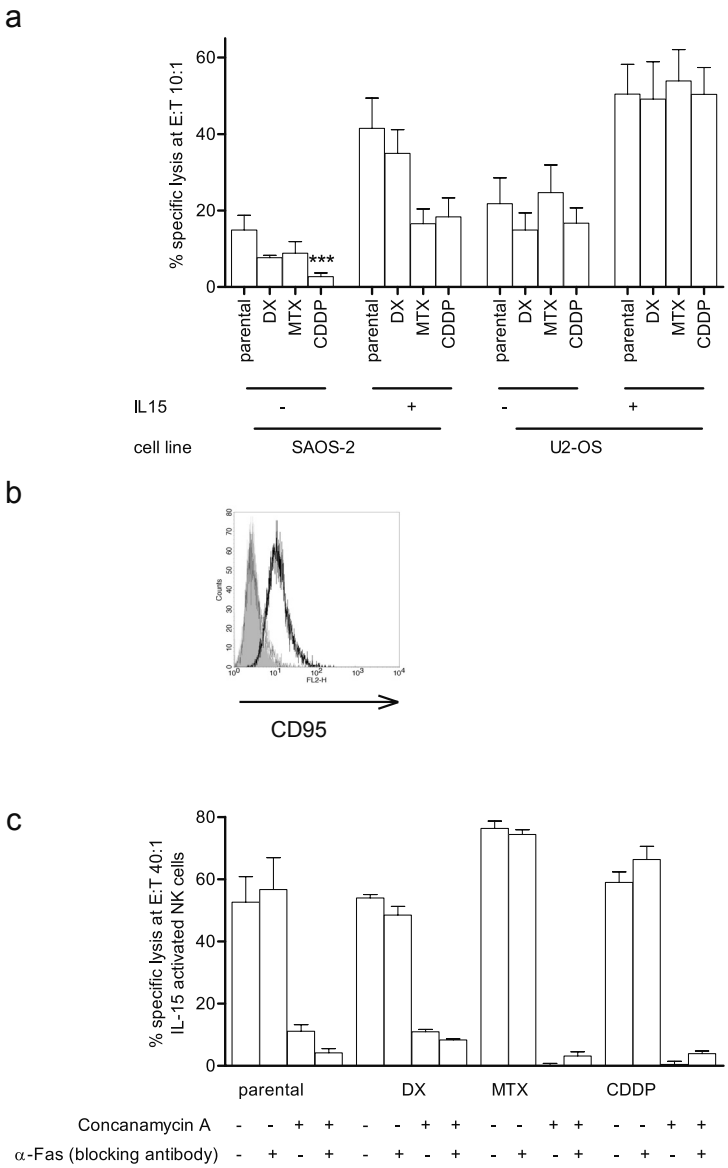

Fig. 5.4 **a**, lysis of U2-OS variants resistant to doxorubicin (DX), methotrexate (MTX) or cisplatinum (CDDP) was comparable to lysis of the parental control. The SAOS-2 CDDP resistant line was less sensitive to lysis by unstimulated NK cells than its parental control (Friedman test *P*-value = 0.001 and Dunns post test compared to parental cell line *P*-value <0.001). NK cell activation ("IL-15 +") increased lysis in all cases. Error bars represent standard error of the mean lysis in at least six independent experiments. **b**, SAOS-2 expressed the death receptor CD95 (Fas; black line) whereas expression was lost in the cisplatinum resistant variant of SAOS-2 (dashed line). Isotype matched control staining is shown in grey. **c**, the granzyme/perforin pathway was the main cytotoxic pathway by which NK cells lysed SAOS-2 and its chemotherapy resistant variants, as shown by inhibition of granule exocytosis by concanamycin A. Blocking Fas resulted in a further decrease in lysis of SAOS-2, but not of the chemotherapy resistant variants. Bars represent mean lysis of experiment performed in triplicate; error bars represent standard error of the mean. Similar results were obtained using NK cells from another donor

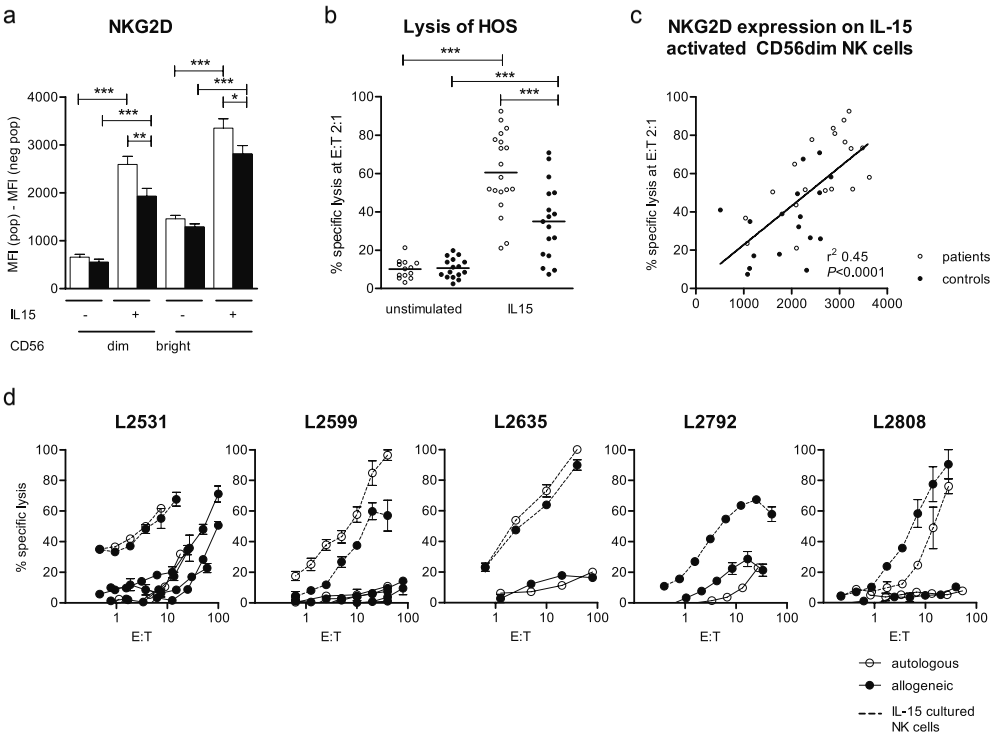

Fig. 5.5 **a**, NKG2D level was similar in unstimulated NK cells of newly diagnosed osteosarcoma patients and healthy controls. Following culture for 3 days in IL15, there was a larger increase in expression level of NKG2Din NK cells of patients. **b**, unstimulated PBMCs of 12/22 patients and 16/23 healthy controls and IL-15 activated PBMCs of 19/22 patients and 17/23 controls were available for functional testing. NK cells of newly diagnosed osteosarcoma patients and of healthy controls lysed HOS at similar levels. Following IL-15 activation, NK cells of osteosarcoma patients showed a larger increase in cytotoxic activity than NK cells of healthy donors. **c**, cytotoxicity of IL-15 activated NK cells correlated with level of NKG2D expression on the CD56dim subset (Pearson correlation coefficient). **d**, primary osteosarcoma cell cultures were tested for sensitivity to lysis by autologous (○) and allogeneic (●) NK cells. NK cells were unstimulated (solid lines) or 3 days IL-15 activated (dashed lines). Autologous IL-15 activated NK cells were available for all patients except L2792. One way analysis of variance (ANOVA) *P*-value <0.0001 for a, b, and c. Bonferroni's multiple comparison post-test; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

better than healthy donor NK cells (ANOVA, Bonferroni's post-test *P*-value <0.0001). Percentage specific lysis of HOS correlated with the level of NKG2D expression on CD56 dim NK cells (Fig. 5.5c; Pearson correlation efficient r^2 0.45, *P*-value<0.0001) and similar results were obtained for the correlation with Granzyme B expression (not shown). To test whether the functional integrity of NK cells from osteosarcoma patients was also preserved towards autologous tumor cells, we took advantage of the fact that we were able to derive short-term cultured cells from fresh biopsies. Autologous, patient derived NK cells lysed short-term cultured tumor cells to a similar degree as allogeneic NK cells from healthy controls (Fig. 5.5d). In all cases, culture of both autologous and allogeneic NK cells in IL-15 resulted in greatly enhanced tumor cell killing.

DISCUSSION

There is increasing interest in the potential for NK cells to be used in the treatment of pediatric solid tumors [41]. Previous studies have shown that osteosarcoma cell lines may be sensitive to cytokine activated NK cell mediated cytotoxicity [104;132;143;156-159;165;191]. However, little is known about the mechanisms involved or the extent to which short-term cultured or chemotherapy resistant osteosarcoma cells are susceptible to NK cell mediated lysis. In addition, there is some evidence for NK cell mediated anti-osteosarcoma activity *in vivo*. Post-operative osteomyelitis-associated inhibition of tumor growth was dependent on the activation of monocytes and NK cells in a murine osteosarcoma model [242]. In human osteosarcoma, treatment with interleukin (IL)-2 in a small cohort of patients resulted in NK cell activation which was correlated with better clinical outcome [151]. Together, these studies suggest that exploitation of NK cell activity may be a suitable therapeutic tool in the adjuvant treatment of osteosarcoma. In the present study, we demonstrate that osteosarcoma cells are highly susceptible to NK cell mediated cytolysis (Fig. 5.1). Osteosarcoma cells expressed activating NKG2D and DNAM-1 ligands *in vivo* as well as *in vitro* and lysis was dependent on the interaction between these ligands on osteosarcoma cells and their receptors on NK cells (Figs. 5.2 and 5.3). Despite expression of the potentially inhibitory KIR ligand HLA class I by osteosarcoma cells, all cell lines and short-term cultures were highly sensitive to lysis by IL-15 activated NK cells (Table 5.1 and Figs. 5.1 and 5.5d). Together, these data suggest that the balance between expression of activating and inhibitory ligands in osteosarcoma is shifted towards activation.

To investigate if NK cell based immunotherapy is also feasible for patients with chemotherapy resistant disease, we tested the susceptibility of *in vitro* selected chemotherapy resistant osteosarcoma cells to NK cell mediated lysis. Methotrexate (MTX), doxorubicin (DX) or cisplatin (CDDP) resistant variants of the cell lines SAOS-2 and U2-OS remained sensitive to lysis by IL-15 activated NK cells (Fig. 5.4a). NK cells kill their targets by the release of perforin and granzyme containing granules and by the ligation of death receptors such as CD95 (Fas) [44;234]. Expression of CD95 was lost in the CDDP and DX resistant SAOS-2 variants (Table 5.1 and Fig. 5.4), but dual blocking studies demonstrated only a minor role for Fas ligation in the lysis of osteosarcoma by cytokine activated NK cells. Expression of Fas is frequently lost in osteosarcoma pulmonary metastases, but our data shows that this will probably not hinder NK cell based immunotherapeutic approaches [86;87;130].

Studies on the feasibility of immunotherapeutic strategies in bone tumors are often hampered by the technical difficulty to isolate viable fresh tumor cells for functional testing. To circumvent this problem, we used short-term cultured cells. Susceptibility to NK cell mediated lysis was determined no later than at passage three. Four out of five cultures originated from patients with chemotherapy resistant disease (L2531, L2792, L2599 and L2808). Still, all were highly sensitive to lysis by cytokine activated autologous and allogeneic NK cells (Fig. 5.5d). Importantly, our experiments on *in vitro* selected chemotherapy resistant cells and on short-term cell cultures generated from patients with chemotherapy resistant disease *in vivo* show that IL-15 activated NK cells are capable of lysis of osteosarcoma cells resistant to chemotherapeutic agents commonly used in high-grade osteosarcoma treatment.

In many tumor types, including Ewing sarcoma, host immune cells have decreased functionality as compared to healthy donor cells [80;128;272]. In these cases, using allogeneic immune cells instead of autologous cells is an attractive option to increase efficacy. However, it also increases the risk of serious complications such as graft-versus-host-disease. Our data shows that NK cells of osteosarcoma patients are as potent as NK cells of healthy controls in lysing osteosarcoma cells. Remarkably, upon activation with IL-15, patient derived NK cells even showed a larger increase in expression of NKG2D and GranzymeB than healthy donor derived NK cells, which correlated with an increased lysis of the osteosarcoma cell line HOS (Fig. 5.5a and Suppl. Fig. 5.2c). This, and the lysis of autologous tumor cells by *ex vivo* IL-15 activated NK cells, indicates that immunotherapeutic strategies employing activated autologous NK cells could be as successful as allogeneic NK cells in the treatment of osteosarcoma. In preclinical validation studies we obtained evidence that IL15 and IL2 stimulated NK cells have similar cytolytic activity against various tumor cell lines [268]

In conclusion, chemotherapy resistant and sensitive osteosarcoma cells were lysed at high levels by NK cells, particularly when NK cells were cytokine activated. Lysis of osteosarcoma cells was dependent on DNAM-1 and NKG2D, ligands of which were expressed by osteosarcoma cells both *in vivo* and *in vitro*. In contrast to what has been reported in patients with other tumor types, there was no intrinsic functional NK cell defect which could hamper anti-tumor activity. Our study shows a potential benefit of either activating NK cells *in vivo* by the administration of cytokines or adoptive transfer of *ex vivo* activated autologous or allogeneic NK cells in the treatment of high-grade osteosarcoma.

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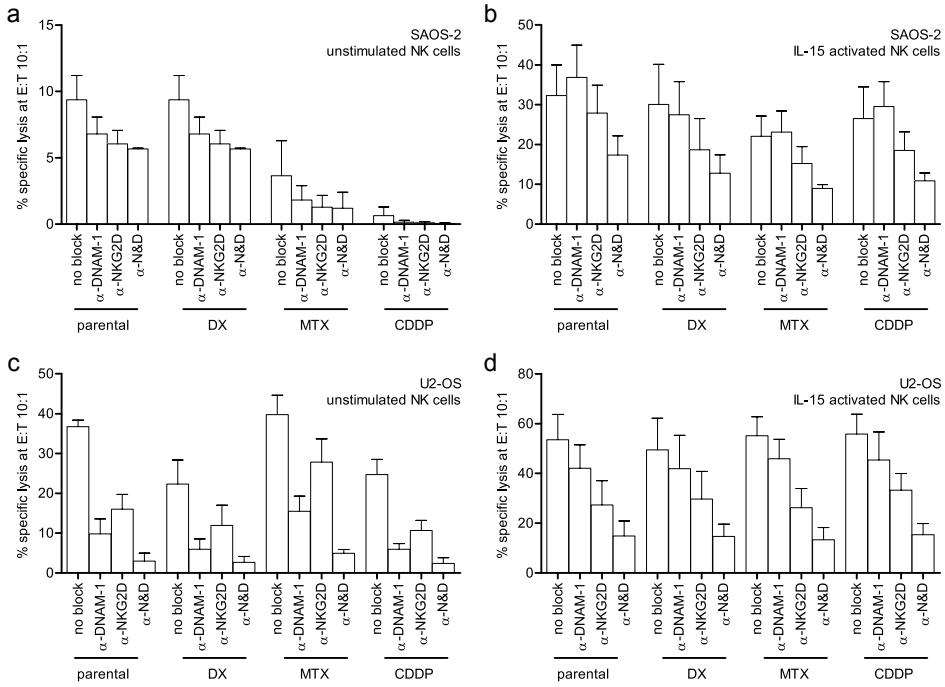
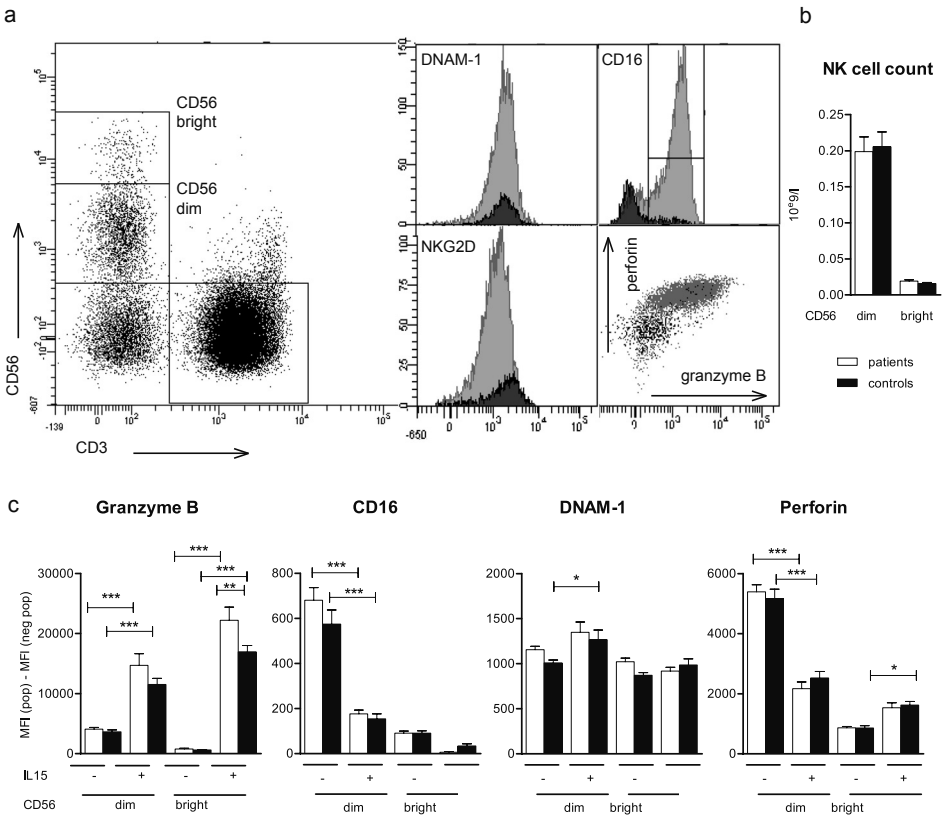


Suppl. Fig. 5.1 The relative contribution of NKG2D and DNAM-1 ligands to the lysis of SAOS-2 and its chemotherapy resistant variants was similar, as demonstrated by blocking DNAM-1, NKG2D or both receptors on unstimulated (**a**) and IL-15 activated (**b**) NK cells. Similar results were obtained in U2-OS (**c** and **d**). Error bars represent standard error of the mean lysis in at least three independent experiments using healthy donor NK cells. DX; doxorubicin resistant variant, MTX; methotrexate resistant variant, CDDP; cisplatinum resistant variant.



Suppl. Fig. 5.2 **a**, representative example of flow cytometry results of unstimulated peripheral NK cell compartment. The CD3 CD56 plot was gated on lymphocytes based on forward and sideward scatter. Monocytes were gated out by excluding CD14 stained cells (not shown). NK cells were defined as CD3-CD56+ cells and were divided in a CD56 bright and a CD56 dim subset. Expression of NKG2D, DNAM-1, CD16, perforin and granzyme B was determined for CD56 bright (black histogram and dots) and CD56 dim (grey histogram and dots) subsets. **b**, patients and controls had similar numbers of NK cells. **c**, following culture for 3 days in IL15, there was a larger increase in expression level of Granzyme B in NK cells of patients than of healthy controls. Patients and controls had similar levels of CD16, DNAM-1 and perforin on NK cells. In both patients and healthy controls, levels of CD16 decreased in CD56dim and bright NK cells and levels of perforin increased in CD56bright and decreased in CD56dim NK cells upon IL-15 treatment. ANOVA, *P*-value <0.0001. Bonferroni's multiple comparison post-test; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***.

Suppl. Table 5.1 Composition of osteosarcoma tissue array

Number of samples (of nr of patients)	Type of sample
73 (73)	Pre-treatment samples of the primary tumor
45 (45)	Post-treatment samples of the primary tumor
20 (13)	Lung metastases
3 (3)	Other metastases (2 bone and one lymph node metastasis)
3 (3)	Local relapses
144 (88)	Total samples

Suppl. Table 5.2 Clinicopathological details of patients in this study

		Peripheral blood NK cell analysis	
	Tissue array	**Osteosarcoma patients**	**Healthy donors**
Total nr of patients	88	22	23
Age in years: median (range)	15.4 (4-44)	16 (9-56)	20 (8-55)
Sex	n (%)	n (%)	n (%)
Male	47 (53.4%)	10 (45.5%)	11 (47.8%)
Female	41 (46.6%)	12 (54.5%)	12 (52.2%)
Location of primary tumor			
Distal femur	40 (45.5%)	11 (50%)	
Proximal tibia/fibula	27 (30.7%)	4 (18.2%)	
Proximal humerus	10 (11.4%)	4 (18.2%)	
Other long bones	9 (10.2%)	3 (13.6%)	
Axial skeleton	1 (1.1%)	0	
Hand	1 (1.1%)	0	
Histological subtype			
Conventional osteosarcoma	71 (80.7%)	18 (81.8%)	
Osteoblastic	59	12	
Chondroblastic	9	3	
Fibroblastic	3	1	
Unusual	8	2	
Telangiectatic	6 (6.8%)	4 (18.2%)	
High-grade surface osteosarcoma	1 (1.1%)	0	
Small cell osteosarcoma	2 (2.3%)	0	
Date of diagnosis:	Jan 1981- Sep 2003	Feb 2007 - Apr 2008	
Treated according to protocols:	EORTC 80831, 80861 and 80931	Euramos-1	

Suppl. Table 5.3 Clinicopathological details of primary cultures

Primary culture	**Origin of primary culture**	**Sex**	**Age (y)**	**Histological subtype of osteosarcoma**	**Location of primary tumor**
L2531	Local relapse	Male	17	Osteoblastic	Distal femur
L2808	Lung metastasis	Male	18	Osteoblastic	Distal femur
L2792	Local relapse	Female	31	Fibroblastic	Proximal humerus
L2599	Diagnostic biopsy	Male	13	Osteoblastic	Distal femur
L2635	Diagnostic biopsy	Female	14	Osteoblastic-sclerosing type	Distal femur

Suppl. Table 5.4 Antibodies used for immunohistochemistry (IHC) and flow cytometry (FC)

Antibody	Catalog/ clone nr	Company	Application
b2-microglobulin	A0072	DAKO (Glostrup, Denmark)	IHC, antigen retrieval (AR): Citrate, 0.01 M, pH 6
HLA-A	HCA-2	Kindly provided by J.Neefjes (NKI, Amsterdam, the Netherlands)	IHC, AR: Citrate, 0.01 M, pH 6
HLA B/C	HC10	Kindly provided by J.Neefjes	IHC, AR: Citrate, 0.01 M, pH 6
MICA	AF1300	R&D systems (Minneapolis, MN)	IHC, AR: Citrate, 0.01 M, pH 6
CD155	HPA012568	Sigma Aldrich (Zwijdrecht, the Netherlands)	IHC, AR: EDTA, 1 mM, pH 8
ULBP-1	HPA007547	Sigma Aldrich	IHC, AR: Citrate, 0.01 M, pH 6
CD112	HPA012759	Sigma Aldrich	IHC, AR: EDTA, 1 mM, pH 8
MICA	MAB1300	R&D systems	FC, cell lines
MICB	FAB1599A	R&D systems	FC, cell lines
ULBP-1	IC1380P	R&D systems	FC, cell lines
ULBP-2	FAB1298A	R&D systems	FC, cell lines
ULBP-3	MAB1517	R&D systems	FC, cell lines
CD48-PE	IM1837U	Beckman Coulter Immunotech (Marseille, France)	FC, cell lines
CD155	IM2755	Beckman Coulter Immunotech	FC, cell lines
CD112-PE	IM3452	Beckman Coulter Immunotech	FC, cell lines
HLA-A/B/C-FITC	555552	BD Pharmingen (San Diego, CA)	FC, cell lines
CD54-PE	555511	BD Pharmingen	FC, cell lines
CD58-PE	555921	BD Pharmingen	FC, cell lines
CD95-PE	340480	BD Pharmingen	FC, cell lines
goat anti-mouse APC	550826	BD Pharmingen	FC, secondary antibody
mIgG1-FITC	639	Beckman Coulter Immunotech	FC, isotype control
mIgG2b	X0944	DAKO	FC, isotype control
mIgG2a	MAB0031	R&D	FC, isotype control
mIgG1-PE	349053	BD Pharmingen	FC, isotype control
mIgG1	MAB002	R&D	FC, isotype control
mIgG2a-PE	349053	BD Pharmingen	FC, isotype control
CD3-PerCPCy5.5	332771	BD Pharmingen	FC, purity of isolated NKs
CD20-FITC	345792	BD Pharmingen	FC, purity of isolated NKs
CD14-APC	340436	BD Pharmingen	FC, purity of isolated NKs
CD56-PE	R7251	DAKO	FC, purity of isolated NKs
CD3-Pacific Blue	558117	BD Pharmingen	FC, PBMCs
CD14-PerCPCy5.5	550787	BD Pharmingen	FC, PBMCs
DNAM-PE	559789	BD Pharmingen	FC, PBMCs
GranzymeB-Alexa700	557971	BD Pharmingen	FC, PBMCs
NKG2D-APC	558071	BD Pharmingen	FC, PBMCs
CD56-PECy7	A21692	Beckman Coulter Immunotech	FC, PBMCs
CD16-FITC	IM0814	Beckman Coulter Immunotech	FC, PBMCs
Perforin-FITC	358-040	Hölzel Diagnostika, Cologne, Germany	FC, PBMCs