Expression of the Immune Checkpoint Modulator OX40 in Acute Lymphoblastic Leukemia Is Associated with BCR-ABL Positivity

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Abstract

OX40 and its ligand are members of the TNF/TNF receptor superfamily, which includes various molecules influencing cellular signaling and function of both tumor and immune cells. The ability of OX40 to promote proliferation and differentiation of activated T cells fueled present attempts to modulate this immune checkpoint to reinforce antitumor immunity. While we recently found evidence for the involvement of OX40 in pathophysiology of acute myeloid leukemia including natural killer (NK) cell immunosurveillance, less is known on its role in acute lymphoblastic leukemia (ALL). In the present study, OX40 expression on ALL cells was significantly associated with positivity for the adverse risk factor BCR-ABL. In line, signaling via OX40 increased metabolic activity of primary ALL cells and resulted in release of cytokines involved in disease pathophysiology. Furthermore, interaction of ALL-expressed OX40 with its cognate ligand on NK cells stimulated ALL cell lysis. The data presented thus not only identify the yet unknown involvement of OX40/OX40L in ALL pathophysiology and NK cell immunosurveillance but also point to the necessity to thoroughly consider the consequences of modulating the OX40/OX40L molecule system beyond its effects on T cells when developing OX40-targeting approaches for cancer immunotherapy.

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Introduction

Immune checkpoint therapy has become a pillar of cancer treatment [1,2]. The first three approved checkpoint antibodies ipilimumab, nivolumab, and pembrolizumab represent a novel strategy for the treatment of a multitude of malignancies by targeting inhibitory pathways that prevent effective antitumor T cell responses [3–7]. Therapeutic concepts for checkpoint modulation utilizing antibodies providing an agonistic signal via activating receptors on T cells are less advanced and presently under investigation. One of the targets is OX40 (CD134), a member of the tumor necrosis factor receptor (TNFR) superfamily [8–10]. This costimulatory molecule is upregulated on effector T cells after activation and supports differentiation, proliferation, and long-term survival. In addition, it

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OX40 in ALL is Associated with BCR-ABL Positivity

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OX40 mediates inhibition of the suppressive activity of regulatory T cells [11], which contribute to evasion of tumor cells from T cell immunity. In line, the frequency of tumor-infiltrating OX40-positive T cells has been reported to correlate with patient survival [12,13]. Application of OX40 agonists, alone or in combination with other checkpoint modulators, stimulated the cytotoxic activity of T cells and caused tumor regression in preclinical models [14–19]. First evidence from early clinical trials also indicates that OX40 stimulation could be effective in cancer patients [20]. A multitude of clinical trials targeting OX40 as monotherapy or in combination with vaccination, radiotherapy, checkpoint blockade, chemotherapy, or targeted therapy are currently ongoing (for review, see [10]). Notably, OX40 was also found to be expressed by T cell–derived leukemic cells and in acute myeloid leukemia (AML). Its counterpart OX40 ligand (OX40L) is upregulated on natural killer (NK) cells following activation and stimulates their reactivity via reverse signaling into the ligand-bearing cells, while forward signaling into AML cells stimulated cellular functions of the leukemic cells [21,22]. So far, less is known regarding the OX40/OX40L system in acute lymphoblastic leukemia (ALL) of B cell lineage and its functional role in ALL cells. Here we report that primary ALL cells and cell lines partially express OX40 and that OX40 surface expression is significantly associated with BCR-ABL status, which constitutes a powerful predictor of treatment outcome and prognosis in ALL. We further show that OX40 stimulation promotes metabolic activity of ALL cells and results in release of cytokines like tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-8 that influence growth and survival of the malignant cells. In line with the stimulatory role of OX40L in NK cells, we further demonstrate that disruption of OX40/OX40L interaction impairs NK cell reactivity against OX40-positive ALL cell lines and provide data on the poor prognostic relevance of OX40 expression.

Material and Methods

ALL Cell Lines

The human ALL cell lines JURKAT, NALM-16, REH, SD-1, SUP-B15, and TOM-1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell lines were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 1% penicillin/streptomycin (Lonza, Basel, Switzerland) and 10% fetal calf serum (FCS, Biochrom) (JURKAT, NALM-16, SD-1, and TOM-1) or 20% FCS (REH). SUP-B15 cells were cultured in IMDM medium (GIBCO, Carlsbad, CA) with 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza), 1% nonessential amino acids (Lonza), 1% sodium pyruvate (Sigma Aldrich, St. Louis, MO), and 10% FCS. Cells were kept in a humidified atmosphere at 37°C and 5% CO₂.

Mycoplasma contamination was excluded by routine testing of cell lines every 3 months. Cell lines were authenticated by single nucleotide profiling.

Patients

Peripheral blood samples of ALL patients were obtained after written informed consent at the University of Tübingen. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll/Paque (Biochrom) density gradient centrifugation and viably stored in liquid nitrogen until analysis. This study was approved by the institutional review board to be in accordance with the ethical standards and the Declaration of Helsinki. Diagnosis of precursor B cell and T cell ALL was confirmed by morphologic analysis, immunophenotyping, and genetic features.

Reagents

For flow cytometry, the following unconjugated monoclonal antibodies were used: OX40 BerAct35, OX40L ANCl0G1 (cat. no. 355-820 and 400-820, both Ancell Corporation, Bayport, MN), and mouse IgG1 isotype control (cat. no. 557273, BD Biosciences, Heidelberg, Germany). Secondary PE goat anti-mouse was from Dako (cat. no. R0480, BIORZOL, Eching, Germany). Fluorescence conjugates were from BD Biosciences (PE-Cy5 mouse anti-human CD3, cat. no. 555334; APC mouse anti-human CD5, cat. no. 555355; APC mouse anti-human CD34, cat. no. 555824; FITC mouse anti-human CD19, cat. no. 555412; FITC mouse anti-human CD34, cat. no. 555821; FITC mouse anti-human CD56, cat. no. 345811), and BioLegend (San Diego, CA) (PE-Cy7 mouse anti-human CD10, cat. no. 312213; FITC mouse anti-human CD7, cat. no. 343104; FITC mouse anti-human CD15, cat. no. 301904) or eBioscience (ThermoFisher Scientific, Waltham, MA) (APC-cFlour780 mouse anti-human CD19, cat. no. 47-0199-42). 7-Aminoactinomycin D (7-AAD, cat. no. 559925) was from BD Biosciences. For stimulation of OX40 on ALL cells, the agonistic antibody M-OX17 was used [22]. To disrupt OX40/OX40L interactions in cytotoxicity assays, F(ab')₂ fragments of the blocking OX40 antibody M-OX2 were applied [23]. Mouse F(ab')₂ IgG1 (cat. no. 0115-14, SouthernBiotech, Birmingham, AL) served as control. Recombinant human IL-2 for NK cell generation was obtained from ImmunoTools (Friesoythe, Germany).

Flow Cytometry

Cells were stained with the respective unconjugated antibody or isotype control (10 μg/ml) followed by PE goat anti-mouse conjugate as secondary reagent (dilution 1:100). Leukemia cells within PBMC of ALL patients were selected according to their prespecified immunophenotype by counterstaining with CD10, CD15, CD19, or CD34 for precursor B cell ALL and with CD5 and CD7 for precursor T cell ALL (Supplementary Figure 1). Analysis was performed using a BD FACScanto II (BD Biosciences). Dead cells were excluded from analysis by 7-AAD staining (1:200). Specific fluorescence indices (SFIs) were calculated by dividing median fluorences obtained with specific monoclonal antibodies by median fluorences obtained with isotype control. Expression was considered positive in case of SFI ≥2.0. Data analysis was performed using FlowJo software (FlowJo LCC, Ashland, OR).

PCR

OX40 primers were 5′-TGTAAACCTCAAGTGGAATGTTG-3′ and 5′-GGTCCCTGTCTCACAGATTG-3′. GAPDH primers were 5′-AGCCACATCGCTCAGACAC-3′ and 5′-GCCCAATCTCC-3′. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously [24]. PCR products were visualized via gel electrophoresis (1.5% agarose gel).

For quantitative PCR, total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and transcribed into cDNA using qScript XLT cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer’s instructions. Amplification of OX40 cDNA was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) on a LightCycler 480 (Roche) instrument. Primer assays (QuantiTect Primer Assay, Qiagen, Hilden, Germany) for OX40 and GAPDH RNA were

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used according to the manufacturer's instructions. Relative mRNA expression was calculated by the ΔΔ cycle-threshold (Ct) method. BCR-ABL status was quantified with the ipsogen mbcr BCR-ABL1 mbcr CE Kit (Qiagen) according to the manufacturer’s instructions.

**Preparation of NK Cells**

Polyclonal NK cells were generated by culturing plastic non-adherent PBMC with irradiated K562-mb15-41BBL feeder cells provided from St. Jude Children’s Research Hospital as described previously [25–27]. Functional experiments were performed when purity of NK cells (CD56+CD3−) was above 80% as determined by flow cytometry.

**Determination of Cytokine Levels and Metabolic Activity**

For analysis of cytokine production, ALL cells were seeded at a concentration of 2 × 10^6 cells/ml followed by incubation at 37°C in a humidified atmosphere. Supernatants were collected and stored at −80°C until analysis for cytokine production by two-site sandwich ELISA using commercially available OptEIA kits from BD Pharmingen according to manufacturer’s instructions.

Effects of OX40 stimulation on metabolic activity were determined using the CellTiter-Glo Assay (Promega, Madison, WI). Therefore, ALL cells were seeded in triplicates at a concentration of 5 × 10^5 cells/ml in 100 μl culture medium followed by incubation at 37°C in a humidified atmosphere. Luminescence was analyzed according to manufacturer’s instructions.

**Cytotoxicity Assays**

Cytotoxicity of polyclonal NK cells against ALL cell lines was analyzed by BATDA Europium assays after 2 hours as described previously [28]. Percentage of lysis was calculated as follows: 100 × [(experimental release) − (spontaneous release)]/[(maximum release) − (spontaneous release)].

**Statistical Analysis**

For statistical analysis, GraphPad Prism 7 software (GraphPad, La Jolla, CA) was used. Mean or median values and standard deviation (SD) are shown. The 95% confidence level was used, and P values were calculated with a two-tailed unpaired Mann-Whitney test for not normally distributed data. Survival analyses were performed using SPSS version 24 (IBM, Ehningen, Germany). Overall survival (OS) was calculated using the Kaplan-Meier estimate and defined as the time from diagnosis to death from any cause. If no event occurred, data were censored, and the time from diagnosis or relapse until last patient contact was considered. Patient characteristics were analyzed using Mann-Whitney test, Kruskal-Wallis, or Spearman Rho tests.

**Figure 1.** mRNA and protein expression of OX40 in ALL cell lines. (A) OX40 surface expression of six different ALL cell lines was investigated by flow cytometry using the monoclonal OX40 antibody BerAct35 (shaded peaks) and mouse IgG1 isotype serving as control (open peaks). Representative results of at least three independent experiments with similar results are shown. (B) OX40 mRNA expression of six different ALL cell lines as determined by RT-PCR with GAPDH and H2O serving as controls. PCR products were visualized by agarose gel electrophoresis. (C) Quantitative RT-PCR analysis of OX40 mRNA relative to GAPDH expression in six different ALL cell lines. (D) For quantitative detection of BCR-ABL mbcr e1a2 transcripts relative to ABL control gene expression ALL cell lines were analyzed by quantitative RT-PCR.
### Results

**OX40 Is Expressed in BCR-ABL–Positive ALL Cell Lines**

OX40 is expressed in different solid tumors and hematological malignancies and was found in leukemic cells of myeloid and T cell origin [22,29], but less is known regarding its expression in precursor B cell ALL. As a first step, we analyzed expression of OX40 in different ALL cell lines. Flow cytometry revealed a variable extent of OX40 cell surface expression. Interestingly, only ALL cell lines carrying a minor breakpoint BCR-ABL fusion gene like SD-1, SUP-B15, and TOM-1 displayed an elevated OX40 expression, whereas the BCR-ABL–negative precursor B cell ALL cell lines NALM-16 and REH as well as the precursor T cell ALL cell line JURKAT showed no relevant surface expression (Figure 1A). Corresponding results were obtained when OX40 mRNA levels were investigated by PCR analysis (Figure 1B). While quantitative PCR revealed higher OX40 mRNA levels in surface-positive compared to surface-negative ALL cell lines, the presence of mRNA in the latter indicates that OX40 expression may also be regulated posttranscriptionally. Interestingly, surface expression

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**Table 1. Clinical Characteristics and OX40 Surface Expression**

| BCR-ABL | ALL type | Risk | Age | Sex | WBC [G/l] | Hb [g/dl] | Plt [G/l] | Karyotype | PB % | BM % | Relapse | SFI | OX40 % | OX40 + cells |
|---------|----------|------|-----|-----|-----------|-----------|----------|-----------|------|------|----------|-----|--------|--------------|
| pro B HR 63 m 39.97 10.1 125 46,XY | 96 | 96 | - | 1.3 | 3.6 |
| pro B HR 36 f 51.99 12.4 247 48,XX, +t(4;11)(q21;q23), **+der(4;11)(q21;q23),+22,46,XX** | 79 | 80 | - | 1.0 | 1.1 |
| common HR 21 m 28.27 11.7 280 NA | 92 | ND | - | 1.1 | 0.7 |
| common HR 31 f 14.23 5.4 80 NA | 68 | 79 | - | 2.9 | 60.3 |
| common HR 66 m 300.95 13.3 121 NA | 88 | ND | - | 1.0 | 0.8 |
| common HR 39 m 33.50 13.3 65 47,XY,+21 | 61 | 88 | - | 2.9 | 21.7 |
| common SR 20 f 11.50 7.1 33 46,XX | 60 | 91 | - | 1.4 | 1.7 |
| common SR 19 m 4.99 5.8 110 46,XY, **del(9)(p21p21),+22,46,XX** | 47 | 59 | - | 1.3 | 0.8 |
| common SR 61 m 7.87 8.5 21 46,XY | 54 | 44 | - | 1.4 | 1.5 |
| common SR 20 m 44.53 10.0 153 NA | 88 | ND | - | 7.5 | 86.2 |
| common SR 61 f 1.80 9.1 7 NA | 99 | ND | - | 1.1 | 1.4 |
| common SR 33 m 13.50 13.0 136 46,XY | 50 | 100 | - | 1.0 | 2.1 |
| pre B HR 61 f 123.81 8.0 40 NA | 98 | 97 | - | 1.3 | 1.4 |
| pre B HR 22 m 74.14 13.2 13 | 95 | 97 | - | 4.6 | 27.9 |
| pre B SR 41 m 11.67 9.1 36 46,XX | 80 | 50 | - | 1.6 | 22.3 |
| pre B SR 45 m 10.35 9.4 21 46,XX | 66 | 98 | - | 1.0 | 0.9 |
| cortical SR 20 f 29.22 10.3 35 46,XX | 86 | 97 | - | 1.2 | 0.9 |
| cortical SR 20 m 54.08 13.4 288 46,XY | 24 | 70 | - | 1.8 | 25.0 |
| cortical SR 20 m 98.62 11.3 76 NA | 63 | 65 | relapse | 1.0 | 11.2 |
| cortical SR 45 m 24.80 8.2 46 46,XY | 95 | 94 | - | 1.4 | 2.1 |
| cortical SR 69 m 37.71 10.7 119 46,XY | 70 | 72 | - | 1.1 | 1.4 |
| cortical SR 29 m 355.70 10.5 81 46,XY | 82 | 64 | relapse | 1.1 | 6.2 |
| cortical SR 32 m 93.31 12.5 57 46,XY | 93 | 95 | - | 2.3 | 8.8 |
| cortical SR 38 HR 34.24 15.0 204 46,XY | 84 | 93 | - | 12.1 | 48.7 |
| cortical SR 74 f 20.86 9.0 22 46,XX | 72 | 97 | - | 2.3 | 38.0 |
| cortical SR 64 m 30.01 13.6 141 46,XY | 57 | ND | - | 3.6 | 48.6 |
| cortical SR 81 f 114.17 8.8 35 46,XX | 97 | ND | - | 50.7 | 87.8 |
| cortical SR 43 m 38.18 13.8 48 46,XX | 65 | 97 | - | 5.7 | 48.0 |
| cortical SR 45 m 10.34 9.3 64 46,XX | 59 | 94 | - | 7.1 | 84.6 |
| cortical SR 25 m 4.69 4.8 15 46,XX | 60 | 81 | - | 2.4 | 21.0 |
| cortical SR 61 m 172.76 7.7 50 46,XX | 80 | ND | - | 14.2 | 45.7 |
| cortical SR 21 m 463.01 11.6 31 NA | 87 | 93 | - | 1.3 | 1.4 |
| cortical SR 49 f 56.81 10.3 48 46,XX | 92 | 94 | - | 10.8 | 22.3 |
| cortical SR 32 m 19.37 7.2 170 NA | 56 | 98 | - | 2.5 | 12.4 |
| cortical SR 31 m 57.83 13.4 270 46,XY | 18 | 37 | - | 2.0 | 16.7 |
| cortical SR 50 f 364.80 8.3 30 46,XX | 97 | ND | - | 2.3 | 10.4 |
| cortical SR 68 m 29.00 13.1 28 46,XX, **del(11)(p10),+2~3mar,inc** | 79 | 83 | - | 6.0 | 37.9 |
| cortical SR 23 m 41.39 12.9 124 NA | 64 | ND | relapse | 1.8 | 38.1 |
| cortical SR 76 m 68.75 9.6 20 46,XY | 82 | ND | - | 75.5 | 71.7 |
| cortical SR 54 f 7.35 7.2 111 46,XX | 28 | ND | - | 4.2 | 28.7 |
| cortical SR 31 m 11.72 10.6 111 NA | ND | 94 | - | 3.8 | 31.4 |

Abbreviations: BCR, breakpoint cluster region; ABL, Abelson murine leukemia viral oncogene homolog 1; –, negative; +, positive; SR, standard risk; HR, high risk; VHR, very high risk; f, female; m, male; WBC, white blood count; G/l, Giga per liter; Hb, hemoglobin; g/dl, gram per deciliter; Plt, platelets; PB, peripheral blood blasts among nucleated cells; BM, bone marrow blasts; NA, not available; ND, not determined; SFI, specific fluorescence index.
did not directly correlate with OX40 mRNA amount (Figure 1C) or BCR-ABL quantity (Figure 1D).

**OX40 Is Expressed in Primary ALL Cells**

Next, primary ALL cells from a total of 44 patients with proven precursor B and T cell ALL were analyzed for OX40 expression. Leukemic cells were classified at the time of diagnosis using the immunological classification proposed by the European Group for the Immunological Characterization of Leukemias [30]. Detailed patient characteristics and individual OX40 expression levels are listed in Table 1.

Leukemic cells within mononuclear cells of patients were identified using different surface marker combinations based on immunophenotyping results at initial diagnosis or relapse. Substantial surface expression of OX40 (SFI ≥ 2.0) was detected in 48% of cases (n=21) with a median expression of 1.9 (SFI range, 1.0-7.5) (Figure 2A).

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**Figure 2.** Primary ALL cells express OX40 on mRNA and protein level. (A and B) Immunophenotypic OX40 expression on ALL patient samples using the monoclonal OX40 antibody BerAct35 or the respective isotype control followed by secondary anti-mouse PE after selection with the predetermined immunophenotype. Combined SFI levels (A) and percentage of OX40-positive cells (B) are shown. Patients with SFI levels ≥ 2.0 or at least 20% OX40-expressing cells were considered positive (cutoff values are indicated as dotted lines). Horizontal bars represent median of results. (C and D) OX40 expression [SFI levels (C); % positive cells (D)] according to ALL subtype. Horizontal bars represent mean. (E and F) PBMC from ALL patients were analyzed by flow cytometry using the monoclonal OX40 antibody BerAct35 (shaded peaks) and isotype control (open peaks) followed by anti-mouse PE. Exemplary histograms (upper panels) and dot plots (lower panels) of OX40-negative (E) and OX40-positive (F) ALL cells of different subtypes are shown. (G and H) Combined SFI levels (G) and combined results showing % OX40 positivity (H) are depicted for BCR-ABL–negative and –positive ALL cells. Horizontal bars represent median of results in each group. *Statistically significant differences, P<.05.
When analyzing the percentage of OX40-positive cells among leukemic cells of each sample, 61% of patients (n=27) showed at least 5% OX40-positive cells with a median expression of 14.6% (range, 0.7-87.8). In 48% of samples (n=21), an OX40 expression above 20% could be detected (Figure 3B). When analyzing different ALL subtypes, no statistically significant differences were observed (Figure 2, C–F). However, significantly differing OX40 expression levels were detected when primary ALL samples were stratified according to BCR-ABL status with positivity being significantly associated with higher OX40 expression, both with regard to SFI levels (P<.0001, Mann-Whitney test) (Figure 2G) and percentage of positive cells (P<.0001, Mann-Whitney test) (Figure 2H).

When OX40 expression in ALL was studied on mRNA level, OX40 amplicons were observed in all analyzed surface-positive but also in surface-negative ALL samples (Figure 2J), which, like the results obtained with cell lines, points to posttranscriptional regulation of OX40 expression. Quantification of OX40 mRNA levels in ALL samples and healthy CD34-positive bone marrow (BM) cells showed significantly higher mRNA levels in surface-positive compared to surface-negative samples (P=.02, Mann-Whitney test) or healthy CD34-positive BM cells (P=.001, Mann-Whitney test).

**OX40 Stimulation Promotes Metabolic Activity and Induces Cytokine Release of Primary ALL Cells**

In order to examine if OX40 plays a functional role in ALL cells, an agonistic antibody recently described to stimulate OX40 was employed [22]. It induced a significant increase of metabolic activity as an indicator of cell proliferation and viability in OX40-positive primary ALL samples (n=10, P=.04, Mann-Whitney test), while metabolic activity remained unaltered in OX40 surface-negative samples (n=3, P=.75) (Figure 3A). Notably, interindividual differences concerning metabolic activity of ALL cells upon OX40 stimulation were detected, but no association of OX40 expression and change in metabolic activity was observed (Supplementary Figure 2).

Antibody-mediated stimulation of OX40 also led to a significant induction of IL-6, TNF, and IL-8 production (P=.003, P=.009, and P=.002; Mann-Whitney test) by OX40 surface-positive ALL cells (n=14), while no significant effect was observed in OX40 surface-negative ALL samples (IL-6, P=.4; TNF, P=.2; IL-8, P=.99; Mann-Whitney test) (Figure 3B). Again, substantial interindividual differences regarding cytokine release of ALL cells upon OX40 signaling were detected (Figure 3C). Notably, no association of OX40 surface expression and basal release of the cytokines was observed (Supplementary Figure 3). In the majority of investigated cases, OX40 stimulation led to an at least 2.5-fold increase in secretion of cytokines, whereas only in two cases could this not be detected in OX40 surface-positive ALL samples (Figure 3, D and E).

**OX40/OX40L Interaction Enhances NK Cell Cytotoxicity Against ALL Cells**

Recently, interaction between OX40 on AML cells and OX40L expressed by NK cells was reported to enhance NK cytotoxicity. Blockade of OX40/OX40L interaction was shown to impair polyclonal NK cell reactivity against leukemic cells [22]. In order to investigate and confirm such consequences of OX40/OX40L interaction with ALL cells, the OX40-negative cell line JURKAT and the OX40-positive cell line SD-1 were employed in cocultures with OX40L-expressing polyclonal NK cells using nonstimulatory OX40 F(ab′)2-fragments [23] to disrupt OX40/OX40L interaction. Blockade of OX40/OX40L signaling significantly (P=.008, Mann-Whitney test) decreased lysis of OX40 surface-positive ALL cell lines, while no effect on lysis was detected employing surface-negative ALL cell lines (P=.97, Mann-Whitney test) (Figure 4, A and B).

**OX40 Expression Is Associated with Clinical Characteristics in ALL**

Next, we set out to study whether OX40 expression was associated with clinical characteristics in our patient cohort. The median age at time of diagnosis was 38 years (range, 19-81), 30% of patients were 55 years or older, and 64% were male. Median white blood cell (WBC) count was 38.17 G/l (range, 1.80-463.01) (Table 1). When we grouped ALL cases into subtypes, we did not observe significant association with OX40 expression (see Figure 2, C and D), but OX40 expression was significantly associated with BCR-ABL status (Figure 5A). Whereas BCR-ABL-negative ALL cells had a median OX40 expression of 1.3 which is below the threshold we defined for surface positivity (range, 1.0-12.1), median OX40 expression was 4.0 (range, 1.3-75.5) in BCR-ABL-positive cases. Furthermore, OX40 expression was significantly associated with very high risk (VHR) status in ALL, while no association was found for high-risk (HR) or standard-risk (SR) patients (%: SR vs. VHR, P<.005; HR vs. VHR, P=.03; SR vs. HR, P=.99; SFI: SR vs. VHR, P=.02; HR vs. VHR; P=.03; SR vs. HR: P=.99 (data not shown); both Kruskal-Wallis test) (Figure 5B). Higher OX40 levels defined as surface expression ≥45% were also associated with significantly shorter overall survival (P=.007 %), log-rank Mantel-Cox) (Figure 5C). Interestingly, when subgrouped according to BCR-ABL status, higher OX40 expression in BCR-ABL–positive patients also resulted in significantly shorter overall survival (P=.009, log-rank Mantel-Cox) (Figure 5D). This effect could not be observed in the BCR-ABL–negative subgroup, where only three patients showed an OX40 value of ≥45% (P=.843, log-rank Mantel-Cox). Higher OX40 levels defined as a cutoff value of SFI ≥5 were also associated with significantly shorter overall survival in the total cohort (P=.02, log-rank Mantel-Cox, data not shown). No association was identified with other genetic abnormalities, disease etiology, clinical parameters such as WBC count, or treatment response.

**Discussion**

During the last decades, OX40 has gained increasing attention due to its function as a potent positive regulatory surface molecule on T cells that is able to reestablish T cell antitumor reactivity [10]. Activation of this stimulatory immune checkpoint leads to sustained T cell proliferation and survival, rendering OX40 a popular therapeutic target for immune-based elimination of tumor cells. The ability of OX40 to potently stimulate both CD4+ and CD8+ T cells makes it a suitable candidate for cancer immunotherapy. Various OX40 targeting approaches were developed in the last two decades [31], and several strategies are being evaluated in clinical trials [10].

In contrast to its use in chimeric antigen receptor T cells, where the costimulatory signaling domain of OX40 is frequently incorporated into chimeric antigen receptors to specifically enhance function of transfected T cells, systemic application of monoclonal antibodies targeting OX40 might also affect other OX40-bearing cells of the immune system or nonimmune cells.

The consequences of OX40 expression on malignant cells and its potential role in pathophysiology and prognosis have even less been taken into account and only investigated in a few studies to our knowledge so far.
Imura et al. demonstrated that 15 out of 17 adult T cell leukemia cases, a peripheral T cell neoplasm associated with infection by the human T-lymphotropic virus type I, expressed significant levels of OX40. While OX40 mRNA was detected by RT-PCR in all tested patient samples, OX40 protein expression could be identified by Western blot in only two out of five adult T cell leukemia cases. The
Figure 3. OX40 signaling induces stimulation of metabolic activity and cytokine release in ALL cells. PBMC of ALL patients were either cultured alone, on immobilized M-OX17 monoclonal antibody or human IgG isotype control (10 μg/ml). (A) Metabolic activity of ALL cells was determined by CellTiter Glo assay after 24 hours. Results of OX40-positive (n=10) and OX40-negative samples (n=3) are shown. For normalization, results obtained with untreated ALL cells were set to 1. Horizontal bars represent mean. (B) Cytokine levels of IL-6 (after 24 hours), TNF, and IL-8 (both after 6 hours) in culture supernatants were determined by ELISA. Results obtained with OX40-positive (n=14, upper panel) and OX40-negative ALL samples (n=3, lower panel) are shown. Horizontal bars represent mean. (C) Fold increase (OX40/isotype) in cytokone production (IL-6, TNF, and IL-8) of ALL cells after OX40 stimulation. Each patient is shown as individual bar. Dotted lines represent a 2.5-fold increase in cytokone production. (D) Quantification of cytokone release by ALL cells after OX40 stimulation. Numbers of patientshowing fold increase exceeding 4% (median 1; range, 1-4) are depicted. *Statistically significant differences, P<.05.

Figure 4. OX40/OX40L interaction increases NK cell cytotoxicity in response to ALL cells. (A and B) OX40L-positive polyclonal NK cells were cultured for 2 hours with OX40-negative JURKAT (left) or OX40-positive SD-1 ALL cells (right) in the absence or presence of blocking OX40 F(ab')2 or isotype control (2 μg/ml each). NK cell reactivity was evaluated by time-resolved fluorometric assays. (A) Exemplary results representing means of triplicates are shown. (B) Combined data of five independent experiments for each ALL cell line at an E:T ratio of 10:1 (control: white bars, OX40 blockade: black bars). Mean values and SD are depicted.*Statistically significant differences, P<.05.

Increased cytokine release was also observed after OX40 signaling. The percentage of samples responding with the indicated cytokine pattern is depicted. *Statistically significant differences, P<.05.

immunonephrotic analyses of different lymphoid malignancies by Koubek et al. showed relevant OX40 surface expression on malignant cells from patients with precursor T cell but not B cell ALL. Six of seven analyzed precursor T cell ALL cases expressed OX40 with a median of 8% (range, 4-31), whereas all eight analyzed precursor B cell ALL cases exhibited only very low levels of OX40 not exceeding 4% (median 1; range, 1-4) [33]. Furthermore, in this study, one of two T cell non-Hodgkin lymphoma (NHL) cases expressed OX40 >5%. B cell chronic lymphocytic leukemia, B NHL, prolymphocytic leukemia with B cell markers, and plasmocytoma cells expressed very low levels of OX40 which did not exceed 3%.

In our study, flow cytometry analyses of OX40 surface expression in a cohort of 44 precursor B and T cell ALL patients revealed that 61% exhibited at least 5% OX40-positive cells with a median expression of 14.6% (range, 0.7-87.8) and in 48%, an OX40 expression above 20% could be detected.

In line with previous studies, five of seven analyzed precursor T cell ALL samples expressed substantial amounts of OX40 in the majority of cases (median 8.8%, range 1.4-48.7). In contrast to earlier reports, in our cohort, OX40 surface expression was also observed for precursor B cell ALL with a median expression of 21.0% (range, 0.7-87.8). The observed discrepancy may be due to the higher number of analyzed cases, the heterogeneity among primary samples, and the use of different OX40 antibodies in our analyses.

Interestingly, also ALL samples without relevant surface expression showed OX40 mRNA expression. One explanation for this observation could be the contamination with OX40-expressing healthy cells that may have influenced the respective PCR results, but also regulatory or mutational blockade of surface expression by posttranscriptional or posttranslational mechanisms. This may comprise, as reported for many other TNF/TNFR members, cell surface shedding and release in soluble form, which is supported by reports on the presence of soluble OX40 in sera of patients with malignant and autoimmune diseases [34–37].

To our knowledge, expression of OX40 in the context of hematological malignancies has further up to now only been reported for AML [22]. Our group recently reported OX40 expression on the surface of primary AML samples in 54% of cases and could show that OX40 stimulation led to enhanced proliferation and release of proleukemic cytokines. When we performed functional analyses using well-characterized monoclonal antibodies with defined specificity and agonistic property, we could show that OX40 signaling results in release of cytokines that act as autocrine/paracrine growth and survival factors also in ALL. Furthermore, like in T cells, OX40 signaling enhanced viability/metabolic activity in a substantial proportion of ALL cases. It seems thus possible that OX40 confers

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a survival benefit for leukemic cells, e.g., upon interaction with OX40L bearing immune or bystander cells. This is in line with increasing evidence regarding the important role of the immune and stromal microenvironment in malignancies [38].

Regarding prognostic impact of OX40 expression, Xie et al. comparatively analyzed immunohistochemistry and RNA sequencing data to assess the biologic relevance of OX40 in hepatocellular carcinoma and could detect higher OX40 expression in malignant cells in comparison to adjacent healthy liver tissue. OX40 expression was associated with elevated serum alpha-fetoprotein level, vascular invasion, and shorter survival [39].

When assessing the biological relevance of OX40 expression in ALL, we grouped ALL subtypes according to risk group. Risk stratification is based on clinical and biological prognostic factors like ALL-subtype, high WBC count at diagnosis, or late achievement of complete remission [40]. Patients with translocation t(9;22) or the...
corresponding molecular aberration BCR-ABL are defined as a separate group because they are treated with tyrosine kinase inhibitors as causal therapy.

Evaluation of these prognostic factors is crucial at initial diagnosis for the development of a suitable treatment strategy governing intensity of treatment. With our study, we identify OX40 as a negative prognostic marker and show its association with BCR-ABL status. Additionally, we demonstrated that disruption of OX40/OX40L interaction between ALL cells and polyclonal NK cells resulted in diminished lysis rates, implying that OX40-targeting approaches should be considered carefully.

Moreover, our findings support our above-mentioned line of argument regarding potential unexpected consequences of a therapeutic application of agonistic OX40 monoclonal antibodies, and it is noteworthy that other investigators reported OX40 expression (without analyzing functionality) on cancer cells of various origins beyond ALL [41]. Another layer of complexity when applying OX40 monoclonal antibodies is added by the issue of whether and how antibody binding to OX40 affects interaction with cells that express its cognate ligand. This is of particular relevance because OX40/OX40L interaction can lead to transduction of bidirectional signals, e.g., into the receptor and the ligand-bearing cell, a characteristic feature of many ligands of the TNF family [42,43]. Besides healthy tissues like endothelial cells, antigen-presenting cells including B cells, and monocytes/dendritic cells express OX40L. Various cellular functions of these cells are affected by OX40L “reverse signaling” [43–46], which may also occur upon their interaction with OX40-expressing ALL cells.

Thus, additional work is warranted to fully unravel the complex role of the OX40/OX40L molecule system, which in turn may help to fully exploit the potential of OX40 stimulation for cancer immunotherapy.

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Author Contributions
K. R. and I. H. designed and performed the experiments, analyzed and interpreted data, and wrote the manuscript. M. R. collected patient samples and provided clinical data. G. B. performed bioinformatic analyses. M. H. and G. J. provided critical reagents. T. N. contributed to the study design, performed experiments, and provided important advice. H. S. contributed to the study design, critically revised the manuscript, and supervised the study. D. D. collected clinical samples and provided patient data, contributed to the study design, wrote the manuscript as lead author, and supervised the study. All authors critically reviewed the manuscript and approved the final version.

Conflict of Interest Disclosure
The authors declare no competing financial interests.

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