The role of the immune checkpoint modulator OX40 and its ligand in NK cell immunosurveillance and acute myeloid leukemia

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Abstract

The TNF receptor family member OX40 promotes activation and proliferation of T cells, which fuels present attempts to modulate this immune checkpoint to reinforce anti-tumor immunity. Besides T cells, NK cells are a second cytotoxic lymphocyte subset that plays an important role in anti-tumor immunity, particularly in leukemia, and multiple approaches to utilize the potential of these cells for cancer treatment, like adoptive transfer of *ex vivo* expanded polyclonal NK cells (pNKC), are presently being clinically evaluated. So far, it is unknown whether and how OX40 and its ligand (OX40L) influence NK cell function and anti-leukemia reactivity. In this study we report that OX40 is expressed on leukemic blasts in a substantial percentage of patients with acute myeloid leukemia (AML) and, as revealed upon stimulation with agonistic OX40 antibodies, can mediate proliferation and release of cytokines that act as growth and survival factors for the leukemic cells. Moreover, we demonstrate that pNKC differentially express OX40L depending on the protocol used for generation. OX40L signaling was found to promote NK cell activation, cytokine production and cytotoxicity, and disruption of OX40-OX40L interaction impaired pNKC reactivity against primary AML cells. Together, our data identify a yet unknown involvement of OX40/OX40L in disease pathophysiology of AML and in NK cell immunosurveillance. Our findings indicate that effects of this molecule system in other immune cell subsets and also malignant cells should be taken into account when developing OX40-targeting approaches for cancer immunotherapy.

Introduction

Modulation of immune checkpoints has become a mainstay in oncological treatment. Besides already approved approaches that block inhibitory molecules like CTLA-4 or PD-1, agonistic antibodies that trigger activating receptors on T cells are presently being developed, one of them being OX40.[1-4] This member of the TNF receptor (TNFR) superfamily is upregulated on effector T cells after activation and promotes their differentiation, proliferation/expansion and longterm survival while inhibiting the suppressive activity of regulatory T cells.[5,6] In cancer patients, the frequency of tumor-infiltrating OX40-positive T cells correlates with survival, and application of OX40 agonists, alone or in combination with other checkpoint modulators, stimulated the cytolytic activity of T cells and caused tumor regression in preclinical models.[7-11] First evidence from early clinical trials, of which most are presently ongoing, indicates that OX40 stimulation is also effective in cancer patients (*e.g.*, Curti *et al.*[12], Linch *et al.*[3]).

Besides T cells which are components of adaptive immunity, NK cells as the major cytotoxic lymphocyte subset of the innate immune system also play an important role in tumor immunosurveillance, particularly in hematological malignancies. This is supported by observations that NK cell counts and activity are reduced in patients with leukemia and that activity levels of autologous NK cells are associated with survival of leukemia patients.[13-15] Their prominent role especially in acute myeloid leukemia (AML) is highlighted by studies on haploidentical stem cell transplantation (SCT), where the resulting KIR mismatch seems to be associated with pronounced *Graft versus Leukemia* reaction and improved clinical outcome.[16] Besides their role in SCT, multiple approaches presently aim to utilize adoptive transfer of allogeneic/KIR-mismatched NK cells for cancer treatment.[17]

Beyond KIR, signals mediated by multiple other activating and inhibitory receptors determine whether NK cell responses against tumor cells are initiated or not. This comprises various members of the TNF/TNFR family which influence NK cell reactivity upon interaction with their counterparts expressed *e.g.* on leukemic cells.[18,19] Notably, OX40L was reported to be

upregulated on NK cells following activation,[20] and its counterpart OX40 was found to be expressed by T cell-derived leukemia cells.[21] However, the influence of the OX40/OX40L system on NK function or its role in AML is so far unknown. Here we report, among others, that AML cells express OX40, and exposure to an agonistic OX40 antibody (mAb) promotes leukemia cell proliferation and release of cytokines that influence growth and survival of the malignant cells.[22,23] Moreover, we found that OX40L is differentially expressed on polyclonal NK cells (pNKC) generated for adoptive transfer depending on the particular protocol utilized, and that OX40L (reverse) signaling alters NK cell function including their reactivity against AML cells.

Material and Methods

Cells

Peripheral blood mononuclear cells (PBMC) and bone marrow (BM) cells of patients and healthy donors were isolated by density gradient centrifugation after informed consent in accordance with the Helsinki protocol. The study was conducted according to the guidelines of the local ethics committee.

pNKC were generated according to standard protocols by incubating non-plastic-adherent PBMC with irradiated RPMI8866 (pNKC-8866) or K562-mb15-41BBL feeder cells obtained from St. Jude´s Children´s Research Hospital (pNKC-SJ) as previously described.[24,25] Functional experiments were performed when purity of NK cells ($CD56^+CD3^-$) was above 90% as determined by flow cytometry. In addition, K562 cells were transfected using the vector pcDNA3 containing the open reading frame of human 4-1BBL (K562-4-1BBL) or empty vector as control (K562-mock) as described previously.[25]

U937 cells were transfected using the vector pcDNA3 containing the open reading frame of human OX40 (U937-OX40) or empty vector as control (mock) and cultured as described previously.[25]

The OX40:Fas reporter cells (Jurkat-JOM2) and their use in cytotoxic assays were previously described.[26]

Isolation of highly pure (purity >95%) NK cells from pNKC and AML cells from patient PBMC was performed by immunomagnetic separation using the NK cell isolation kit and negative selection using microbeads CD3, CD14, CD19 and CD56 from Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturers instructions.

Reagents

OX40 mAb BerAct35, OX40L mAb ANC10G1 and mouse IgG1 isotype control were from Ancell Corporation (Bayport, MN) and BD Biosciences (Heidelberg, Germany), respectively. OX40L

mAb lk-1 and 11C3.1 were from BD Biosciences and Biolegend (San Diego, CA), respectively. All fluorescence conjugates were from BD Biosciences, secondary goat anti–mouse-PE was from Dako (BIOZOL, Eching, Germany). Fusion proteins consisting of human OX40 with a murine (OX40-Fc) and human (OX40-huFc) Fc-part were from R&D Systems (Minneapolis, MN) and Ancell, respectively. RhIL-2 was from ImmunoTools (Friesoythe, Germany).

In addition, antibodies against human OX40 were raised by immunization of C57BL/6 mice by repeated injection of $20x10^6$ OX40-transfected CHO cells. Then spleen cells were fused with SP2/0-Ag14 cells, and hybridoma cells secreting OX40 mAbs were cloned by limiting dilution. mAbs were purified from hybridoma supernatants using Protein A Agarose columns (GE Healthcare, Muenchen, Germany). $F(ab')_2$ fragments were generated using previously described standard protocols.[27]

Flow cytometry

Analysis of OX40 and OX40L surface expression was performed using specific mAb or isotype control followed by anti–mouse-PE using a BD FACSCanto™ II.

Leukemic cells in patient samples were selected by FSC/SSC and employing the surface markers CD33/CD34/CD14/CD117 based on the individual immunophenotype defined upon routine diagnosis. Specific fluorescence indices (SFI) were calculated by dividing median fluorescences obtained with specific mAb by median fluorescences obtained with isotype control. Expression was considered positive in case of SFI≥1.5. Intracellular staining was performed using the Fixation/Permeabilization Solution Kit with BD GolgiStop™ from BD Biosciences according to manufacturer's instructions.

PCR analysis

OX40 primers were 5'-TGTAACCTCAGAAGTGGGAGTG-3' and 5'-GGTCCCTGTCCTCACAGATTG-3'. 18S rRNA primers were

5’-CGGCTACCACATCCAAGGAA-3’ and 5’-GCTGGAATTACCGCGGCT-3’. OX40L primers were 5’-CTGCTCCTGTGCTTCACCTAC-3’ and 5’-TCCAGGGAGGTATTGTCAGTG-3’. GAPDH primers were 5’-AGCCACATCGCTCAGACAC-3’ and 5’-GCCCAATACGACCAAATCC-3’. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously.[18]

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 instrument. Primer assays (QuantiTect Primer Assay, Qiagen, Hilden, Germany) for OX40 and 18S ribosomal RNA were used according to the manufacturer's instructions. Relative mRNA expression was calculated by the ΔΔ cycle-threshold (Ct) method.

Cytotoxicity assays

Cytotoxicity of NK cells against primary leukemia cells and U937-transfectants was determined by ^{51}chromium release assays after 4h or 24h as previously described.[28]

Determination of cytokine levels and metabolic activity

Cytokine determination was performed by ELISA according to manufacturer’s instructions using OptEIA sets from BD Pharmingen or DuoSet ELISA development systems from R&D Systems. Metabolic activity was measured using the cell proliferation reagent WST-1 set (Roche) according to manufacturer’s instructions.

Results

OX40L is differentially expressed on pNKC

As OX40L can be upregulated on NK cells upon activation and *ex vivo*-preactivated pNKC are being evaluated for cancer treatment,[17,20] we characterized OX40L expression in pNKC generated according to two differing standard protocols (pNKC-8866[25] and pNKC-SJ[24]). RT-PCR revealed substantial and similar OX40L mRNA levels in both pNKC preparations (Fig. 1A). Next we employed various commercially available OX40L mAb to study surface expression using NK-92 cells, which do not express OX40L mRNA (Fig. 1A) as negative control and OX40-Fc fusion protein to ascertain specificity. Comparative FACS analyses revealed that only mAb ANC10G1 can reliably be used to determine OX40L expression, while the other two mAbs (but not OX40-Fc) unspecifically bound to NK-92 cells as exemplified in Fig. 1B. While resting NK cells of healthy donors were never found positive for surface OX40L and pNKC-8866 displayed only low or no relevant levels, pNKC-SJ displayed substantial and significantly higher OX40L surface expression. Notably, this difference between pNKC-8866 and pNKC-SJ held true for preparations generated with PBMC of the same or independent donors and despite the considerable variation among individual donors (Fig. 1C, $P<0.05$, Mann-Whitney test and data not shown). Expression in pNKC-SJ peaked after 4-6 days of culture and declined thereafter but OX40L was still profoundly expressed at the time point when the pNKC are usually used for functional experiments and clinical application (usually beginning day 8 of culture) by us and others.[29] In some cases a slight upregulation of OX40L was observed early during culture of pNKC-8866, but expression levels always were clearly lower than that of pNKC-SJ and OX40L was never detectable after day 9 (Fig. 1D). In line with previous findings,[20] activation of NK cells with IL-2 alone was not sufficient for induction of OX40L; upregulation rather was dependent on 4-1BB stimulation as revealed by coculture experiments involving K562-41BBL and mock transfectants and transwell settings (Fig. 1E).

Signaling via OX40L modulates NK cell reactivity

In addition to acting as a ligand for OX40, OX40L can, like several other TNF family members, itself transduce signals into the ligand-bearing cell.[30] To determine whether and how such reverse signaling via OX40L affects NK cells, we cultured pNKC on immobilized OX40-Fc or Fc-control to allow for OX40L crosslinking in the absence of a second, OX40-expressing (target) cell population. FACS analysis of the activation markers CD69 and NKp44 revealed a significant (both $P<0.05$; Mann-Whitney test) upregulation on pNKC-SJ following OX40L signaling, while the OX40L-negative pNKC-8866 were not affected (Fig. 2A). In addition, triggering OX40L also led to a significant ($P<0.001$, Mann-Whitney test) induction of IFN-γ release by OX40L-positive but not -negative pNKC, which again confirmed that signals were mediated via OX40L. The effect of OX40L-signaling was observed both in the absence and presence of IL-2, the latter serving to mimic a generally augmented state, which indicates that OX40L signaling may further enhance the activity of activated NK cells (Fig. 2B).

To determine whether OX40L also affects NK lysis, we next transfected U937 cells to express high levels of OX40 (U937-OX40) and generated mock-transfectants (U937-mock) as control. When the transfectants were employed in cytotoxicity assays, we observed significantly ($P<0.05$, Student's *t*-test) higher lysis rates for the OX40-positive targets (Fig. 2C). Next we aimed to disrupt receptor-ligand engagement in this experimental setting to confirm that OX40-OX40L interaction enhances NK lysis of target cells. We reasoned that for this purpose a blocking OX40 mAb would be ideally suited, because thereby (in contrast to using OX40L mAb) binding to and potential induction of signaling in NK cells would be avoided. In addition, to exclude potential Fc-mediated effects/ADCC after mAb binding to OX40 on target cells, we wanted to utilize $F(ab')_2$-fragments. As no OX40 mAb with distinct blocking capacity was commercially available, we generated mouse OX40 mAb as described in the methods section and, after production and definition of specificity, employed these in cross-competition experiments using OX40-huFc. As depicted in Fig. 2D, our mAb clones M-OX2 and M-OX17 specifically and comparably bound to

the U937-OX40 transfectants, but only M-OX2 clearly disrupted OX40-OX40L interaction as revealed by reduced binding of OX40-huFc to OX40L on pNKC-SJ. We then produced $F(ab')_2$-fragments of M-OX2 according to standard protocols[27] and employed these in cytotoxicity assays with pNKC-SJ and U937-transfectants. While no effect on NK lysis of mock-transfectants was observed, blocking OX40 significantly ($P<0.05$, Student's *t*-test) decreased the per se higher cytotoxicity observed with the OX40-transfectants, which confirmed the stimulatory effect of OX40-OX40L interaction on NK cell reactivity (Fig. 2E).

Expression of OX40 on AML cells

Next we employed FACS analysis to study whether OX40 is expressed on the surface of leukemic cells using a total of 111 different AML patient samples and also $CD34^+$ progenitor cells obtained from peripheral blood and BM of healthy donors. Leukemic blasts within PBMC were selected as described in the methods section. The clinical characteristics of each patient and individual SFI levels are given in Table 1. While no surface expression was observed on healthy $CD34^+$ cells, their malignant counterparts displayed relevant OX40 expression in a substantial proportion of AML cases (SFI≥1.5, n=60 (54%); SFI≥2.0, n=41 (37%) (Fig. 3A, B). Interestingly, $CD34^+$ cells from patients with chronic myeloid leukemia (CML, n=10) and myelodysplastic syndrome (MDS, n=6) showed no relevant OX40 expression (suppl. Fig. 1A). In AML, OX40 expression was significantly associated with the t(15;17) translocation (PML/RARA) and FLT3-ITD mutation (both $p<0.05$, Mann-Whitney-U-test), whereas no association with other genetic abnormalities, risk according to the ELN classification, FAB classification, disease etiology (i.e. secondary AML from MDS) or clinical parameters like extent of BM fibrosis, treatment response or survival was observed (table 2 and data not shown). OX40 expression by AML cells was also confirmed on mRNA level using RT-PCR: Amplicons of OX40 were detected in all 8 investigated samples of patients with at least 80% blast count (Fig. 3C). Notably, this also comprised samples of three patients without detectable surface expression on leukemic cells.

Very low or no OX40 mRNA was detected in the CD34-enriched BM cells of healthy donors (Fig. 3D). Quantitative PCR revealed significantly (P<0.05, Mann-Whitney test) lower mRNA levels in healthy $CD34^+$ BM cell samples and surface-negative compared to surface-positive AML samples (Fig. 3E). Next we cultured primary AML cells in the presence of OX40L, G-CSF, GM-CSF, IFN-γ, TNF, IL-6, IL-8 and IL10 for various times (6h, 24h and 48h) and then analysed OX40 expression by FACS using an (non-competing) OX40 mAb. Interestingly, we found that TNF significantly induced OX40 expression beginning already after 6h with a peak at 24h, while none of the other factors had any effect (Fig. 3F and data not shown).

OX40 can induce cytokine release and promote proliferation of AML cells

To elucidate the role of OX40 on AML cells we functionally characterized our newly generated OX40 mAb using a reporter cell assays in which Jurkat-JOM2 cells expressing a human OX40-Fas chimeric receptor are killed upon engagement of the OX40 portion of the receptor.[26] OX40 mAb clone M-OX17 killed these reporter cells in a dose dependent manner, indicating that it has agonist activity (Fig. 4A). The same mAb also stimulated a robust IL-8 production in U937-OX40 transfectants, but not in mock controls, which further confirmed its specific stimulatory property (Fig. 4B).

Next we aimed to determine whether OX40 on leukemic cells of AML patients was functional. No clear association of constitutive OX40 expression with the basal release of the cytokines TNF, IL-10, IL-6 and IL-8 in vitro, the extent of fibrosis in BM samples, metabolic activity/proliferation of leukemic cells or apoptosis/death in vitro was observed (suppl. Fig. 2 and data not shown). However, M-OX17-induced OX40 signaling resulted in a significant (all P<0.05, Wilcoxon signed rank test) induction of TNF, IL-10, IL-6 and IL-8 by OX40-positive AML cells (both in cases with de novo and secondary AML from MDS), while no effects were observed with OX40-negative leukemic cells (Fig. 4C, suppl. Fig. 3A and data not shown). Notably, we observed substantial inter-individual differences concerning the cytokine release of AML cells upon OX40 signaling.

None of the 19 investigated samples released all four cytokines. Release of TNF, IL-10, IL-6 and IL-8 was observed with 15, 10, 15, 3 of the 19 samples, respectively (Fig. 4D). Analysis of intracellular IL-8 and TNF levels by FACS and gating for $CD33^+/CD19^-/CD3^-$ cells served to ascertain that in fact the leukemic cells among patient-PBMC produced the respective cytokines upon OX40-stimulation (Fig. 4E). WST-1 assays then further revealed that OX40 signaling can also significantly ($P<0.05$, Wilcoxon signed rank test) enhance AML cell proliferation/viability: in OX40-positive AML patient samples, 8 (53%) and 5 (33%) out of 15 investigated patients responded to OX40 stimulation with a 1.5-fold and 2-fold increase, respectively, while no effect was observed with OX40-negative samples as controls (Fig. 4F, suppl. Fig. 3B and data not shown).

OX40-OX40L interaction enhances NK cell cytotoxicity in response to AML cells

Finally we aimed to determine the outcome of OX40-OX40L interaction for NK cell anti-leukemia reactivity. As a first step, we studied the frequency and activation state of NK cells within AML patients with different OX40 expression levels on leukemic blasts, which however did not reveal a clear correlation (suppl. Fig. 4). This can be attributed to the fact that NK cell reactivity is influenced by a multitude of activating and inhibitory receptors as well as cytokines and other immune cell subsets far beyond the newly identified OX40/OX40L molecule system.{Handgretinger, 2016 #11} Accordingly we next aimed to delineate the specific role of this molecule system by employing our blocking OX40-$F(ab')_2$-fragments in long term (24h) chromium release assays with OX40L-positive pNKC-SJ of 9 different healthy donors and OX40-positive primary AML cells from 8 different patients (at least 80% blast count). Blocking OX40 significantly ($P<0.0001$, Wilcoxon signed rank test) decreased lysis of primary AML cells, while the isotype control had no relevant effect (Fig. 5A, B). To further exclude a potential influence of other immune effector cells remaining in AML patient samples or the pNKC preparations, we conducted lysis assays using primary AML samples after MACS-depletion of

292 CD3/CD14/CD19/CD56 cells as targets with highly purified NK cells obtained by MACS-isolation
293 from bulk pNKC-SJ as effectors. Again we found that blocking OX40-OX40L interaction reduced
294 AML cell lysis, which confirmed the stimulatory role of this molecule system in AML-NK cell
295 interaction (Fig. 5C).

Discussion

The therapeutic inhibition of immune checkpoints to reinforce anti-tumor immunity of T cells has meanwhile become a mainstay of cancer treatment. However, many patients do not benefit from the presently available checkpoint blockers that target CTLA-4 and PD-1/PD-L1, or for limited time only.[2] New strategies are thus needed and are presently being developed to better exploit the immune system's potential to combat malignant disease. Novel approaches beyond blocking immune-inhibitory molecules comprise, among others, therapeutic stimulation of activating immune receptors on T cells, but also *ex vivo* manipulation/expansion and subsequent transfer of cytotoxic lymphocytes, like chimeric antigen receptor (CAR) T cells[31] or pNKC.[29]

The TNFR family member OX40 is a prominent example of an activating receptor that can reinforce T cell anti-tumor-reactivity in the sense of a "stimulatory" immune checkpoint. Preclinical studies with agonistic mAb revealed that the ability of OX40 to stimulate T cells is comparable to *e.g.* CTLA-4 blockade.[12] Due to its profound ability to sustain T cell proliferation/survival, OX40 is also frequently used in the costimulatory signaling domain of CAR.[32,33] The latter confines the effects of OX40 activation to the transfected T cells, while systemic application of agonistic mAb may also affect other cellular components of the immune system and the many non-immune cells that express OX40.[4-6] Our results obtained by FACS analysis of 111 primary patient demonstrate that AML cells (but not $CD34^+$ cells of healthy donors or patients with MDS or CML) express OX40 on the cell surface in a substantial proportion of cases. Notably, OX40 mRNA expression was also observed in AML samples without relevant surface expression. While contamination with OX40-expressing healthy cells may have influenced the respective PCR results, it appears rather likely that regulatory or mutational blockade of surface expression by posttranscriptional and/or posttranslational mechanisms may have contributed to the same. This may comprise, alike 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] Our finding that OX40 expression can be upregulated by exposure of AML cells to TNF indicates that environmental stimuli in the micromilieu may affect OX40 expression via these or other yet unidentified mechanisms.

Functional analyses using newly generated mAb with defined specificity and agonistic property revealed that OX40 signaling can induce the release of cytokines that act as autocrine/paracrine growth and survival factors in AML and are associated with development and progression of the disease.[22,23] Notably, OX40 signaling did not always induce release of the same cytokines. Rather, we found distinct patterns of cytokine release upon OX40 signaling, and whereas TNF and IL-6 were released in more than 70% of the investigated cases, IL-10 and IL-8 were only released by about half and less than 20% of the patient samples, respectively. With none of the AML samples, release of all four cytokines was observed, but all investigated OX40-positive AML patient samples responded to OX40 signaling by release of at least one of the cytokines. OX40 may thus (variably) contribute to the cytokine milieu associated with AML. Furthermore, alike in T cells, OX40 signaling enhanced viability/metabolic activity in a substantial proportion of the AML cases. It seems thus possible that OX40 confers 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 in general, which also holds true for AML.[38] Moreover, these findings support our above-mentioned line of argument regarding potential unexpected consequences of a therapeutic application of “untargeted” agonistic OX40 mAb, and it is noteworthy that other investigators reported on OX40 expression (without analyzing functionality) on cancer cells of various origins beyond AML.[39] Another layer of complexity when applying OX40 mAb is added by the issue of whether and how mAb-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, *i.e.* into the receptor and the ligand-bearing cell, a characteristic feature of many ligands of the TNF family.[30,40] Besides healthy tissues like

endothelial cells, antigen-presenting cells including B cells, monocytes/dendritic cells express OX40L, and various cellular functions of these cells are affected by OX40L "reverse signaling",[30,41-43] which may also occur upon their interaction with OX40-expressing AML cells.

Notably, Zingoni and colleagues reported that also NK cells, which play an important role in the immune surveillance particularly of leukemia,[44] express OX40L following activation and stimulate OX40-expressing T cells via this molecule system.[20] Despite the fact that presently multiple approaches evaluate the clinical efficacy of *ex vivo* expanded/activated NK cells upon adoptive transfer,[17] the expression and function of OX40L by such cell preparations has so far not been analyzed. When we studied OX40L expression on pNKC generated as described in the work of Zingoni *et al.*[20] (pNKC-8866) or generated according to a protocol employed for large-scale clinical grade expansion (pNKC-SJ),[24,29,45] we observed no or very low OX40L expression on the first which are generated by culture with RPMI8866 feeder cells in the presence of IL-2. In contrast, OX40L was highly expressed on pNKC-SJ that are expanded in the presence of K562 cells transfected with 4-1BBL and membrane-bound IL-15 as well as soluble IL-2. In line with the findings of Zingoni *et al.*[20], cytokine activation of NK cells alone was not sufficient to induce OX40L expression, and additional expression of 4-1BBL as revealed by analyses involving transfectants and transwell settings was required. Notably, in a clinical study with pediatric solid tumors that received IL-15/4-1BBL-activated NK cells after allogeneic SCT, acute GVHD was observed despite the fact that the T cell dose in the grafts was below the threshold usually required for GVHD in this setting.[46] The mechanism underlying this observation remained unclear, but it is tempting to speculate that the above described ability of NK cells to stimulate T cells via OX40-OX40L interaction may have contributed to the same. This hypothesis is supported by observations in mouse models of GVHD, where OX40 signals in effector T cells play a crucial pathophysiological role.[47-49]

When we analyzed how OX40L reverse signaling affected NK cells, we found that OX40L-induced activation and production of IFN-γ, a cytokine which participates in cancer elimination by

inhibiting cellular proliferation and angiogenesis, promoting apoptosis, and stimulating the adaptive immune system.[50] Analyses with OX40-transfectants revealed that OX40-OX40L interaction also enhanced NK lysis of target cells. This was further confirmed in experiments using $F(ab')_2$-fragments of a blocking OX40-antibody, which substantially reduced NK cell killing. Notably, 17 independent experiments with primary OX40-expressing AML cells and OX40L-positive pNKC of different patients/donors also clearly revealed that disruption of OX40-OX40L interaction reduces target cell lysis despite the observed substantial donor variability. The latter might be due to differing OX40 and OX40L-expression levels (or differences in other immunoregulatory molecules that influence NK function) on AML cells and pNKC, respectively. Thus, even when the intracellular pathways and molecular mechanisms that mediate OX40L signaling in NK cells remain so far unclear and require further elucidation, our results clearly demonstrate that OX40L enhances the reactivity of NK cells.

Taken together, the results presented in this study unravel the yet unknown expression of OX40 in AML and point to its involvement in disease pathophysiology. In addition, we report on the consequences of reverse signaling via its counterpart OX40L for NK cells including their anti-leukemia reactivity. A limitation of our study is that we were not able to demonstrate expression of OX40L on NK cells of AML patients directly ex vivo, but it needs to be considered that all OX40L antibodies available for FACS analysis compete with OX40 for OX40L binding. NK cell-expressed OX40L could thus be masked *e.g.* by soluble OX40.{Taylor, 2001 #1450;Komura, 2008 #19} Moreover, OX40L can be released by shedding, which might particularly occur after binding to its AML-expressed counterpart. So far, the exact reason for our failure to detect OX40L on NK cells in our ex vivo analyses remains unclear. In addition, it still remains to be determined whether and how OX40/OX40L interactions endogenously affect AML cells or NK cells, as the latter themselves can express OX40.

Nevertheless, our findings deserve consideration with regard to potential consequences of bidirectional signaling following OX40-OX40L when designing approaches to utilize adoptive

transfer of *ex vivo* expanded pNKC. They also may be of relevance for therapeutic strategies aiming for checkpoint stimulation by agonistic OX40 mAbs, as they provide further evidence that OX40 can affect multiple cell types beyond T cells including malignant cells, and the effects of OX40 agonists may thus be more complex than anticipated: the treatment may also (i) directly influence cellular properties of the malignant cells, (ii) their interaction with the microenvironment, (iii) other OX40-expressing immune cells (including NK cells), and, in case of blocking properties of the applied mAb, (iv) reactivity of other OX40L-expressing immune cells in general and NK cell immunosurveillance in particular. 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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Conflict of interest

The authors declare that there are no competing financial interests.

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552

553 **Figure legends**

554 **Figure 1. OX40L expression on human NK cells.**

555 pNKC were generated using standard protocols (comprising medium containing 25U/ml IL-2)
556 with either RPMI 8866 (pNKC-8866) or K562-mb15-41BBL (pNKC-SJ) as feeder cells. NK cells
557 within PBMC of healthy donors (HD-NKC, selected by counterstaining for $CD56^{+}CD3^{-}$) and NK-
558 92 cells served as controls.

559 (A) OX40L mRNA expression in pNKC-8866 and pNKC-SJ generated with PBMC of three
560 different donors and NK-92 cells was determined on day 8 by RT-PCR of equal mRNA levels
561 with 18S rRNA or GAPDH serving as control.

562 (B) OX40L surface expression was analyzed on day 8 by FACS using OX40-Fc or the anti-
563 OX40L mAb clones ANC10G1, ik-1, 11C3.1 (shaded peaks) and their respective controls (open
564 peaks) followed, in case of unlabeled antibodies, by secondary PE-conjugates.

565 (C) SFI levels of OX40L surface expression as obtained by analysis of pNKC-SJ, pNKC-8866
566 and HD-NKC (obtained from seven independent healthy donors each) on day 7 or 8 using mAb
567 ANC10G1 as described above.

568 (D) SFI levels of OX40L surface expression on NK cells were determined at the indicated time
569 points of coculture of PBMC with RPMI8866 or K562-mb15-41BBL as described in (C).

570 (E) To unravel the molecular mechanism involved in OX40L upregulation, PBMC of healthy
571 donors were cultured and analysed by FACS as described above on day 7 after culture with
572 K562-mb15-41BBL (separated by a transwell insert (TW) to prevent cell contact where
573 indicated), K562-mock or K562-4-1BBL as indicated.

574 Data of one representative experiment of a total of three with similar results are shown.

575 *Statistically significant differences, $P<0.05$.

576

577 **Figure 2. OX40L stimulates reactivity of human NK cells.**

578 (A,B) OX40L-negative pNKC-8866 and OX40L-positive pNKC-SJ were cultured alone, on

immobilized OX40-Fc or isotype control for 24 hours. Where indicated, 25 U/ml IL-2 was added during culture. Exemplary results (left) and combined data of at least 6 experiments with pNKC-SJ of independent donors after setting results with pNKC alone to 1 for normalization to account for donor variability (right) are shown. Horizontal bars represent the mean of the results within each culture condition. (A) Expression of CD69 and NKp44 was analyzed by FACS of $CD56^{+}CD3^{-}$ pNKC-8866 (upper two panels) or pNKC-SJ (lower two panels) using specific fluorescence conjugates and isotype control. (B) IFN-γ levels in culture supernatants were determined by ELISA.

(C) Expression of OX40 on transfectants (U937-OX40) and mock controls (U937-mock) was ascertained by FACS (left panels; shaded peaks, OX40 mAb; open peaks, isotype control) prior to use in 4-hour ^{51}chromium release assays with pNKC-SJ (right panel). Exemplary results of one experiment out of five with similar results are shown.

(D) Upper panels: U937-OX40 and U937-mock transfectants were analyzed by FACS using the OX40-mAbs M-OX17 and M-OX2 followed by secondary PE-conjugate. Shaded peaks, specific mAb; open peaks, isotype control. Lower panels: The OX40 mAbs M-OX17 and M-OX2 or isotype control (10µg/ml each) were preincubated with OX40-huFc or huFc-control (both at 2 µg/ml) for 1h. Then pNKC-SJ cells were incubated with pretreated OX40-huFc/huFc-control followed by anti-human PE-conjugate and FACS analysis. Cross-competition and thus blocking properties of the antibodies were identified by the reduction of OX40-huFc binding to pNKC-SJ; dark grey peaks, OX40-huFc preincubated with isotype control; light grey peaks, OX40-huFc preincubated with the OX40mAb; dashed line, huFc-control.

(E) U937-mock (left) or U937-OX40 (right) cells were incubated with pNKC-SJ in the presence or absence of blocking M-OX2 F(ab)2-fragments or isotype control (both at 2µg/ml) and cytotoxicity was evaluated by 4h ^{51}chromium release assays. Data represent means of triplicates with SD, and one representative experiment of a total of at least 3 with similar results is shown.

*Statistically significant differences, $P<0.05$.

Figure 3. OX40 is expressed on AML cells

(A, B) PBMC from AML patients (upper panel) and $CD34^+$ bone marrow and peripheral blood cells of healthy donors (middle and lower panels) were analyzed by FACS using the OX40 mAb BerAct35 (shaded peaks) and isotype control (open peaks) followed by anti-mouse PE. Exemplary (A) and combined (SFI levels, B) results with AML cells of different FAB types are shown.

(C, D) PBMC of AML patients with >90% blast count (C) and CD34-enriched bone marrow cells from healthy donors (D) were analyzed by RT-PCR for OX40 mRNA; 18S rRNA served as control.

(E) Quantitative real time PCR analysis of OX40 mRNA expression in PBMC samples of different OX40 surface-positive and -negative AML patients (both n=8) and CD34-enriched bone marrow cells from 7 different healthy donors.

(F) PBMC of 7 AML patients were exposed to *s*OX40L [2 µg/ml], G-CSF, GM-CSF, IFN-γ, TNF, IL-6, IL-8 and IL10 [all 100 ng/ml] for 24h followed by FACS analysis with a non-competing OX40 mAb (M-OX17).

*Statistically significant differences, $P<0.05$.

Figure 4. OX40 induces cytokine release and metabolic activity of AML cells.

(A) OX40: Fas reporter cells (Jurkat-JOM2) were exposed to the indicated concentrations of the anti-OX40 antibody M-OX17 or isotype control and metabolic activity was measured by WST-1 assays. Representative data from one out of four experiments with similar results are shown.

(B) U937-OX40 and U937-mock transfectants were cultured alone, on immobilized OX40 mAb (M-OX17) or isotype control for 24h. Then IL-8 levels in culture supernatants were determined by ELISA. Representative data from one out of four experiments with similar results are shown.

(C-F) AML patient cells were cultured alone, on immobilized M-OX17 mAb or isotype control for 6h (TNF and IL-8) or 24h (IL-10 and IL-6). Then (C) levels of the indicated cytokines in culture

supernatants were determined by ELISA. Data of n=19 investigated OX40+ patients (UPN 3, 4, 5, 15, 27, 30, 33, 52, 55, 61, 70, 72, 76, 81, 83, 86, 94, 96, 106) are shown. Horizontal bars represent the mean of the results in each culture condition. (D) The results obtained in (C) were analyzed with regard to release of specific cytokine combinations. Positive response (+) was defined as >2-fold increase of each individual cytokine upon OX40-signaling. The percentage of samples responding with the indicated cytokine pattern is depicted. (E) Intracellular cytokine levels were analysed by FACS using specific mAbs and isotype control after 12h. AML cells within PBMC were selected as $CD3^-/CD19^-/CD33^+$. Numbers in upper right quadrants indicate the percentage of $IL\text{-}8^+$ and TNF^+ AML cells. (F) Metabolic activity was measured by WST-1 assays after 24h. Data of n=15 investigated OX40+ patients (UPN 3, 10, 15, 21, 25, 27, 33, 46, 56, 61, 71, 72, 81, 91, 106) are shown.

Horizontal bars represent the mean of the results in each culture condition. *Statistically significant differences, $P<0.05$.

Figure 5. OX40-OX40L interaction enhances NK-cell reactivity against AML cells.

(A, B) OX40L-positive pNKC-SJ were cultured with PBMC of the indicated OX40-expressing AML patients with more than 80% blast count in the presence or absence of blocking OX40-$F(ab')_2$ or isotype control and cytotoxicity was evaluated by 24h ^{51}chromium release assays. In (A), three representative results are depicted, (B) shows data of 17 independent experiments at an E:T ratio of 40:1.

(C) Lysis was analyzed as described above using primary AML cells after MACS-depletion of CD3/CD14/CD19/CD56 cells contained in the samples as targets and NK cells isolated by MACS-isolation from bulk pNKC-SJ as effectors. Three representative results from 9 experiments with similar results are shown.

*Statistically significant differences, $P<0.05$.

Table 1. Patient characteristics and levels of cell-surface OX40

UPN	OX40	FAB	Age	Sex	PBB [%] Diff.	Karyo	WBC [G/l]	Hb [g/dl]	Plt [G/l]	ELN	NCCN	MRC	Etiology
1	1.4	M0	78	m	84	45,XY, -7,+13,-21 [28], 46,XY [2]	85	6.3	20	Adverse	Poor	Poor	pAML
2	1	M0	46	m	97	46, XY	60.5	7	39	Favorable	Favorable	Better	pAML
3	3.5	M0	49	m	83	46, XY, t(1;20)(p32;p13),del(12)(p12p13)[20]	29.2	12.9	39	Intermediate II	Intermediate	Intermediate	sAML
4	2.4	M1	50	f	93	46, XX	270	8	18	Intermediate I	Intermediate	Poor	pAML
5	5.1	M1	40	m	100	complex	81.3	10.8	51	Adverse	Poor	Poor	pAML
6	1.4	M1	27	m	95	46, XY	135.1	8.1	20	Favorable	Favorable	Better	pAML
7	1.3	M1	58	m	93	46,XY	23.9	8.2	42	Intermediate I	Intermediate	Intermediate	n.a.
8	1	M1	83	f	88	n.d.	128.9	7.5	15	n.a.	n.a.	n.a.	pAML
9	2.6	M1	67	m	78	complex	11.1	8.9	48	Adverse	Poor	Poor	pAML
10	2.3	M1	76	f	76	n.d.	15.6	10.7	46	n.a.	n.a.	n.a.	pAML
11	1.3	M1	76	m	64	complex	12180	7,3	134	Adverse	Poor	Poor	tAML
12	1.1	M1	61	m	63	46,XY[25]	59.4	13	69	Intermediate I	Intermediate	Intermediate	tAML
13	1.5	M1	68	m	86	46,XY	84.1	6.7	332	Intermediate I	Poor	Poor	pAML
14	1.5	M1	75	f	84	46, XX	150	10.8	174	Favorable	Favorable	Better	pAML
15	3.8	M1	80	f	93	n.d.	34.9	11.2	28	n.a.	n.a.	n.a.	pAML
16	1.5	M1	72	f	80	46 ,XX [20]	25.8	7.8	50.0	Intermediate I	Intermediate	Intermediate	pAML
17	2.8	M1	64	f	94	n.d.	222	9.2	44	n.a.	n.a.	Poor	pAML
18	1.9	M1	63	f	60	46,XX, del(9)(q22) [5]/46,XX [25]	11.3	11.3	292	Favorable	Intermediate	Intermediate	pAML
19	1.4	M1	50	m	98	46,XY	19.7	10.4	55	Favorable	Favorable	Better	pAML
20	1.4	M1	67	m	99	n.d.	67.7	9.8	23	n.a.	n.a.	n.a.	n.a.
21	1.5	M1	62	m	77	46,XY	45	7.1	270	Intermediate I	Poor	Poor	pAML
22	1.1	M1	50	f	89	46,XX, del(9)(q13;q22)[4]/ 46,XX [16]	13.8	8.8	25	Intermediate II	Intermediate	Intermediate	pAML
23	1	M1	44	m	75	46, XY	10.7	7.9	234	Intermediate I	Poor	Poor	pAML
24	2.2	M1	82	f	81	46, XX	130	10.1	127	Intermediate I	Intermediate	Intermediate	pAML
25	2.7	M1	47	f	98	n.d.	56.3	9.4	60	n.a.	n.a.	n.a.	pAML
26	0.9	M1	76	f	81	46,XX[20].	10.7	7.4	145	Favorable	Favorable	Better	pAML
27	6.9	M1	47	f	91	47,XX, +8	27.6	10.0	26	Intermediate II	Intermediate	Intermediate	sAML
28	1.1	M1	62	m	89	n.a.	18.3	12.3	18	n.a.	n.a.	Poor	pAML
29	1.4	M1	52	m	98	46,XY	153.3	9.2	23	Intermediate I	Poor	Poor	pAML
30	3.8	M1	56	f	87	46, X, t(X;12)(p11;p13)[14];46, XX [7]	4.14	8.3	111	Intermediate II	Intermediate	Intermediate	pAML
31	0.9	M1	68	m	93	n.d.	42.5	9.8	80	n.a.	n.a.	n.a.	pAML
32	1.7	M1	21	f	95	46,XX [16], 46,XX, del(9)(q13q22) [4]	84	7.1	30	Intermediate II	Intermediate	Poor	pAML
33	4.5	M1	41	f	92	46,XX	68.7	8.1	55	Intermediate I	Poor	Poor	pAML
34	1.1	M1	34	m	75	46, XY	33.3	10.2	11	Favorable	Favorable	Better	pAML
35	1.1	M1	38	f	60	46, XX	26	9.6	153	Intermediate I	Poor	Poor	pAML
36	1.2	M2	84	m	79	n.d.	115.2	7.8	107	n.a.	n.a.	n.a.	pAML
37	2.3	M2	30	m	53	46, XY	24.5	7.4	54	Intermediate I	Poor	Poor	pAML
38	1.1	M2	54	f	71	46,XX,t(8;21)(q22;q22)[21]	9	8.9	40	Favorable	Favorable	Better	tAML
39	1.2	M2	38	m	28	46, XY	8.8	8.7	52	Intermediate I	Intermediate	Intermediate	sAML
40	1.1	M2	72	m	97	complex	56.9	8.9	30	Adverse	Poor	Poor	sAML
41	1.4	M2	56	m	63	46, XY	8.8	9.7	311	Favorable	Favorable	Better	pAML
42	1.2	M2	81	m	96	n.d.	10	10	46	n.a.	n.a.	n.a.	sAML
43	1.2	M2	66	m	80	n.d.	27.8	10.1	49	Favorable	Better	Better	pAML
44	2.3	M2	29	m	70	46,XY	9.6	8.5	17	Intermediate I	Poor	Poor	tAML
45	1.3	M2	73	f	83	46,X,inv(X)(p22.3q13) [14]; 46,XX [6]	5	6.3	95	Intermediate II	Intermediate	Intermediate	pAML
46	1.8	M2	73	m	83	46,XY	33.8	8.1	80	Intermediate I	Poor	Poor	pAML
47	1.4	M2	68	m	96	46,XY	85.5	9.5	146	Intermediate II	Intermediate	Intermediate	pAML
48	1.6	M2	72	m	81	46, XY, del(17)(q11q21) [6]/47,XY,+8 [3] 46, XY [16]	78.9	6,4	47	Intermediate II	Intermediate		pAML
49	1.4	M2	51	f	43	46,XX,t(2;2)(q21;q23)[21]	35.3	9.3	51	Intermediate II	Intermediate	Better	pAML
50	1.2	M2	48	m	87	46,XY	36.9	7.1	66	Favorable	Favorable	Better	pAML
51	1.8	M2	63	m	78	46,XY	180	8	30	Favorable	Favorable	Better	pAML
52	1.5	M2	64	m	82	47, XY+8[16]/47,XY,+8,del(9)(q22)[1]/46,XY,del(9)(q22)[1]/46,XY[7]	338.5	8.1	19	Intermediate II	Intermediate	Intermediate	pAML
53	0.4	M2	58	f	92	46,XX	62	7.4	25	Intermediate I	Poor	Poor	sAML
54	1.1	M2	78	m	99	45, XY, -7[7], 46, XY[15]	26	9.7	36	Adverse	Poor		sAML
55	6.4	M2	68	m	84	47,XX,+11[23]	165.5	3.8	222	Intermediate II	Intermediate	Poor	pAML
56	1.6	M2	71	f	94	46, XX	105.4	10.4	83	Intermediate I	Poor	Poor	pAML
57	1.7	M2	58	m	20	complex	39.1	9.3	23	Adverse	Poor	Poor	pAML
58	1.3	M2	23	f	69	47,XX, +21 [2]/46,XX [23]	50	9.9	135	Intermediate II	Intermediate	Intermediate	pAML
59	1.1	M2	71	f	81	47, XX +11	26.1	9.4	10	Intermediate II	Intermediate	Intermediate	sAML
60	1.3	M2	45	m	80	complex	17.1	9.0	51	Favorable	Favorable	Better	pAML
61	2.5	M2	44	m	94	46,XY	138.5	10.3	57	Intermediate I	Poor	Poor	pAML
62	1.1	M2	45	m	94	47, XY, +8	44.4	8.7	11	Intermediate II	Intermediate	Poor	pAML
63	2.2	M2	45	m	94	complex	44.4	8.7	11	Adverse	Poor	Poor	pAML
64	1.3	M2	68	m	93	47, XY, +11	110.9	8	27	Intermediate II	Intermediate	Intermediate	pAML
65	0.9	M2	70	f	20	47,XX,+8,del(17)(p11-12)[5]/46,XX[20]	4	13,6	182	Adverse	Poor	Poor	sAML
66	5.7	M3	46	m	51	complex	23.1	13.3	25	n.a.	n.a.	n.a.	pAML
67	1.1	M3	67	f	94	46,XX	99	9.9	128	Intermediate II	Intermediate	Intermediate	sAML
68	14.3	M3	65	m	40	46, XY, t(15;17)(q22;q12)[20]	6.9	8.5	17	Favorable	Favorable	Better	pAML
69	5.2	M3	58	f	96	46,XX,t(15;17)(q22;q12)[20].	42.1	8.4	17	Favorable	Favorable	Better	pAML
70	3	M4	70	f	71	45, XX, -7	282.3	5.7	68	Adverse	Poor	Poor	sAML
71	1.8	M4	49	m	72	46,Y,t(X;17)(p11;p17),add(21)(q22)[13]	61.9	5.8	18	Adverse	Poor	Poor	pAML
72	2.9	M4	71	m	93	47, XY, + 11 (6)	79.7	9.9	40	Intermediate II	Intermediate	Intermediate	pAML
73	1.4	M4	55	m	72	46,XY	17.2	8.6	162	Favorable	Favorable	Better	pAML
74	9.7	M4	61	m	70	46, XY	85.4	8.5	12	Intermediate I	Poor	Poor	pAML
75	6.1	M4	76	f	94	46, XX, t (9;11) (q22; q23) [12]/52, XXX, +3, +6, +8 t (t;11) (q22; q23) +12, +13, +18 [13]	141	12	70	Adverse	Poor	Poor	tAML
76	7.3	M4	43	f	86	46, XX	63.5	9.2	178	Favorable	Favorable	Better	pAML
77	1.3	M4	41	f	73	complex	112.7	8.5	30	Adverse	Poor	Poor	pAML
78	5	M4	63	m	96	46,XY	92.3	10.5	431	Intermediate I	Poor	Poor	pAML
79	2.2	M4	73	f	85	46,XX (25)	52.6	7.5	48	Intermediate I	Poor	Poor	tAML
80	1.2	M4	71	m	75	46, XY,del(20)(q11)[6],47,idem,+11[14];	161.1	8.6	61	Intermediate II	Intermediate	Intermediate	sAML
81	1.6	M4	83	f	95	46,XX add(14)(p11)[8],/46, XX[5]	155.8	11.6	144	Intermediate II	Intermediate	Intermediate	pAML
82	2.3	M4	78	f	39	51 ,XX,+6,+9,+9,+11 ,+13[20]	8.8	9.2	146	Adverse	Poor	Poor	pAML
83	2.7	M4	64	m	22	46,XY	62.4	7.5	40	Intermediate I	Poor	Poor	n.a.
84	1.5	M4	36	m	78	46, XY	207.4	6.1	55	Intermediate I	Poor	Poor	pAML
85	2	M4	68	m	82	46, XY	148.7	9.1	134	Intermediate I	Poor	Poor	pAML
86	4.4	M4	67	m	75	46,XY,?dup(9)(p13p22)c	64.4	9.1	33	Intermediate II	Intermediate	Intermediate	pAML
87	0.6	M4	72	f	81	46,XX	26.1	8.2	70	Intermediate I	Intermediate	Intermediate	pAML
88	2	M4	54	f	46	46, XX	17.2	10.6	167	Intermediate I	Poor	Poor	pAML
89	1	M4	57	m	68	n.d.	334	9.4	293	n.a.	n.a.	n.a.	pAML
90	2.2	M4	57	f	29	46,XX	17.5	8.0	117	Intermediate I	Intermediate	Intermediate	pAML
91	3.3	M4	56	f	64	46, XX, inv(16)(p13.1q22)[11]	56.6	10.9	99	Favorable	Favorable	Better	pAML
92	0.8	M5	70	m	90	46,XY	190	7.1	65	Intermediate I	Intermediate	Intermediate	pAML
93	1.6	M5	70	m	4	46, XY	73.6	13.2	25	Favorable	Favorable	Better	pAML
94	1.6	M5	26	f	18	46, XX	51	6,2	17	Favorable	Favorable	Better	pAML
95	7.3	M5	73	m	97	47, XY,+8[2]: 46,XY[23]	78.9	6.4	47	Intermediate II	Intermediate	Poor	tAML
96	28.5	M5	69	f	80	n.d.	275	7.1	47	n.a.	n.a.	Poor	pAML
97	1.3	M5	65	m	91	n.d.	394.2	7.9	189	n.a.	n.a.	n.a.	sAML
98	1.7	M5	69	m	11	n.d.	22.1	8.7	53	n.a.	n.a.	n.a.	pAML
99	1.1	M5	24	m	90	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.	pAML
100	1.3	M5	78	m	49	complex	11.7	8	13	Adverse	Poor	Poor	pAML
101	1.3	M5	74	m	>90	46,XY	239	5.7	122	Intermediate I	Poor	Poor	pAML
102	9.5	M5	52	f	8	46, XX	15.7	7.2	21	Favorable	Favorable	Better	pAML
103	1.2	M5	54	m	89	46, XY, del(9)(q13q22)[8]/48, XY[16]	109.4	8.2	78	Intermediate II	Intermediate	Intermediate	pAML
104	4.6	M5	72	m	40	46,XY	14.5	5.3	28	Intermediate I	Intermediate	Intermediate	sAML
105	31.6	M5	82	m	35	n.d.	61.3	11.7	110	n.a.	n.a.	n.a.	pAML
106	1.5	M5	23	m	83	48,XY,+8,+13[17]/46,XY[3]	145.1	7.6	34	Intermediate II	Intermediate	Poor	pAML
107	1.3	M5	58	f	70	46,XX	0.6	9.1	42	Intermediate I	Poor	Poor	pAML
108	0.7	M5	45	f	80	46,XX	248.1	7.3	46	Favorable	Favorable	Better	pAML
109	18.7	M5	53	m	85	46,XY	105.6	8.1	35	Intermediate I	Poor	Poor	pAML
110	7	M5	50	m	3	46, XY	27.3	11.3	85	Intermediate I	Poor	Poor	pAML
111	0.9	M5	54	m	96	46,XY	70.3	8.1	139	Favorable	Favorable	Better	pAML

UPN, uniform patient number; OX40 SFI, specific fluorescence index; FAB, French-American-British classification; F, female; M, male; PBB, peripheral blood blasts among nucleated cells; WBC, white blood count; Hb, hemoglobin; Plt, platelets; ELN, European LeukemiaNet; NCCN, National Comprehensive Cancer Network; MRC, Medical Research Council; Etiology: pAML = de novo AML, tAML = therapy related AML, sAML = secondary AML evolving from MDS/MPS; n.d., not determined and n.a., not available.

Table 2. Association of OX40 Expression with genetic landscape and clinical parameters

Characteristic	Analyzed Patients	P Value two-sided*	Cox-Regression	Correlation**
PML/RARA t(15;17)	96	<0.05		
RUNX1/RUNX1T1 t(8;21)	94	n.s.		
CBFB/MYH11 inv(16)	93	n.s.		
subtype of FLT3 mutation				
FLT3-ITD	84	<0.05		
FLT3-TKD	83	n.s.		
NPM1	84	n.s.		
CEBPA	55	n.s.		
MLL-PTD	78	n.s.		
ELN	90	n.s.		
ELN 2017	88	n.s.		
Survival	105		n.s.	
CR	63	n.s.		
Etiology	108	n.s.		
pAML	87			
sAML	14			
tAML	7			
Relapse		n.s.		
FAB	105	n.s.		
age at diagnosis	110			n.s.
sex	110	n.s.		
PBB [%]	109			n.s.
WBC	108			n.s
Plt[G/l]	108			n.s.
HB [g/dl]	108			n.s.
CRP [mg/dl]	108			n.s.

ELN, European LeukemiaNet classification; ELN 2017, modified European LeukimiaNet classification; CR, complete remission; pAML, de novo AML; tAML,therapy related AML; sAML, secondary AML evolving from MDS/MPS; FAB, French-American-British classification; PBB, peripheral blood blasts among nucleated cells; WBC, white blood count; Plt, platelets; HB, hemoglobin; CRP, C-reactive protein; n.s. not significant

* all P values are two-sided and were calculated either with Mann-Whitney or Kruskal-Wallis test or, for categorial variables, with the chi-square tests.

** Correlation Spearman r

Figure 1

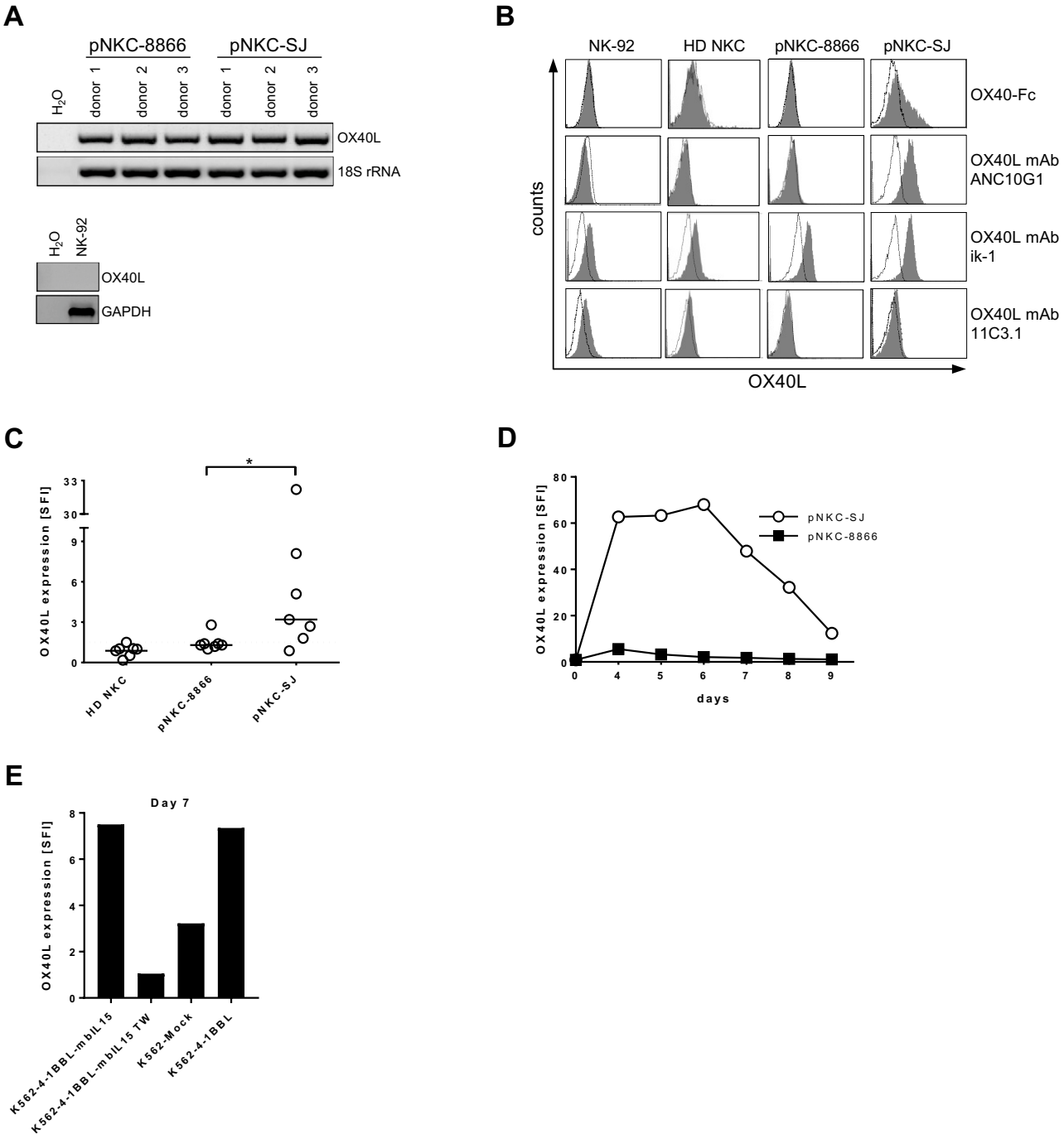

A
pNKC-8866
pNKC-SJ
H2O
donor 1
donor 2
donor 3
donor 1
donor 2
donor 3
OX40L
18S rRNA
H2O
NK-92
OX40L
GAPDH
B
NK-92
HD NKC
pNKC-8866
pNKC-SJ
OX40-Fc
OX40L mAb ANC10G1
OX40L mAb ik-1
OX40L mAb 11C3.1
counts
OX40L
C
OX40L expression [SFI]
33
30
9
6
3
0
*
HD NKC
pNKC-8866
pNKC-SJ
D
OX40L expression [SFI]
80
60
40
20
0
pNKC-SJ
pNKC-8866
0
4
5
6
7
8
9
days
E
Day 7
OX40L expression [SFI]
8
6
4
2
0
K562-4-1BBL-mbIL15
K562-4-1BBL-mbIL15 TW
K562-Mock
K562-4-1BBL

Figure 2

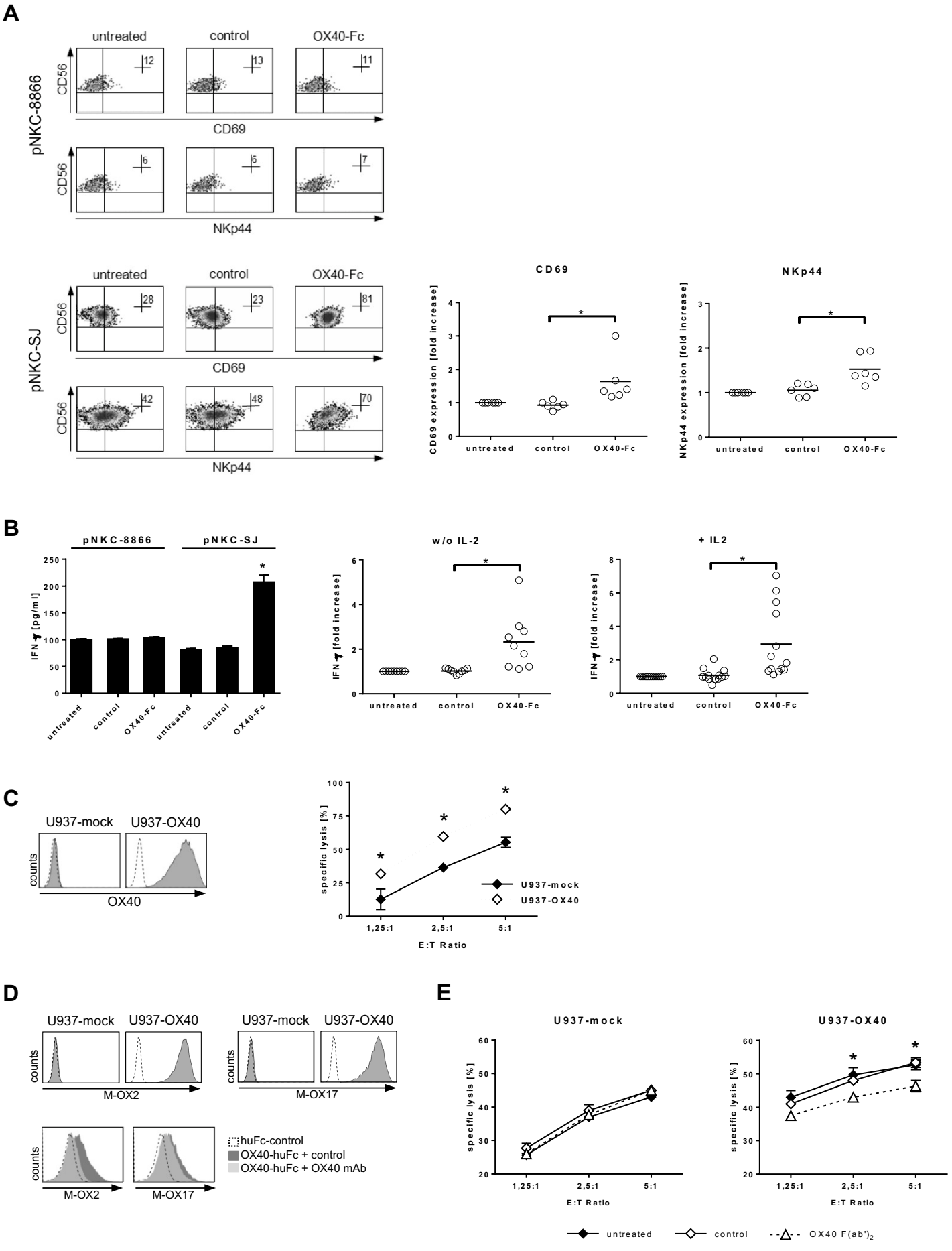
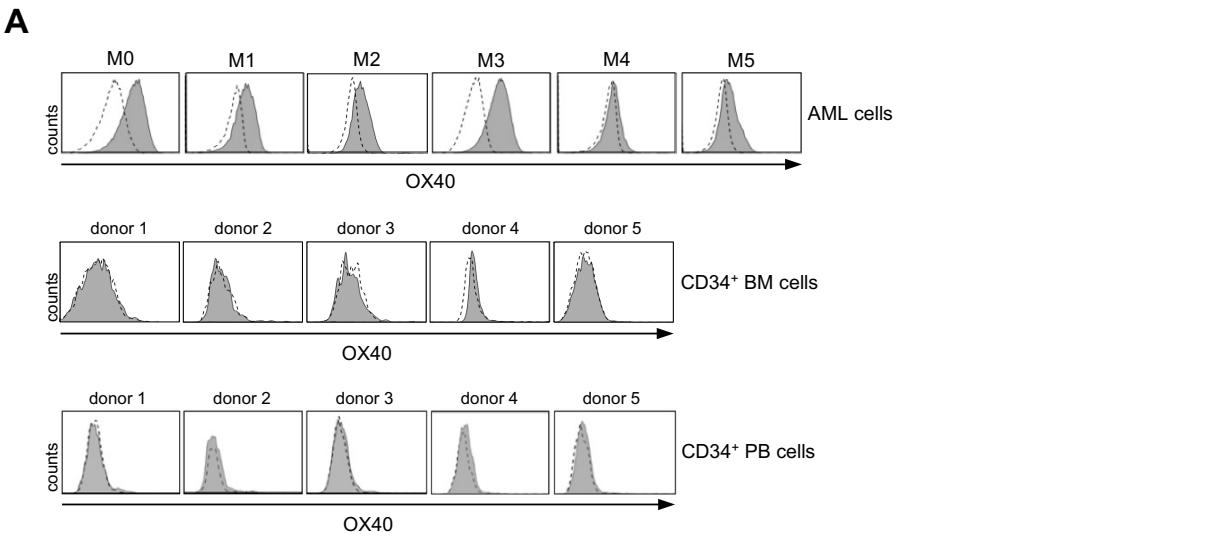
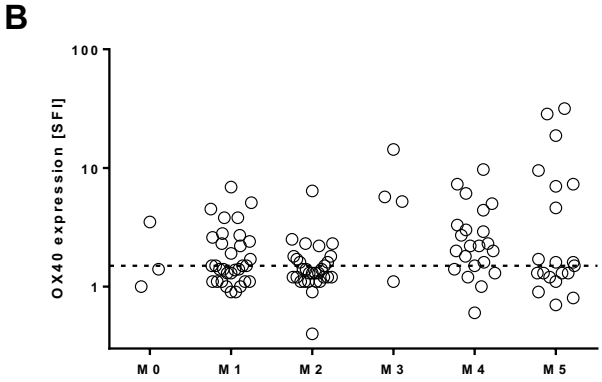
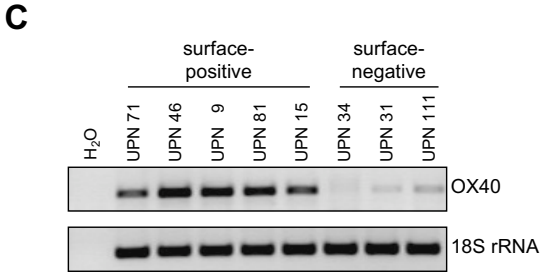
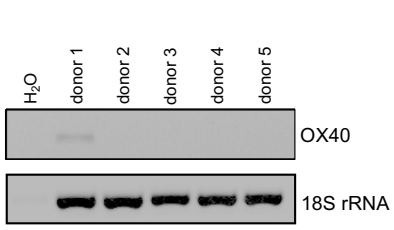

A
untreated
control
OX40-Fc
pNKC-8866
CD56
CD69
NKp44
12
13
11
6
6
7
pNKC-SJ
28
23
81
42
48
70
CD69
NKp44
CD69 expression [fold increase]
NKp44 expression [fold increase]
B
pNKC-8866
pNKC-SJ
IFN-γ [pg/ml]
w/o IL-2
+ IL2
IFN-γ [fold increase]
C
U937-mock
U937-OX40
counts
OX40
specific lysis [%]
E:T Ratio
1,25:1
2,5:1
5:1
D
M-OX2
M-OX17
huFc-control
OX40-huFc + control
OX40-huFc + OX40 mAb
E
U937-mock
U937-OX40
untreated
control
OX40 F(ab')2

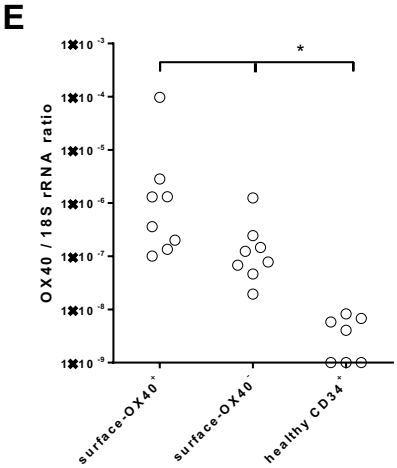
Figure 3


A
M0
M1
M2
M3
M4
M5
counts
AML cells
OX40
donor 1
donor 2
donor 3
donor 4
donor 5
CD34+ BM cells
CD34+ PB cells


B
OX40 expression [SFI]
100
10
1
M 0
M 1
M 2
M 3
M 4
M 5


C
surface-positive
surface-negative
H2O
UPN 71
UPN 46
UPN 9
UPN 81
UPN 15
UPN 34
UPN 31
UPN 111
OX40
18S rRNA


D
H2O
donor 1
donor 2
donor 3
donor 4
donor 5
OX40
18S rRNA


E
OX40 / 18S rRNA ratio
*
surface-OX40+
surface-OX40-
healthy CD34+

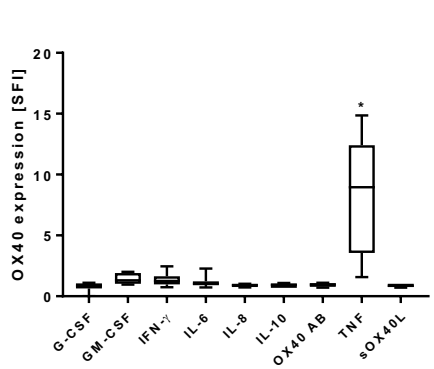

F
OX40 expression [SFI]
20
15
10
5
0
*
G-CSF
GM-CSF
IFN-γ
IL-6
IL-8
IL-10
OX40 AB
TNF
sOX40L

Figure 4

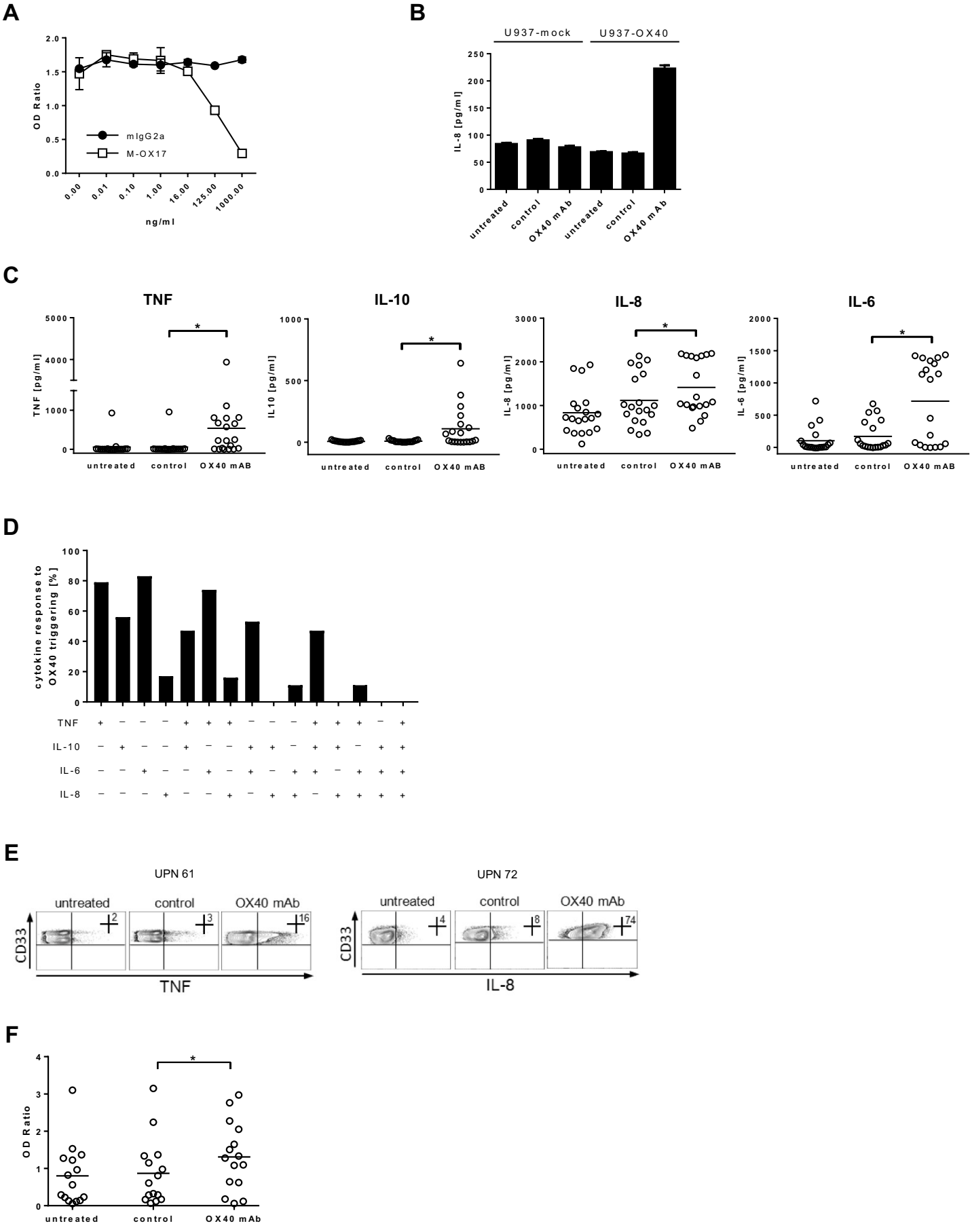
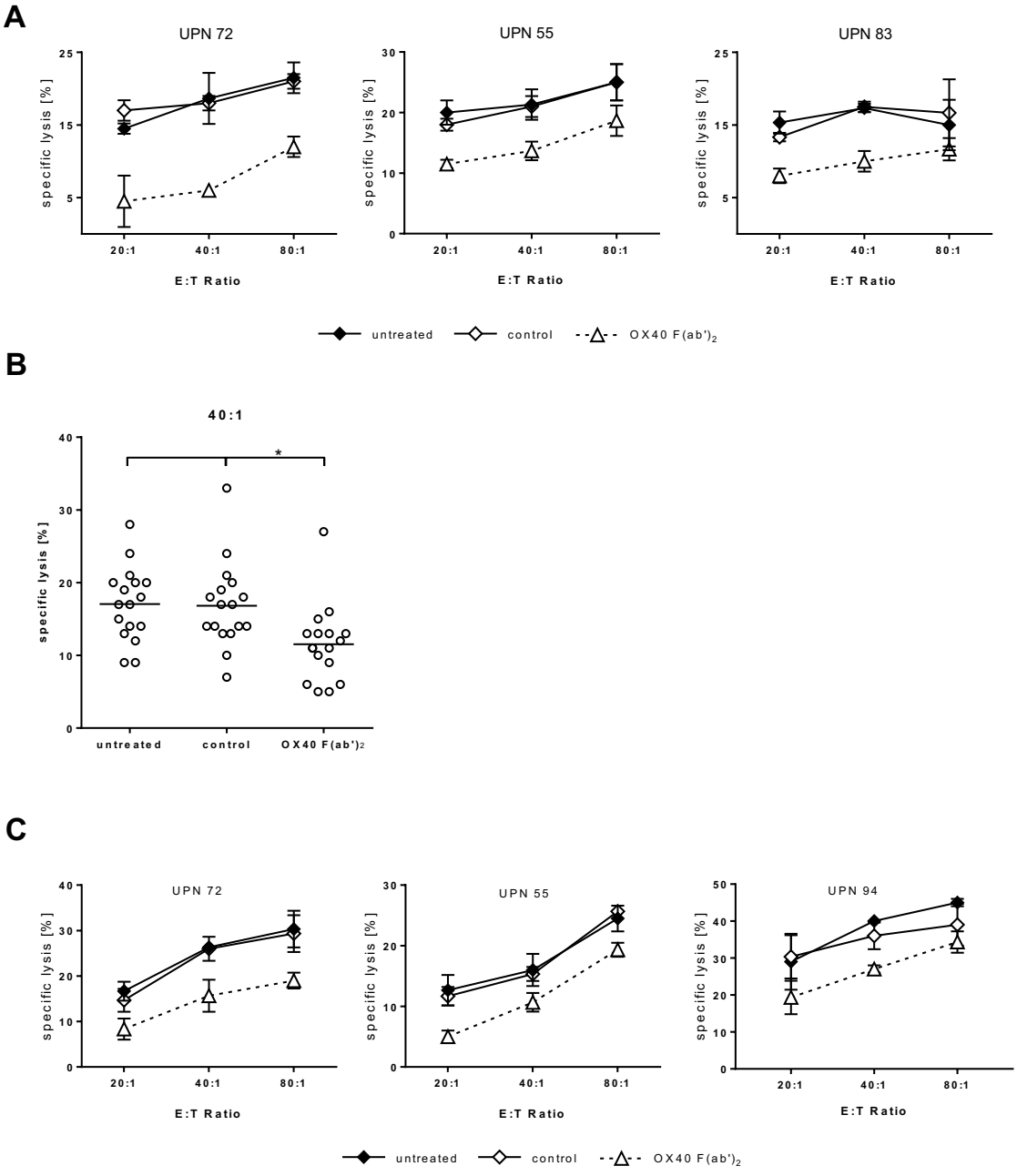

A
OD Ratio
mIgG2a
M-OX17
ng/ml
B
U937-mock
U937-OX40
IL-8 [pg/ml]
untreated
control
OX40 mAb
C
TNF
IL-10
IL-8
IL-6
TNF [pg/ml]
IL10 [pg/ml]
IL-8 [pg/ml]
IL-6 [pg/ml]
D
cytokine response to OX40 triggering [%]
TNF
IL-10
IL-6
IL-8
E
UPN 61
UPN 72
CD33
TNF
IL-8
F
OD Ratio

Figure 5


A
UPN 72
UPN 55
UPN 83
specific lysis [%]
E:T Ratio
20:1
40:1
80:1
untreated
control
OX40 F(ab')2
B
40:1
*
specific lysis [%]
untreated
control
OX40 F(ab')2
C
UPN 72
UPN 55
UPN 94
specific lysis [%]
E:T Ratio
untreated
control
OX40 F(ab')2