Expression of 4-1BB and its ligand on blasts correlates with prognosis of patients with AML

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ABSTRACT

Costimulatory ligands (COLs) and their receptors (COR) regulate immune reactions and cellular survival and might be relevant in acute myeloid leukemia (AML). This study evaluated the clinical relevance of 4-1BBL, glucocorticoid-induced TNFR-related protein (GITR) and ligand (GITRL), CD80, and CD86 in case of expression on AML blasts. 98 patients were evaluated at initial diagnosis. Immunophenotypically evaluated specific fluorescence index (SFI) levels of COR and COL on blasts were correlated with morphological, cytogenetic, and several prognostic parameters. Significantly higher COR expression was seen in monocytic versus non-monocytic AML subtypes; GITR, p=0.05; GITRL, p=0.005; CD86, p=0.001). Cut-off values for two COR and their ligands were evaluated: cases presenting with 4-1BB values above cut-off 1.2 SFI levels correlated (tendentially) significantly with a higher probability for disease-free survival (DFS, p=0.06) and a favorable HR of 0.2; p=0.04 for relapse. HR for death was also significantly lower in this group (0.12; p=0.04). In contrast, a lower probability for DFS and overall survival was seen in cases with 4-1BBL expression above 2.2 SFI levels (p=0.08 and p=0.09). In addition, multivariate analysis showed a significantly higher probability of death in this group (HR 10.3, p=0.04). Expression of CD80 and CD86 did not show significant prognostic relevance. On initial diagnosis, 4-1BB and 4-1BBL qualify as markers for prediction of patients' course and represent a valuable screening target for patients with AML at initial diagnosis.

INTRODUCTION

Members of the tumor necrosis factor (TNF) receptor (R) family play a central role in cellular regulations like activation, proliferation, and differentiation of cells. With regard to hematologic diseases such as acute myeloid leukemia (AML), immunological interactions of TNFR with ligands and the capability of TNFR to promote intracellular apoptosis and survival might be of importance with respect to patients' (pts') prognosis, as described for markers such as TNF-related apoptosis-inducing ligand receptors 2, 3, TNFR1,[1] and other costimulatory receptors and ligands (COR and COL).[2] The characteristics of COR

Significance of this study

What is already known about this subject?

- Glucocorticoid-induced TNFR-related protein (GITR) and ligand (GITRL) are expressed on blasts in acute myeloid leukemia (AML) cells[16] and in other hematological diseases such as chronic lymphatic leukemia (CLL) and chronic myeloid leukemia. In AML and CLL, we showed evidence that expression on tumor cells might contribute to impaired immune response in the context of inhibition of natural killer-cell performance as a mechanism of immune escape.[12]
- 4-1BB and 4-1BBL are known to be expressed on antigen-presenting cells as well as on carcinoma cells, AML blasts, and non-Hodgkin's lymphoma and can be induced to activate T-cells. The soluble form of 4-1BBL is present at high levels in sera from patients with hematological diseases and is of prognostic value: low levels of release correlate with better prognosis (longer disease-free survival or probability to achieve complete remission in myelodysplastic syndromes and AML), but not in non-Hodgkin's lymphoma.[8 9 24]
- High expression of CD80 and CD86 on AML blasts correlated with 'non-responders' to TAD/HAM therapy and cut-off values could be evaluated, which allowed a significant separation of cases with high expression (correlating with worse progression-free survival), although no correlation with unfavorable karyotype was found.[45]
- The presented costimulatory markers and ligands in this study have a known impact on immunological interactions and can influence cancer defense, thus might also be prognostically relevant in patients with AML.

What are the new findings?

- 4-1BB and 4-1BBL expressed on blasts at initial diagnosis are found to be relevant markers to predict prognosis as shown by multivariate analysis.

Significance of this study

- In contrast to chronic lymphatic leukemia, GITR and its ligand do not have an impact on patients' survival if expressed on the surface of AML blasts at initial diagnosis.
- CD80 and CD86 do not have an influence on patients' survival if expressed on blasts surface at initial diagnosis.

How might these results change the focus of research or clinical practice?

- We showed that standard flow cytometry screening for 4-1BB and 4-1BBL and other COR/COL on blasts of initially diagnosed AML provides a simple method to predict patients' prognosis. This screening method might provide a basis for a prompt and patient-specific treatment plan adjusted for prognostic data gained from AML blasts' surface expression.

4-1BB, glucocorticoid-induced TNFR-related protein (GITR), known to perform immunological regulation, and COL 4-1BBL, and GITRL, which interact with immune effectors of the host (table 1), might be critical for prognostic evaluations and for clinical understanding when expressed on AML blasts. Furthermore, CD80 and CD86 show also evidence of being prognostically valuable, however indicated in only small groups of AML pts.

TNFR 4-1BB (CD137) is expressed on the surface of CD4+ and CD8+ T-cells during activation processes and interacts with its ligand (L) 4-1BBL, expressed on antigen-presenting cells (APC) (c4-1BBL), thereby mediating and regulating immune responses.[3 4] Stimulation of 4-1BB by monoclonal antibodies (mAbs) has been shown to eradicate established tumor tissue in mice, suggesting a crucial role in cancer defense and is best characterized by interaction with T-cells leading to enhanced immune responses as reviewed elsewhere.[5] Choi *et al* reported a complex relationship between immunological response and receptor expression of myeloid cells and dendritic cells (DC), natural killer (NK) cells and tissue cells. These cells are proven to have an impact on T-cell performance by negatively regulating T-cell responses via their 4-1BB expression and interaction.[6] Impact of 4-1BB expression on AML blasts on the immune system remains unclear.

4-1BBL is also known to mediate important functions of apoptosis and regulates development of solid tumor (seen in colon carcinoma cell lines HT-29, Colo205, and HCT116), as well as AML in cases of coexpression.[7 8] Membrane-bound (c) 4-1BBL was previously reported to be expressed on AML blasts.[8] The soluble (so) 4-1BBL form in sera of AML pts was shown to correlate negatively with progression-free survival and positively with bone marrow (BM)-blast counts, unfavorable French-American-British (FAB) categorization, and cytogenetic risk,[9] whereas no correlation was found for c4-1BBL[8] in small AML cohorts.

The GITR is a costimulatory receptor predominantly expressed on CD4+CD25+ natural regulatory T-cells (T_{reg}) and NKs. While there is a consensus of a costimulatory role after engagement of GITR in T-cells,[10] the role in NK cells remains controversial and can be activating or inhibitory (as reviewed in ref. 11). In chronic lymphatic leukemia (CLL), GITR expression is also documented, shown to induce TNF production, and considered a survival factor when stimulated.[12] Studies suggest that GITR is also expressed on myeloid cells such as macrophages with TNF induction[13] promoting macrophage-related survival.[14] GITR stimulation in mice suffering from carcinoma leads to improved overall survival (OS) due to enhanced host-related immune reactivity.[15] The prognostic impact when expression on cells' surface of human hematological diseases such as AML is not yet clear; however, signaling via GITRL in AML pts was reported to induce TNF and interleukin (IL)-10, implicating physiological activity of GITR in case of AML blasts.[16]

GITRL is a membrane associated protein mainly expressed on macrophages and other APC. Engagement of the GITR–GITRL pathways activates T-cell-related proliferation[17] and NK-cell performed lysis.[18] In macrophages, GITRL stimulation induces proinflammatory mediators such as NF-κB and promotes survival.[13] GITRL is also expressed on the surface of AML blasts;[16] however, significance remains unclear.

CD80 and CD86 are expressed on APC. Constitutive expression of CD80 and CD86 is known for B-cell leukemia and lymphoma cells. Transfection of AML blasts with CD80 is shown to induce immune defense and

Table 1 Overview of physiologic receptor expression

Receptors/CD	Expression profile	Function
4-1BB (CD137)	Expressed on T-cells, natural killer (NK) cells, antigen-presenting cells (APC) like dendritic cells (DC), B-cells, monocytes	Induction of interleukin (IL)-6 and IL-12 secretion by DC. Central element in activation of nuclear factor kB
4-1BBL (CD137 ligand)	Expressed on APC like DC, B-cells, monocytes, splenic DC	Mediates contact to cytotoxic lymphocytes. Interaction with its receptor on T cells leads to production of IL-2 and IL-4 by CD4+ T-cells and T-cell proliferation
GITR (CD357)	Expressed on T-cells and APC (B-cells, macrophages, and DC)	Important for T-cell activation and regulation. Influence on 'non-T-cells' not known
GITRL (CD357 ligand)	Expressed on APC (B-cells, DC)	Modulates immune stimulation of T-cells and regulates inflammation and self-tolerance
B7-1/B7-2 (CD80/CD86)	Expressed on APC (B-cells, DC)	Ligands for CD28 and CTLA-4. Costimulatory or regulatory signals of CD80 and CD86 for T-cell proliferation and cytokine production

blast destruction, suggesting relevance in tumor immunogenicity.[19]

The TNFR family is important for intracellular regulation as well as for interaction with immune effectors. Evidence of clinical significance of blast expression in the complex composition found in AML pts initially diagnosed is still lacking. To further elucidate the clinical significance of 4-1BB, GITR, and ligands, as well as to improve weak data regarding significance of CD80 and CD86 expressed on AML blasts, we used flow cytometry-based SFI evaluation[20] to quantify receptor/ligand expression on AML blasts from initially diagnosed pt samples and correlated results with clinical parameters.

MATERIALS AND METHODS

Pts' characteristics and sample collection

Peripheral blood (PB) samples from 98 consecutive AML pts at initial diagnosis were collected after pts declared written informed consent in accordance with the Helsinki protocol of 1975, as revised in 2013 and the local Ethics Committee (13/2007V). All pts were included into the study at initial diagnosis. Diagnosis and classification of AML cases were based on morphology and cytochemistry of BM according to the FAB classification[21] as well as by using cytogenetic and molecular-genetic evaluations. All samples were obtained before treatment.

Twenty pts presented with an undifferentiated leukemia (M0: n=4, M1: n=16), 41 pts with an immature granulocytic leukemia (M2: n=34, M3: n=7), and 34 pts with a monocytic leukemia (M4: n=23, M5: n=11); 2 pts had an erythroleukemia (M6) and 1 pt was not FAB categorized. Furthermore, subdivision of cases was performed in cases with monocytic (n=34) and non-monocytic (n=63), and one pt was not classified. In 83 pts, a primary (p) was diagnosed; in 12 pts, a secondary (s) AML was diagnosed; and 3 pts were not categorized. The median age was 59 years (range: 18–85 years). The male:female ratio was 1:0.56. Pt characteristics are shown in supplementary appendix 1.

Anthracyclin-based induction therapy (idarubicin or daunarubicin) was applied in 55 of 98 pts. Of these 55 pts, 34 pts achieved allogeneic stem cell transplantation. In 43 of 98 remaining pts, other approved therapies or supportive therapy was used. Response to induction chemotherapy was defined for pts achieving complete remission (CR) 25–35 days after start of first induction chemotherapy. CR was determined in case of normocellular BM, containing <5% blasts, when platelet count was over 100,000/μL and neutrophil granulocytes in PB had recovered to 1500/μL according to Cancer and Leukemia Group criteria.

Relapse was defined when >5% BM blasts were counted or leukemic infiltration occurred at any other site. The investigated samples contained on average 73% (range: 14–99%) leukemic blasts in the whole mononuclear fraction of PB. White cell count (WCC) fraction was on average 64.2 G/L (range: 0.91–394.2 G/L), hemoglobin 9.0 g/dL (range: 3.3–14.7 g/dL), and platelets 74.2 G/L (range: 8–347 G/L).

Cytogenetic

Cytogenetic analyses were performed by standard methods at the University of Ulm, Muenchner Leukaemie Labor GmbH, or by Dr Eberhard & Partners Dortmund, Germany. Samples were stratified according to the National Comprehensive Cancer Network (NCCN) guidelines.[22] According to NCCN, a better risk abnormality was defined by the presence of t(8;21), t(15;17), or inv/t(16); *NPM1*; or mutated *CEBPA* without *FLT3-ITD*, and an intermediate risk was stated if normal cytogenetics, trisomy 8, t(9;11) or t(8;21), inv (16), and t(16;16) with cKIT mutation was detected. A poor risk abnormality was considered in pts with 11q23 (other than t(9;11)), del5/5q, del7/7q aberrations, t(6;9), inv(3), t(3;3), t(9;22) aberrations or a complex karyotype (three or more numerical and/or structural abnormalities), and normal cytogenetics with FLT3-ITD mutation.

According to European Leukemia Network (ELN) criteria,[23] a favorable abnormality was defined with the presence of t(8;21), inv(16), t(16;16), a mutated *NPM1*, or a mutated *CEBPA* gene (normal karyotype). Intermediate subtypes (I and II) were combined and categorized by a mutated NPM1 and FLT3-ITD (normal karyotype), wild-type NPM1 and FLT3-ITD (normal karyotype), wild-type NPM1 without FLT3-ITD (normal karyotype), t(9;11) (p22;q23), and cytogenetic abnormalities not classified as favorable or adverse. An adverse abnormality was defined in case of an inv(3) (q21;q26.2), t(3;3) (q21;q26.2), t(6;9) (p23;q34), MLL rearrangement, −5 or del (5q), −7, abnl (17p), or a complex karyotype (three or more numerical and/or structural abnormalities). Study cohort showed the following distribution according to ELN/NCCN: favorable/intermediate/adverse: 20%/50%/17% of pts.

Cells were characterized by flow cytometry

Mononuclear cells (MNC) were isolated from whole PB samples by density gradient centrifugation with Ficoll-Hypaque (Biochrom, Berlin, Germany), then washed and resuspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany). Cell counts were quantified by Neubauer counting chambers, and cells frozen with standardized procedures and stored in liquid nitrogen until use. The surface expression of the respective receptors and ligands on blasts of AML pts was determined by flow cytometry. Since fluorochrome-labeled mAbs were not available for every given receptor/ligand and in order to amplify potentially weak fluorescence signals, we applied sequential staining steps (indirect staining): after blocking of unspecific binding sites with human immunoglobulin G1 (10 μg/mL), MNC were incubated with anti-human mAbs specific for 4-1BB/4-1BBL, GITR/GITRL, CD80 or CD86, or their respective isotype control. Afterwards incubation with species-specific phycoerythrin conjugates was performed. After a washing step, AML cells were selected by staining with fluorochrome-conjugated mAbs specific for CD33, CD34, or CD117, or a combination of the above-mentioned mAbs depending on each individual patient's blast phenotype as determined by immunophenotyping at initial diagnosis. Antibodies were provided by Ancell, Stillwater, Minnesota, USA[a]; R&D, Minneapolis, Minnesota, USA,[b] and BD Biosiences, San Jose, California, USA[c]: 4-1BB[a], GITR[b], GITRL[b], CD80[c], CD86[c], CD33[c], CD34[c], CD117[c], and isotypes[c]. The antibody against 4-1BBL was described previously.[24] Expression patterns in healthy controls were also performed using BM samples from healthy donors after gating

on CD34+ cells. A negative expression profile could be documented in all four control cases with all COR/COL markers (see supplementary appendix 2). Analyses were performed using a FC500 (Beckman Coulter, Krefeld, Germany). Specific fluorescence indices (SFIs) were calculated by dividing median fluorescence obtained with specific mAb by median fluorescence obtained with control.[20]

Statistical analyses

Data were presented as mean or median±SD as appropriate. Statistic comparisons for two groups were performed using the t-test, Mann-Whitney-Wilcoxon test, Fisher's exact test, or spearman correlation coefficient. The statistical analysis was performed with JMP 10.0 statistical software (SAS Institute, Cary, New York, USA). Significant differences were considered in cases with *p=0.05–0.1 (tendentially significant), **p<0.05 (significant), and ***p≤0.005 (highly significant). DFS and OS analyses were performed by the Kaplan-Meier method in combination with a log-rank test. Multivariate analyses were performed using a Cox proportional hazards model. Values evaluated by flow cytometry are median SFI levels. Cut-offs were evaluated that allowed an optimal separation of cases in those with higher/lower probability for DFS. Data are presented in combination with the corresponding clinical data (eg, cytogenetic values, response to therapy, and age).

RESULTS

We studied expression profiles of GITR, GITRL, 4-1BB, 4-1BBL, CD80, and CD86 on blasts from AML pts at initial diagnosis. In some pts, not all COR/COL analyses could be performed due to limited cell counts in samples or incomplete records. All results are also shown in supplementary appendix 3.

Expression profiles in AML subtypes

Blasts of monocytic AML subtypes showed significantly enhanced expression of GITR, its ligand, and of CD86

Significant differences were observed in expression profiles of monocytic versus non-monocytic subgroups in the expression of GITR (FAB M3 AML subtypes included 1.8 vs 1.3; p=0.05*, excluded 1.8 vs 1.2, p=0.015**), GITRL (with FAB M3 AML subtypes included 2.7 vs 1.4; p=0.005***, excluded 2.7 vs 1.3; p=0.005***). CD80 expression showed a difference in the rank sum test, which was not visible after exclusion of AML M3 (see supplementary appendix 3), and CD86 showed a higher expression in the monocytic subtype (FAB M3 AML subtypes included 5.9 vs 1.3; p=0.001***, excluded 5.9 vs 1.3; p=0.0006***) (see figure 1A–C). 4-1BB (FAB M3 AML subtypes included 1.1 vs 1.1; p=0.59; excluded 1.1 vs 1.0; p=0.52) and 4-1BBL (FAB M3 AML subtypes included 1.3 vs 1.2; p=0.23; excluded 1.3 vs 1.1; p=0.16) did not show significant difference.

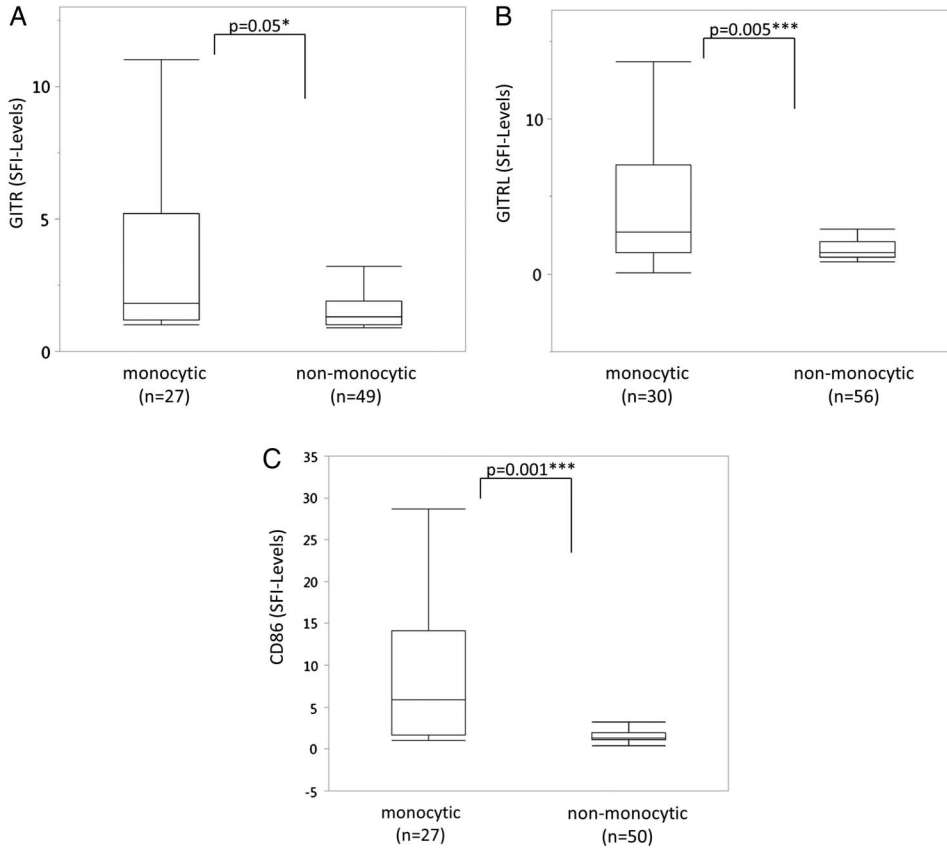

Figure 1 Blasts of monocytic AML subtypes showed significantly enhanced expression of GITR, its ligand GITRL, and of CD86. Significantly enhanced expression of GITR, its ligand GITRL (A, B), and of CD86 (C) was found in monocytic compared to non-monocytic AML (AML M3 cases included). AML, acute myeloid leukemia; GITR, glucocorticoid-induced TNFR-related protein; GITRL, GITR ligand; SFI, specific fluorescence index.

Increased expression of GITR, its ligand GITRL, and of CD86 in differentiated and prognostically good FAB subgroups

Dividing cases in those with 'good' (without M0 and M6) and 'bad' (M0, M6) prognosis FAB subtypes according to Goldberg *et al*[25] and Drexler[26] (M7 cases were not present in our cohort), expression of GITR and its ligand on blasts was increased (tendentially significant for GITR) in the 'good' prognosis group (1.4 vs 1.0; p=0.09*; 1.6 vs 1.1; p=0.1). After exclusion of M3 cases, no significant results were seen. In mature AML subtypes (M2–M6), expression levels of GITR were not different compared to immature FAB M0 and M1 groups; however, GITRL was significantly increased in differentiated AML subtypes and remained significant after exclusion of M3 cases (1.7 vs 1.3; p=0.02**; 1.7 vs 1.3; p=0.03**).

4-1BB and its ligand did not show correlations neither with prognostic FAB subgrouping nor with the status of morphological differentiation. CD80 was not differently expressed in the 'good' versus 'bad' prognosis group or dependent on differentiation status according to FAB subtypes. CD86 showed significantly increased expression in the 'good' prognosis groups (with M3 cases included 1.7 vs 1.1; p=0.03**, excluded 1.7 vs 1.1; p=0.04**). Enhanced expression in differentiated subgroups was seen with M3 cases included (1.95 vs 1.2; p=0.001***) and excluded (2.6 vs 1.2; p=0.002***) (see also figure 2A, B).

CD86 expression on blasts correlated with high-peripheral blast count

Blast counts in PB or BM smears at first diagnosis did not show a correlation with blasts' coexpression of GITR/L, 4-1BB (−0.05, p=0.69; −0.24, p=0.11), and CD80; however, a spearman analysis showed a direct correlation of 4-1BBL (tendentially significant) (0.19, p=0.09*; −0.13, p=0.38) and CD86 expression on blasts with peripheral blast counts (0.31, p=0.005***). In the BM smear, tendency of a correlation was seen (0.26, p=0.07*). No correlation of markers' expression (GITR/L, 4-1BB/L, CD80, and CD86) with age and pAML versus sAML groups was found.

COR and COL expression not different in NCCN/ELN risk groups or in pts responding or not responding to induction chemotherapy

No significant differences in 'favorable' versus 'adverse' risk NCCN/ELN groups were found for GITR and GITRL (1.1 vs 1.3; p=0.6; 1.4 vs 1.6; p=1.0); however, after exclusion of M3 cases, a significantly higher expression for GITR in the adverse group was visible (1.3 vs 1.1, p=0.04**). For 4-1BB and 4-1BBL (with FAB M3 AML subtypes included 1.2 vs 1.0; p=0.88, excluded 1.1 vs 1.0; p=0.92; with FAB M3 AML subtypes included 1.2 vs 1.1; p=0.69, excluded 1.1 vs 1.1; p=0.47) or CD80 and CD86, no significant differences were seen. Pts responding to anthracyclin-based induction chemotherapy were not characterized by differences in expression of COR/COLs compared to those pts without response.

Prognostic evaluations

No marker was associated with relapses

We linked expression levels of COR and COL with pts' risk to relapse. Pts were included which had an observation

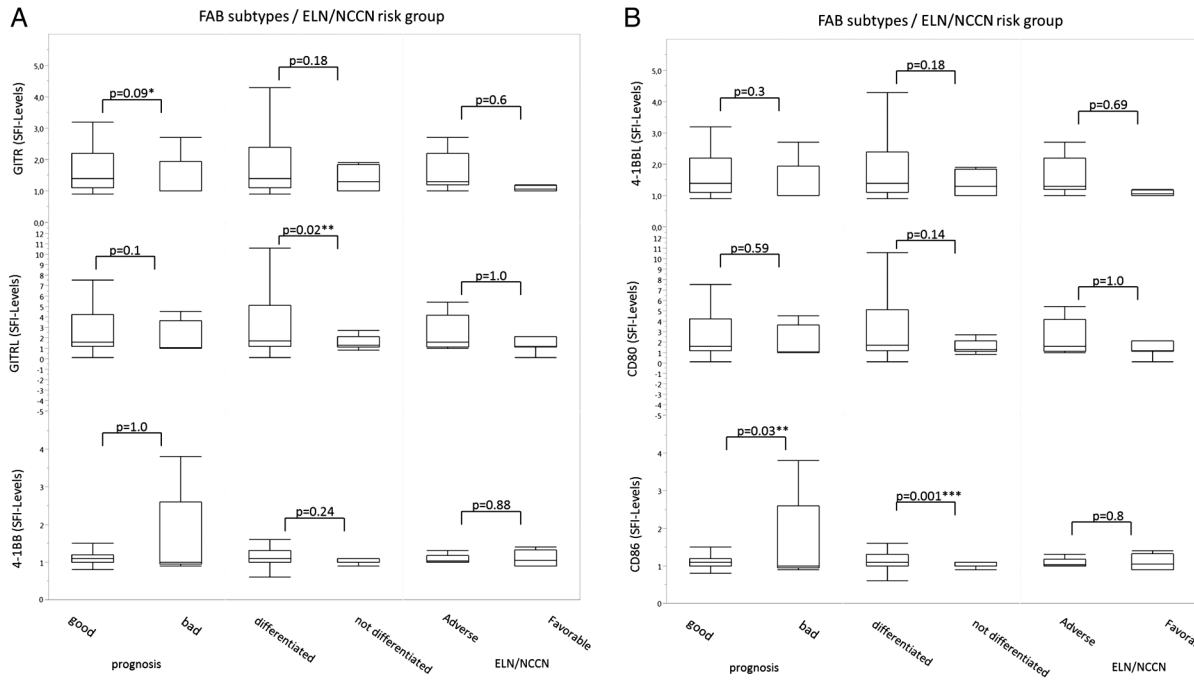

Figure 2 Increased expression of GITR, its ligand GITRL, and of CD86 in prognostically 'good' and differentiated FAB subgroups. SFI levels according to 'good' versus 'bad' prognosis FAB types, differentiated (M2–M6) (M7 not present in our cohort) versus not differentiated (M0, M1) FAB types, and ELN/NCCN risk groups (adverse vs favorable) of all COR and COL are displayed. Data are presented including M3 AML cases. ELN, European Leukemia Network; FAB, French-American-British; GITR: glucocorticoid-induced TNFR-related protein; NCCN, National Comprehensive Cancer Network.

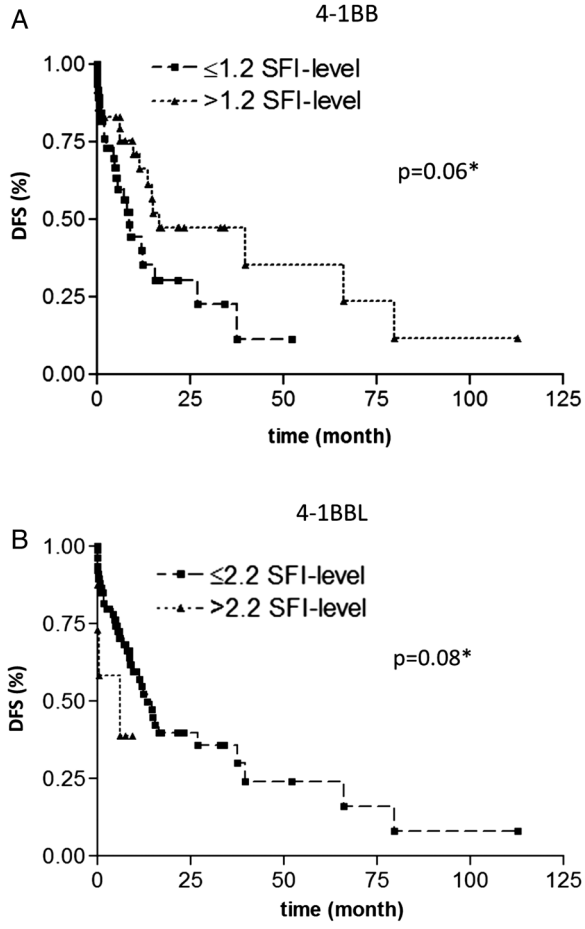

Figure 3 Higher expression of 4-1BB and lower expression of 4-1BBL might be associated with longer disease-free survival (DFS). (A) 4-1BB showed evidence for enhanced DFS when expression exceeded 1.2 SFI levels (n=87). (B) 4-1BBL could be discriminated by a cut-off value of 2.2 SFI levels showing evidence for dividing the groups into longer DFS when expression was below the cut-off (n=87). DFS, disease-free survival; SFI, specific fluorescence index.

time of at least 100 days after initial diagnosis and relapsed within 365 days in order to ensure relation between marker expression at first diagnosis and clinical outcome. All markers did not show significantly different expression profiles (relapse yes vs no: GITR 1.7 vs 1.4, p=0.3; GITRL 1.9 vs 1.4, p=0.8; 4-1BB 1.05 vs 1.1, p=0.27; 4-1BBL 1.1 vs 1.2, p=0.09; CD80 1 vs 1, p=0.45; CD86 1.7 vs 1.35, p=0.43). Dividing cases that experienced no or two relapses saw a significantly higher expression of CD80 in those cases that later on suffered two relapses (1 vs 1.45, p=0.03**, n=28/2), whereas no differences were seen in cases that relapsed or not relapsed. In other markers also, no differences between the numbers of relapses were obvious.

Cut-off analyses to predict prognosis

We evaluated cut-off values that allowed a separation of pts in better or worse prognostic groups by using Kaplan-Meier analysis in order to identify differences in prognostically relevant parameters. Duration of DFS was used for differentiation and generation of groups separated by cut-offs. For GITR, GITRL, CD80, and CD86, no cut-offs could be evaluated.

Cut-off values were found for 4-1BB and 4-1BBL

Cut-offs were evaluated, that allowed the best separation in cases with 'high' or 'low' probability of DFS. 4-1BB showed a (tendentially significant) higher probability for prolonged DFS when expression exceeded 1.2 SFI levels (35.7 vs 14.6 months; p=0.06*; n=87) (figure 3A). Results were confirmed by showing a tendentially significant higher probability for a longer time from first diagnosis to relapse in the group exceeding cut-off-defined SFI levels (453 vs 240 days, p=0.06*). We could confirm these results also in a multivariate approach including the factors age, p/sAML, WBC, NCCN/ELN together with 4-1BB and 4-1BBL expressions: a high expression of 4-1BB correlated with a lower risk to relapse (HR 0.2, CI 0.02 to 0.95, p=0.04**, table 2). For 4-1BBL, a discriminating cut-off value of 2.2 SFI levels could be defined: a lower expression of 4-1BBL (below the cut-off value) correlated tendentially significant with the probability for a prolonged DFS (27.9 vs 3.7 months, log rank p=0.08*, Wilcoxon 0.05**, n=87) (figure 3B). In an univariate evaluation for influences on OS, we did not detect an impact of 4-1BB expression between the cut-off groups. The multivariate analysis showed, however, that a high expression of 4-1BB (above the cut-off value) significantly correlated with a lower risk to die (HR 0.12; CI 0.005 to 0.9; p=0.04**). Univariate analysis of 4-1BBL expression showed a tendentially higher probability for longer OS in the group with values below the cut-off (35.6 vs 4.5 months, p=0.09*, n=86). Using

Table 2 Multivariate analysis of 4-1BB, 4-1BBL

	Relapse			Death		
Variables	**HR**	**95% CI**	**p Value**	**HR**	**95% CI**	**p Value**
Age (years)	1.0	0.97 to 1.03	0.76	1.02	0.98 to 1.08	0.3
Primary vs secondary AML	1.86	0.36 to 0.11	0.35	0.62	0.11 to 2.64	0.54
White blood cell count (G/L)	1.01	1.0 to 1.01	0.05*	1.0	1.79 to 118.53	0.01**
4-1BB (>1.2 vs ≤1.2 SFI levels)	0.2	0.02 to 0.95	0.04**	0.12	0.005 to 0.9	0.04**
4-1BBL (>2.2 vs ≤2.2 SFI levels)	4.6	0.5 to 27.78	0.14	10.3	1.14 to 73.68	0.04**
NCCN/ELN (favorable vs adverse)	0.3	0.06 to 1.69	0.16	0.05	0.002 to 0.51	0.01**

**significant.
AML, acute myeloid leukemia; ELN, European Leukemia Network; NCCN, National Comprehensive Cancer Network; SFI, specific fluorescence index.

multivariate analyses, we observed (in addition to the influence of the WBC and the NCCN/ELN classification) also a significant correlation of a higher expression of 4-1BBL with a higher probability to die (HR 10.3; CI 1.14 to 73.68; p=0.04**, see table 2). For all cut-off analyses, we could exclude influence of FAB subcategories such as M3 cases by consecutive subgrouping according to groups below and above the cut-off. Cases showed a homogenous distribution for both groups.

DISCUSSION

The development of tumor cells in humans is dependent on their survival and immunoescape in the human body. By avoiding recognition and elimination through innate and adaptive immune effectors (immune escape), tumor cells gain evolutionary advantages leading to clonal proliferation and establishment of cancer. T-cells and NK-cells are main mediators of tumor defense. Several receptor groups, for example, of the TNFR family can influence these cell types. COR and COL are expressed on immune effectors (table 1), known to regulate immune responses and thereby cancer defense. COR and COL expression on AML blasts might influence anticancer response and thus be of importance for pts' outcome. The original contribution of this work was to elucidate the significance of COR/COL expression on AML blasts with respect to prognostic and clinical parameters in order to identify screening targets to predict pts' course.

In vivo assays with recombination-active gene mice pretreated with agonistic anti-4-1BB revealed an increase in activation of DC and consecutively of T-cells,[27] resulting in suppression of tumor growth in hematological diseases. 4-1BB expression on cancer cells might play a relevant role by being expressed on solid tumor cells like osteosarcoma and lung cancer[28 29] as well as on several subtypes of lymphoma. Expressed on granulocytes, its appearance on AML blasts is not surprising; however, the role in interaction with immunological effectors and intracellular blast-regulation is not yet clarified. Despite the fact that 4-1BBL is known to influence blasts' maturation processes,[30] we could not confirm that for 4-1BB. 4-1BB expression did not correlate with maturation stages, prognostically classified FAB subgroups or p/sAML. However, 4-1BB is known to mediate T-cell stimulation and might contribute to mediate immunological interaction with T-cells when expressed on blasts. Using an animal model, Choi *et al*[6] showed that in vivo 4-1BB signaling of myeloid cells negatively regulates peripheral T-cell responses, which might implicate that 4-1BB expression on AML blasts might lead to reduced T-cell-associated tumor defense and thereby contributes to an adverse course of the disease. Confirming this theory, we found a tendentially significant enhancement in the probability of prolonged DFS in pts with a higher 4-1BB expression. Furthermore, a significantly lower HR for relapse in the performed multivariate analysis indicates importance of this finding.

4-1BBL is the ligand for 4-1BB and serves as a secondary signal for T-cells: It induces T-cell division, sustains their survival, and enhances their effector function.[31] Interaction between 4-1BB and its ligand leads to production of several immunorelevant cytokines such as interferon-γ, IL-2, and IL-4 and occurs in a so4-1BBL and c4-1BBL form. In general, so4-1BBL is known to be significantly higher released in hematological malignancies like Morbus Hodgkin and non-Hodgkin's lymphoma compared to healthy donors.[32] In previous studies, we revealed that the release of so4-1BBL from AML-blast samples leads to a higher plasma level and expression correlated with prognostically adverse AML subtypes what might be explained by a binding of so4-1BBL to T-cells, thereby inhibiting T-cell-c4-1BB binding to tumor cells. Moreover, c4-1BBL is known to be expressed on AML blasts' surface[8] and was stated to have an impact in the blasts' maturation processes due to its reverse signaling capability when stimulated as shown in 15 of 21 not closer specified AML samples in a study of Cheng *et al*[30] that could not be confirmed in our study, probably due to a different composition of our pt cohort. In cut-off analyses, we correlated significantly higher 4-1BBL expression with shorter (but not significant) DFS and OS times, thereby providing evidence of correlation not only of a higher so4-1BBL release but also of a higher c4-1BBL expression in the case of unfavorable prognosis. Multivariate analysis indicated statistically significant higher probability of death.

Shimizu *et al* showed a GITR expression in a mouse model predominantly on CD25+CD4+ and CD25+CD8+ T-cells known to characterize proliferative and also T_{reg}. Stimulation of GITR abrogated CD25+CD4+-T-cell-mediated suppression of immune reaction. Additionally, eradication of tumor tissues was observed and also related to longer survival by an enhanced tumor elimination.[33–36] No data exist about relevance of GITR expression on solid tumor cells or lymphoma cells like multiple myeloma although an impact of GITR deficiency was associated with an increased cell proliferation and reduced apoptosis.[37] AML blasts are also known to express GITR on their cell surface, but no data exist about the role of GITR expression on intracellular as well as on extracellular interactions.[16] With our data, we revealed that GITR expression was associated with monocytic AML subtypes, more favorable FAB subgroups, if M3 AML cases are excluded with an adverse NCCN/ELN-related cytogenetic subgroup; however, no correlations with DFS or OS were found assuming no relevant prognostic significance of a higher or lower GITR expression.

Previous studies revealed that not only GITR but also GITRL can signal inside a cell and induce activation as seen in macrophages.[38] Ex vivo analyses of human tumors from different histological origin revealed substantial expression of GITRL, which was not found in corresponding healthy tissues.[10] Previous publications of our study group revealed frequent expression of GITRL on CLL cells[12] with association to NK-cell inhibition. Baessler *et al*[16] investigated 60 AML pts with GITRL expression on blasts and showed cell surface positivity in 57% of the pts and GITRL mRNA was found to be blocked by mutational changes in surface negative cases. Moreover, GITRL was shown to represent a monocytic AML marker that could be confirmed by our finding of a GITRL overexpression in monocytic as well as in more differentiated AML subtypes. Up to now, no detailed expression analyses of GITRL in correlation with AML pts' prognosis have been performed. We are the first to show that although GITRL is highly expressed on AML blasts, its expression has no relevance as a prognostic marker in AML.

In DC maturation, CD80 is upregulated after phagocytosis and known to induce T-cell response.[39 40] Its expression is also known on several human solid cancer cell lines like melanoma and colorectal carcinomas and on hematological malignant cells such as myeloma and AML blasts.[41 42] Data about the prognostic relevance of a CD80 expression are controversially discussed: some groups correlated a high expression with an 'improved immunity', resulting in tumor rejection and an improved response to chemotherapy,[43 44] whereas other groups (including our own[45]) correlated a high CD80 expression with unfavorable risk groups and worse progression-free survival. Here, we show a significantly higher CD80-expression level in cases that later on experienced two relapses, thereby characterizing an unfavorable subgroup (although only a few samples were available). With the remaining results, however, we could not confirm a correlation of CD80 expression with unfavorable prognosis. Reasons for these discrepant data and the unclear prognostic relevance could be the multifactorial functioning of CD80 in the mediation or inhibition of tumor-immunological reactions. Moreover —especially compared to our preliminary publication—the great heterogeneity of pt cohorts included in the two studies might explain the differences.[45] In summary, this means that the prognostic role of an expression of CD80 on the cell surface of AML blasts for the further course of the disease could not be clarified in our study.

The second tandem receptor evaluated in this study was CD86. CD86 has been discovered of being expressed in solid tumors like melanoma and gastrointestinal cancer;[46 47] however, no data exist showing function in these cancer diseases. CD86 is also known to be expressed on malignant hematopoietic cells such as chronic myeloid leukemia, Burkitt lymphoma and myeloma[48] and on AML blasts. The clinical significance of this expression on blasts is controversially discussed. In a previous study, we correlated the CD86 expression on AML blasts with (non-significantly) associated worse progression-free survival or OS, reduced response to TAD/HAM therapy, and higher risk to relapse.[45] Maeda *et al*[42] did not compare expression levels but grouped pts with and without CD86 surface expression and associated 'worse prognosis' with the group expressing CD86 on blasts. In contrast, Whiteway *et al*[49] correlated a longer duration of remission with a higher expression of CD80 and CD86. Here, we show a significantly enhanced CD86 expression in 'good' prognosis and differentiated FAB subtypes; however, we could not correlate CD86 expression with a treatment response after anthracyclin-based induction I or with higher probability of DFS or OS. Moreover, we could not evaluate a predictive cut-off point—in contrast to our preliminary trial. This could be due to the great heterogeneity of pt cohorts included in the two trials, resulting in higher proportions of pts with 'unfavorable prognostic' characteristics in the present study. Additionally performed comparison between the subgroups in our previous study[45] might result in a high sensitivity of the composition of pts due to highly limited pt cases. Maeda *et al*[42] also correlated a high expression of CD86 with unfavorable prognosis. However, since he used different screening methods (flow-based threshold of 20% CD86-positive cells to differentiate between positive and negative subgroups), a direct comparison to our study using SFI levels and cut-off analyses could not be performed and may explain differences in the results. Taken together, our data could not clarify the prognostic role of CD86 in our study.

With this study, we provide further evidence that refined expression profiles on AML blasts may be valuable for prediction of pts' prognosis when performed at initial diagnosis: we provide strong evidence that 4-1BB is important for pts' prognosis and correlate negatively in its surface expressed form with an unfavorable prognosis, whereas 4-1BBL expression correlated with an unfavorable risk profile when highly expressed. As shown in our study, GITR and GITRL are monocytic markers that did not correlate with prognostic subtypes and therefore do not qualify as a prognostic marker. The prognostic relevance of CD80 and CD86 expression has to be studied in more detail to allocate expression profiles to prognostic groups.

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