Chapter VII

High expression of decoy receptor TRAIL-R3 on AML blasts is associated with poor clinical outcome and induces apoptosis resistance which can be overcome by targeting TRAIL-R2

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Abstract

Activation of the TNF-related apoptosis-inducing ligand (TRAIL) pathway can induce apoptosis in a broad range of human cancer cells. Four membrane-bound receptors have been identified: TRAIL-R1 and TRAIL-R2 contain a functional death domain. TRAIL-R3 and TRAIL-R4 lack a functional death domain and function as decoy receptors. Flow-cytometric determination of TRAIL receptors revealed that acute myeloid leukemic (AML) blasts expressed significantly more pro-apoptotic receptors compared to normal blasts ($p<0.0005$). However, about 20% of AML patients had a high expression level of the anti-apoptotic TRAIL-R3, which was strongly correlated to a shortened overall survival (log rank, $p=0.002$). In multivariate analysis, TRAIL-R3 expression was a stronger prognostic factor for overall survival than age. Cell death induction of primary AML samples with sTRAIL/Apo2L was 14% (0-54%) and could be enhanced by down-modulation of TRAIL-R3, confirming its decoy function on AML blasts. Bypassing of TRAIL-R3 by treatment with antibodies directly targeting R2 resulted in high rates of induced cell death (mean 30%, 0-80%).

In conclusion, AML blasts do express pro-apoptotic TRAIL receptors. However, co-expression with the decoy receptor TRAIL-R3 results in significant shortened overall survival. AML blasts are sensitive to targeting the pro-apoptotic TRAIL-R2 receptor, yielding a new therapeutic option for AML patients.

Introduction

Since chemotherapy and stem cell transplantation can only cure approximately 30% of patients with acute myeloid leukemia (AML), there is urgent need for complementary and targeted treatment modalities. Use of recombinant TRAIL (TNF (tumor necrosis factor)-related apoptosis-inducing ligand) could be included in the treatment of AML patients. Lymphocytes, NK cells, dendritic cells and monocytes are able to upregulate membrane bound TRAIL (mTRAIL) and to secrete a soluble form of TRAIL (sTRAIL) after stimulation with interferons or IL-2[1-4]. In human, four membrane bound receptors for mTRAIL and sTRAIL have been identified: two of them, TRAIL-R1 (formerly known as DR4) and TRAIL-R2 (formerly known as DR5) contain a functional death domain and are capable of initiating the apoptotic cascade, while two others TRAIL-R3 (formerly known as DcR1) and TRAIL-R4 (formerly known as DcR2) lack a functional death domain and function as decoy (antagonistic) receptors. Binding of TRAIL to one of the functional receptors TRAIL-R1 (R1) or TRAIL-R2 (R2) results, after clustering, in the formation of a Death-Inducing Signaling Complex (DISC) consisting of TRAIL, its receptor, the FAS associated death domain adaptor protein (FADD) and caspase 8[5;6]. Activated caspase 8 can directly activate caspase 3 leading to apoptosis and also, by cleaving Bid, activate the mitochondria-intrinsic pathway[7]. Binding of TRAIL to a decoy receptor will not induce apoptosis. TRAIL-R3 (R3) and TRAIL-R4 (R4) inhibit TRAIL induced apoptosis in distinct ways. R3 is a glycosyl-phosphatidy-linositol (GPI) linked protein and lacks an intracellular domain[8]. The death domain of R4 is truncated. R3 prevents TRAIL-R2-DISC assembly, while R4 impairs DISC processing to caspase activation[9].

Several important functions for TRAIL induced apoptosis have been reported. First, TRAIL mediated cytotoxicity plays an important role in innate and adaptive immune responses[10]. Second, TRAIL exerts a regulatory function on erythroid and myeloid maturation in normal hematopoiesis[11-14]. Moreover, senescent neutrophils are eliminated by TRAIL induced apoptosis upon their return to the bone marrow[15]. Finally, and most intensively studied, TRAIL has an important function in tumor immune surveillance. TRAIL deficient mice are more susceptible to tumor development and metastasis[16]. Inhibition of TRAIL induced apoptosis by administration of a neutralizing antibody leads to tumor progression in mice[17]. Recombinant soluble TRAIL (rsTRAIL) constructs are able to induce apoptosis in many cancer cell lines (reviewed in[18]). An explanation for the tumor selective activity of TRAIL has been the observation that normal cells mostly express the decoy receptors R3 and R4, while many tumor cells express the functional receptors R1 and R2 (reviewed in[19]).

Studies on TRAIL receptor expression on myeloid leukemic cells and clinical outcome of AML patients did not reveal a correlation between receptor expression and prognosis[20]. Moreover, low sensitivity of leukemic blasts to rsTRAIL has

been reported[21-23]. This could be either due to a relative high expression of the decoy receptors on the cell surface or to intracellular high levels of anti-apoptotic proteins or low expression of pro-apoptotic proteins. Indeed, pre-treatment of leukemic cells with sensitizing agents (kinase inhibitors, triptolide, chemotherapy) increases the susceptibility to rsTRAIL[24-28].

An alternative strategy to employ the TRAIL pathway could be to circumvent the decoy receptor expression by directly targeting the pro-apoptotic receptors R1 and R2 by selective antibodies[29-32]. These antibodies have entered phase I and phase II clinical trials (reviewed by Ashkenazi[33]).

In this study we explored the possible applications of employing the TRAIL pathway in therapy regimens for patients with AML. We analyzed the receptor expression on hematopoietic precursor cells of a large cohort of AML patients, compared them to expression levels of healthy donors, and correlated them to clinical outcome. We have found that a high expression of the decoy receptor R3 was correlated to a poor clinical outcome. Also, decoy function of R3 was confirmed in vitro by treating leukemic cells lines and fresh primary AML samples with rsTRAIL or with antibodies directly targeting R1 and R2. By confirming the decoy function of R3 in myeloid leukemic cells and by demonstrating highest levels of cell death after targeting R2, we have found new evidence for directly targeting the pro-apoptotic receptors as an option in the treatment of AML patients.

Materials and Methods

Patients' samples

After informed consent and according to the Helsinki declaration, blood and bone marrow samples were collected from 92 patients with *de novo* AML and from 11 healthy donors. Patients were classified according to the French-American-British (FAB)-classification[34]. Patients received therapy according to HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols (available at www.hovon.nl). Patients received two cycles of chemotherapy (containing cytarabin, combined with idarubicin or amsacrine) followed by autologous stem cell transplantation or a third cycle of chemotherapy (mitoxantrone and etoposide). Patients with promyelocytic leukemia (FAB-M3) were treated differently and were excluded from this study. Cytogenetic risk group was determined as favorable (translocation(8;21), or inv(16)), standard (neither favorable nor adverse) or adverse (complex karyotype, -5 or -7, deletion(5q), abnormality(3q), t(6;9), t(9;22) or abnormality 11q23). Overall survival (OS) was defined as the time period between date of diagnosis and either date of death or last date of follow-up. Disease free survival (DFS) was defined as the time period between

achievement of complete remission (CR) and either moment of relapse or last date of follow up in non-relapsed patients.
Bone marrow mononuclear cells and peripheral blood mononuclear cells were collected through density gradient centrifugation (Ficoll-Paque^{TM}PLUS, Amersham Biosciences). Samples were analyzed immediately or cryopreserved in liquid nitrogen until analysis.

Flow cytometry analysis

The following mouse antibodies were used: unlabeled anti-TRAIL (clone 5D5), anti-TRAIL-R1 (clone HS101), anti-TRAIL-R2 (clone HS201), anti-TRAIL-R3 (clone HS301) and anti-TRAIL-R4 (clone HS402) all from Alexis (Lausen, Switzerland).

Table 1. Characteristics of 92 patients with de novo AML.

	total
Number	92
Male/female	47/45
Age in years at diagnosis, median (range)	48 (16-65)
WBC at diagnosis, median (range)	43 (1-300)
follow-up in months, mean (range)	32 (1-160)
disease free survival in months, mean (range)	35 (1-158)
complete remission rate, number (%)*	74 (80)
Fab classification, number (%)	
AML M0	6 (6)
AML M1	13 (14)
AML M2	20 (22)
AML M4	24 (26)
AML M5	24 (26)
AML M6	2 (2)
RAEB-t	2 (2)
Not classified	1 (1)
Cytogenetic risk group, number (%)	
Favorable	7 (7)
Standard	53 (60)
Adverse	16 (17)
No metaphasis	13 (14)
Not done	2 (2)

Patients were classified according to FAB (French-American-British) classification and to cytogenetic risk group (for detail see materials and methods). *A significant difference between patients either or not achieving CR was observed in white blood cell count (WBC) at diagnosis (p=0.001).

Unlabeled IgG1 isotype (Beckton Dickinson, (BD, New Jersey, USA)); FITC labeled CD34 and CD38 (BD); PercP labeled CD45 (Beckman Coulter, Fullerton, USA); APC labeled CD34 and CD38 (BD).
Mononuclear cell fractions were preincubated with 10% human gammaglobuline (6 mg/ml, Sanquin, the Netherlands) followed by incubation with directly labeled

antibodies. For TRAIL and TRAIL receptor detection, cells were incubated with the unlabeled antibodies and subsequently with PE-conjugated rabbit-anti-mouse immunoglobulin (Dako, Glostrup, Denmark). A mixture of non-relevant mouse antibodies of different isotypes was added to avoid aspecific binding of subsequently directly labeled antibodies. All incubations were performed at room temperature during 15 minutes. Cells were washed after every incubation step with PBS/0.1% BSA/0.05% sodiumazide and analyzed on a FACS Calibur (BD). 25,000 living cells on a forward scatter were analyzed using CellQuest software (BD). Cell viability was measured by combined Annexine V (VPS diagnostics, Hoeven, The Netherlands) and 7AAD staining (Via-Probe, BD Pharmingen). Absolute cell numbers were counted by using beads (flow-count ^{TM} fluorospheres, Beckmann Coulter).

Apoptosis induction of cell lines and fresh AML samples with sTRAIL/Apo2L, mapatumumab and lexatumumab

The cell lines MM6 (ACC 124), Kasumi-1 (ACC 220) and ME1 (ACC 537) were purchased from DSMZ. Cell lines U937 (CRL-1593.2), HL60 (CCL-240), KG1-a (CCL-246.1) and THP1 (TIB-202) were purchased from ATCC. sTRAIL/Apo2L was kindly provided by Amgen/Genentech. Agonistic fully human monoclonal antibodies specific for TRAIL-R1 (mapatumumab, formerly HGS-ETR1) and TRAIL-R2 (lexatumumab, formerly HGS-ETR2) were kindly provided by Humane Genome Sciences, Inc (Rockville, MD, USA).

250,000 cells from indicated cell lines and from patient samples were incubated in 500 µl in 48 wells flat bottom plates with different concentrations sTRAIL/Apo2L (10, 100, 300 and 1000 ng/ml) for different time periods (2, 4, 8, 18, 24 and 48 hours) at 37ºC in a humidified incubator. Optimal conditions for apoptosis induction in cell lines with sTRAIL/Apo2L were found after 18 hours at 37ºC at a concentration of 100 ng/ml sTRAIL/Apo2L (data not shown).

Fresh AML patient samples were incubated for 18 hours at 37ºC, with different concentrations of sTRAIL/Apo2L, mapatumumab and lexatumumab. All cells were cultured in RPMI supplemented with 10% fetal calf serum (FCS), except for the MM6 cell line which was cultured in special medium[35]. In some experiments, cells were pretreated with the pancaspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD) (Alexis, 10 µmol final concentration) for 30 minutes. Absolute viable cells were defined by AnnexinV and 7AAD negativity and counted by using fixed amounts of beads in every sample.

Modulation of cell surface R3 expression

To remove R3, which is a GPI linked protein, we used phosphatidyl-inositol phospholipase C (PI-PLC) (Molecular Probes (Eugene, USA)). To optimize R3 removal we tested various PI-PLC concentrations (0.5 and 3.0 µg/ml) and various

incubation times, temperatures (20 min. at a rock plate at 4ºC, 1 and 4 hour at 37ºC), and media (PBS, RPMI/10% FCS for HL60, for MM6 special medium (see above)). Moreover, we added different concentrations (0.5, 1 and 10 µg/ml) of cycloheximide (CHX, (Sigma, St Louis, USA)) to prevent de novo synthesis of R3. Incubation with PI-PLC 0.5 µg/ml, for 4 hours at 37 ºC in RPMI/10% FCS or MM6 medium yielded best results.
Addition of CHX induced additional apoptosis in all cultures. Control experiments without CHX showed no re-synthesis of cell surface R3 after 24 hours, so CHX was withdrawn in subsequent experiments from cultures. After treatment with PI-PLC, cells were directly incubated for 18 hours with sTRAIL/Apo2L, mapatumumab or lexatumumab.

Statistical analyses

Statistical analyses were conducted with SPSS software program (version 15.0). To analyze associations between variables Spearman's correlation coefficient was used. Differences between patient characteristics were analyzed with Mann-Whitney U test. Paired samples t-test was used to measure differences in induced apoptosis in cell lines. For survival data, Kaplan-Meier curves were constructed and compared by means of the log-rank test. To explore the simultaneous effect of several variables on survival the Cox regression model was used.

Results

TRAIL receptor expression on myeloid leukemic blasts and correlation to clinical outcome.

Patient characteristics are shown in table 1 and reflect a representative AML patient group. Mean follow-up of all patients was 32 months (range 0-158, median 11 months). 18 patients (20%) did not achieve CR during induction therapy. A significant difference between patients CR or not was observed in white blood cell count (WBC) at diagnosis (p=0.001). 6 of 7 (86%) patients with a favorable cytogenetic risk group achieved CR versus 5 of 16 (31%) patients with an unfavorable cytogenetic risk group (difference not significant due to small groups).
Myeloid leukemic blasts were defined by $CD45^{dim}$ and low side scatter expression. In contrast to earlier reports on TRAIL receptor expression levels[23], we have presumably found (pro-apoptotic) R1 and R2 expression (mean percentage positive cells 16% and 34%, range 0-79% and 0-97% respectively) versus (anti-apoptotic) R3 and R4 expression (mean 9% and 10%, range 0-71 % and 0-45%) on myeloid leukemic blasts (figure 1). When compared to normal blasts, AML blasts had significantly higher expression of R1 and R2 (p= 0.001 and p=0.006

Table 2. TRAIL receptor expression levels on seven myeloid leukemic cell lines and sensitivity to sTRAIL of five of these cell lines.

	TRAIL R1	TRAIL R2	TRAIL R3	TRAIL R4	% vital cells after incubation with sTRAIL/Apo2L
	percentage positive cells				
ME1 (M4eo)	6	11	2	2	82
U937 (histiocytic)	10	88	6	2	45
THP1 (monocytic)	76	53	8	4	66
MM6 (monocytic)	74	93	74	2	68
HL60 (M3)	23	97	86	4	72
Kasumi (M2)	26	56	5	0	nd
KG1A (M0)	50	10	5	1	nd

Results are the mean of at least three experiments. Induced cell death was determined by counting the $7AAD^{-/}AnnexinV^{-}$ viable cells. As expected, ME1 cells lacking R1 and R2 expression, showed barely induced cell death. U937 cells that only expressed R2 showed highest levels of cell death. Cell death was reduced in MM6 cells and HL60 cells expressing high levels of both R2 and R3, suggesting that TRAIL effects are inhibited by binding to R3. nd (not done).

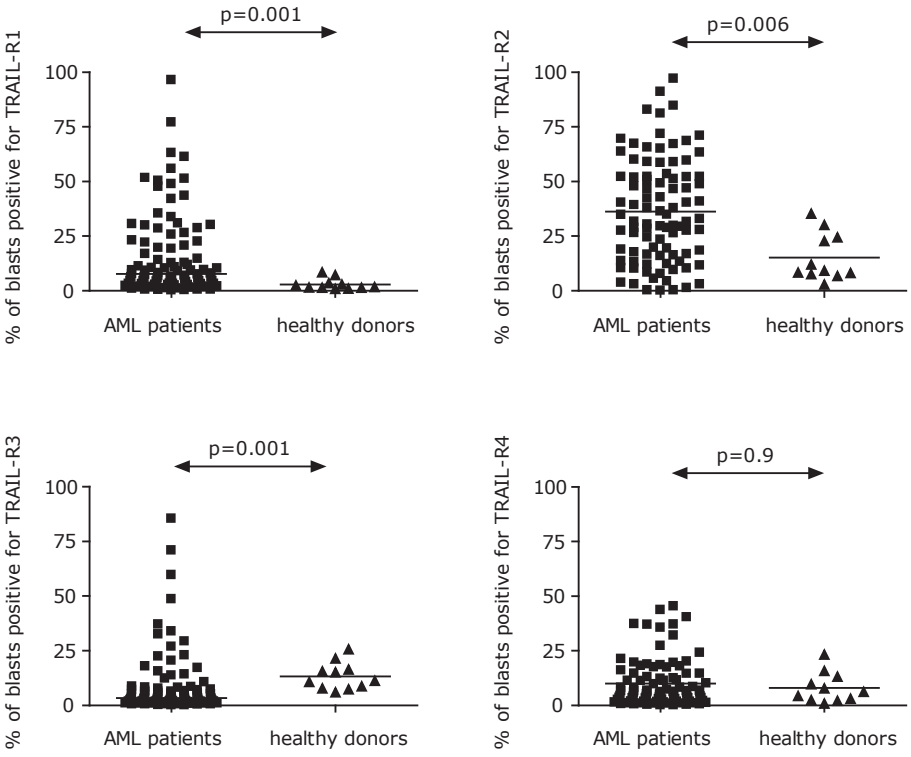

Figure 1. TRAIL-receptor expression on myeloid leukemic blasts of 92 patients with de novo AML and from 11 healthy donors. Myeloid blasts were defined by CD45dim and side scatter low expression. Myeloid leukemic blasts have an increased expression of R1 and R2 (mean percentage positive cells 16% and 34%, range 0-79% and 0-97%, respectively) versus R3 and R4 expression (mean 9% and 10%, range 0-71 % and 0-45%). When compared to normal blasts, AML blasts had significantly higher expression of R1 and R2 (p=0.001 and p=0.006 respectively) and significantly lower expression of R3 (p=0.006). R4 expression did not differ between AML and normal blasts. Horizontal bars indicate mean expression levels.

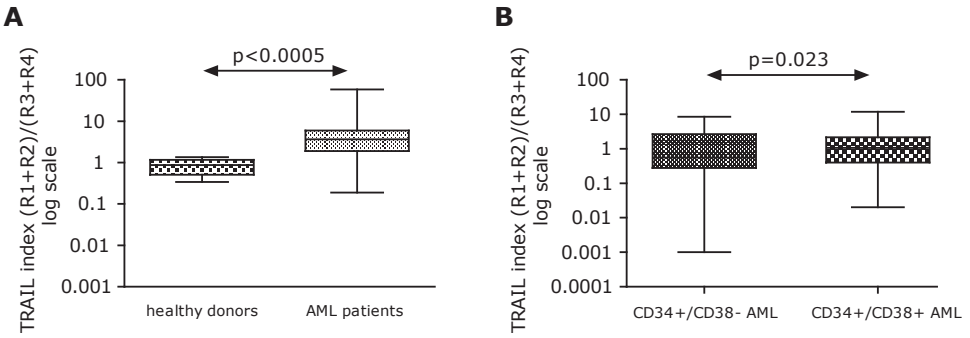

Figure 2A. Pro-apoptotic and anti-apoptotic profiles of leukemic blasts from 92 de novo AML patients and 11 healthy donors determined by dividing the percentage of positive leukemic blasts for R1 and R2 by the percentage positive blasts for R3 and R4 (R1+R2)/(R3+R4) , the TRAIL index. When comparing healthy donors and AML patients, blasts from AML patients revealed a significant higher ratio and thus a higher pro-apoptotic receptor profile than healthy donors ($p<0.001$). Horizontal bars indicate mean levels.

Figure 2B. Pro-apoptotic and anti-apoptotic profiles of leukemic blasts of 14 de novo AML patients. Analysis were done on the $CD34^+/CD38^-$ compartment (which harbors the leukemic stem cells) and compared to the $CD34^+/CD38^+$ compartment (harboring the bulk of leukemic cells). Profiles were determined by dividing the percentage of positive leukemic blasts for R1 and R2 by the percentage positive blasts for R3 and R4 (R1+R2)/(R3+R4), the TRAIL index. Comparing the $CD34^+CD38^-$ blasts to the $CD34^+CD38^+$ blasts, the pro-apoptotic index was lower on $CD34^+/CD38^-$ blasts, indicating that the stem cell containing compartment has relatively more decoy receptor expression to protect itself to TRAIL mediated apoptosis.

respectively) and significantly lower expression of R3 ($p=0.006$). R4 expression did not differ between AML and normal blasts (figure 1).

Although mean expression of R3 on AML blasts is lower than on healthy donors, figure 1 shows that 20% of patients have R3 expression above mean plus 1 standard deviation (SD) (19 % = mean normal expression (13%) plus SD (6%)). 10% of patients have a higher R3 expression than mean + 2SD (=25%).

We determined a pro- or anti-apoptotic profile of leukemic blasts by dividing the percentage of positive leukemic blasts for R1 and R2 by the percentage positive blasts for R3 and R4 (R1+R2)/(R3+R4). When comparing healthy donors and AML patients, blasts from AML patients revealed a significantly higher ratio and thus a higher pro-apoptotic receptor profile than healthy donors ($p<0.005$, figure 2A).

In a univariate analysis, the percentage of R1, R2 and R4 positive blasts did not correlate to OS ($p=0.6$, $p=0.2$ and $p=0.2$)) or DFS ($p=0.2$, $p=0.5$ and $p=0.3$ respectively). On the other hand, the percentage of the anti-apoptotic R3 positive blasts significantly correlated to OS ($p= 0.036$) and to DFS ($p=0.04$). Patients in which > 25% (mean normal value + 2SD) of leukemic blasts were positive for R3 had a significantly shortened OS ($p=0.002$), as demonstrated in a Kaplan-Meier curve (figure 3).

In a multiple regression model (Cox), high R3 expression was the most significant prognostic factor for OS next to age at diagnosis ($p=0.003$ vs.

p=0.03, respectively). WBC was not a significant prognostic factor (p=0.174). As the patient groups with a poor and favorable risk profile were small (n=7 and n=16 respectively), we also performed the regression analysis in the patient group with an intermediate risk profile (n=53). WBC was also in the intermediate cytogenetic risk group not a significant predictor (p=0.128). In patients with an intermediate cytogenetic risk profile, high R3 expression was a better predictive parameter for OS (p=0.007) than age (p=0.038).

In conclusion, although leukemic blasts did express higher levels of pro-apoptotic R1 and R2 than normal blasts, these clinical data suggest that simultaneously expressed anti-apoptotic R3 strongly influences OS. We hypothesize that high expression of R3 prohibits effective apoptosis by naturally occurring native mTRAIL or sTRAIL.

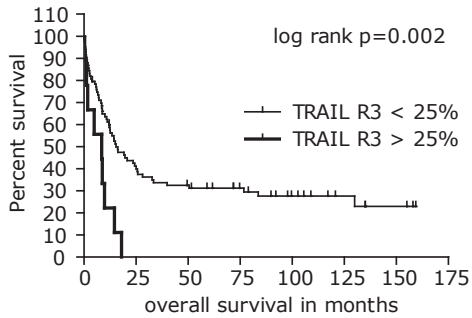

Figure 3. Kaplan-Meier survival curve of 92 de novo AML patients. Patients in which > 25% (mean normal value + 2SD) of leukemic blasts were positive for R3 had a significantly shortened OS (p=0.002 log rank).

TRAIL receptor expression on the leukemic $CD34^+CD38^-$ stem cells

In CD34 positive ($CD34^+$) AML, the leukemia-initiating event originates from the $CD34^+/CD38^-$ stem cell compartment. Survival of these cells after chemotherapy may lead to minimal residual disease (MRD) and subsequently to relapse. In AML patients, a high percentage of $CD34^+CD38^-$ stem cells at diagnosis significantly correlated with a high MRD frequency after chemotherapy and directly correlated with poor survival[36]. It is generally accepted that treatment of AML should aim to target the leukemic stem cell. We analyzed the TRAIL receptor expression directly on the $CD34^+/CD38^-$ compartment of 14 AML patients and 4 healthy donors. The higher pro-apoptotic receptor profile of AML blasts when compared to normal blasts was confirmed in these groups. When comparing the $CD34^+CD38^-$ cells to the $CD34^+CD38^+$ cells, the apoptotic ratio was lower on the $CD34^+CD38^-$ cells (figure 2B). This difference in ratio of pro-apoptotic versus anti-apoptotic receptor expression levels was significant on leukemic blasts (p=0.023). Probably, normal but especially leukemic stem cells are assigned to prevent themselves to TRAIL induced apoptosis by higher decoy receptor expression.

In conclusion, both the clinical correlation between high R3 expression and worse survival and the relative high expression of anti-apoptotic receptors on leukemic stem cells suggest a possible role for decoy receptor R3 in causing immune-escape of leukemic blasts.

TRAIL receptor expression on leukemic cell lines and in vitro sTRAIL/Apo2L induction

The function of R3 as a decoy receptor has been clearly addressed on Jurkat cells (T cells), HeLa (human adenocarcinoma), and 293 (human embryonic kidney) cell lines[37], but never on human myeloid cells. To confirm the anti-apoptotic function of R3 in myeloid leukemic cells we determined the expression of TRAIL receptors on different myeloid leukemic cell lines (table 2). Subsequently, we induced 5 cell lines with distinct receptor profiles with sTRAIL/Apo2L. The amount of induced cell death was determined by counting the 7AAD-/AnnexinV- viable cells. As expected, ME1 cells that lack R1 and R2 expression, barely showed induced cell death. U937 cells that only expressed R2 showed highest levels of cell death. Cell death was reduced in MM6 cells and HL60 cells that express high levels of both R2 and R3, which suggests that sTRAIL/Apo2L effects are inhibited by binding to R3 (table 2).

We then further explored the receptor expression on the cell lines with high R3 expression (HL60 and MM6). TRAIL receptor expression was determined for several days on HL60 and MM6 cells. Receptor expression on HL60 cells fluctuated over time and turned out to be cell cycle and differentiation status dependent[38], but was constantly expressed on the cell surface of MM6 cells. Induction of HL60 cells on different time instants with different receptor expression levels demonstrated a correlation (although not significant) between receptor expression defined as (R1+R2)/(R3+R4) and amount of induced cell death ($p=0.08$, $R=-0.6$). To elucidate the decoy receptor role of R3 the following experiments were performed with MM6 cells which showed stable receptor expression levels in time.

In conclusion, in myeloid cell lines we have found a correlation between the cell surface TRAIL receptor profile and the amount of induced cell death.

Modulation of R3 restores sTRAIL/Apo2L sensitivity of cell lines

To further elucidate the decoy receptor role of R3 we treated MM6 cells with PI-PLC. PI-PLC selectively removes GPI-linked proteins from the cell surface. Among the TRAIL receptors, R3 is the only GPI linked protein. Treatment with PI-PLC reduces R3 efficiently (>90%, figure 4). Expression levels of the other receptors were not significantly influenced by this treatment (data not shown). Treatment with PI-PLC alone did not induce significant cell death. Induction with

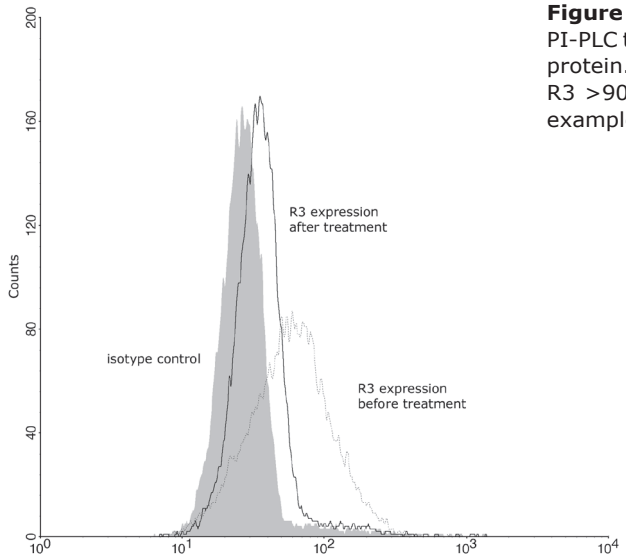

Figure 4. MM6 cells were treated with PI-PLC to remove R3, which is a GPI linked protein. Treatment with PI-PLC reduces R3 >90%. Histogram is a representative example of 3 different experiments.

sTRAIL/Apo2L (for 18 hours using the same protocol as above) induced cell death significantly (p=0.004, figure 5A). However, directly after removing R3, cell death could be enhanced significantly when compared to induction with sTRAIL alone (p=0.001, figure 5A). Addition of the pancaspase inhibitor Z-VAD-fmk 1 hour prior to induction with sTRAIL/Apo2L fully abrogated sTRAIL/Apo2L induced apoptosis indicating caspase dependent killing (with or without pre-treatment with PI-PLC).

In conclusion, these experiments confirmed both caspase dependency of sTRAIL/Apo2L induced apoptosis and the anti-apoptotic role of R3 in myeloid cell lines.

Treatment of primary AML leukemic blasts

We then treated 10 fresh primary AML samples (with different receptor expression profiles) with increasing levels of sTRAIL/Apo2L (20-1000 ng/ml) for 18 hours. Spontaneous cell death after 18 hours varied from 0-95%. We excluded from further analysis 3 samples that showed >80 % spontaneous cell death. Mean percentage of vital cells from the remaining samples was 69.6% (median 62.7%).

Results of the remaining 7 samples are shown in figure 6A. sTRAIL/Apo2L induced cell death > 20% in only 3 out of 7 patients (induced cell death ranged from 0-52 % (mean 19.8%, median 12.9%)).

In contrast to cell line derived myeloid cells, no clear correlation between TRAIL receptor expression level and the amount of induced cell death by sTRAIL/Apo2L could be demonstrated in freshly derived patient samples. However, in one patient with high R3 expression we could enhance cell death by removing R3

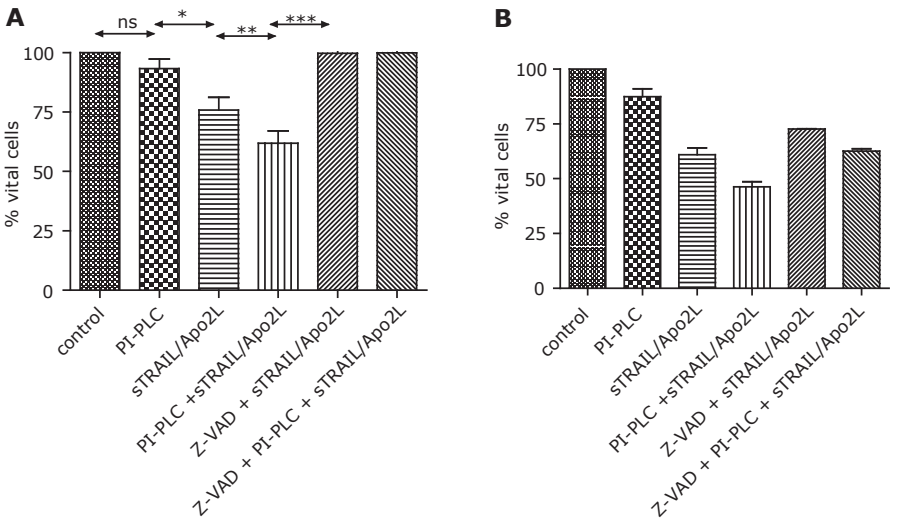

Figure 5A. MM6 cells treated with PI-PLC, sTRAIL/Apo2L and Z-VAD. Treatment with PI-PLC alone did not induce significant cell death. Induction with sTRAIL/Apo2L alone significantly induced cell death (* p=0.004). However, directly after removing R3, cell death could be enhanced significantly when compared to induction with sTRAIL alone (** p=0.001, figure 6). Addition of the pancaspase inhibitor Z-VAD-fmk 1 hour prior to induction with sTRAIL/Apo2L) fully abrogated (*** p=0.001) sTRAIL/Apo2L induced apoptosis indicating caspase dependent killing (with or without pre-treatment with PI-PLC). Results are mean of 7 different experiments.

Figure 5B. Fresh de novo AML sample treated with PI-PLC and sTRAIL/Apo2L. Treatment with PI-PLC alone did not induce significant cell death. Induction with sTRAIL/Apo2L alone significantly induced cell death. However, directly after removing R3, cell death could be enhanced when compared to induction with sTRAIL alone. (no statistics possible, experiment was performed in triplo).

with PI-PLC. By adding Z-VAD we could also confirm that induced cell death was caspase dependent (figure 5B).

In conclusion, fresh AML patient samples were not very sensitive to sTRAIL/Apo2L induced cell death, possibly due to high decoy receptor expression as demonstrated in one sample, in which cell death could be enhanced by removal of R3.

Directly targeting pro-apoptotic TRAIL receptors R1 and R2

As, in vivo, removal of R3 will also affect other GPI linked proteins, we explored other ways to bypass R3 expression. We treated AML samples with monoclonal antibodies that target specifically R1 and R2 (mapatumumab and lexatumumab, respectively)[32]. Twelve fresh AML samples were incubated with different concentrations of these antibodies (figure 6B and 6C). Mapatumumab induced cell death > 20% in only one out of twelve patients (induced cell death ranging from 0 to 39.2% (mean 5.9%, median 2.7%)). No clear correlation with R1 expression on the cell surface could be demonstrated; the patients that showed 35% and

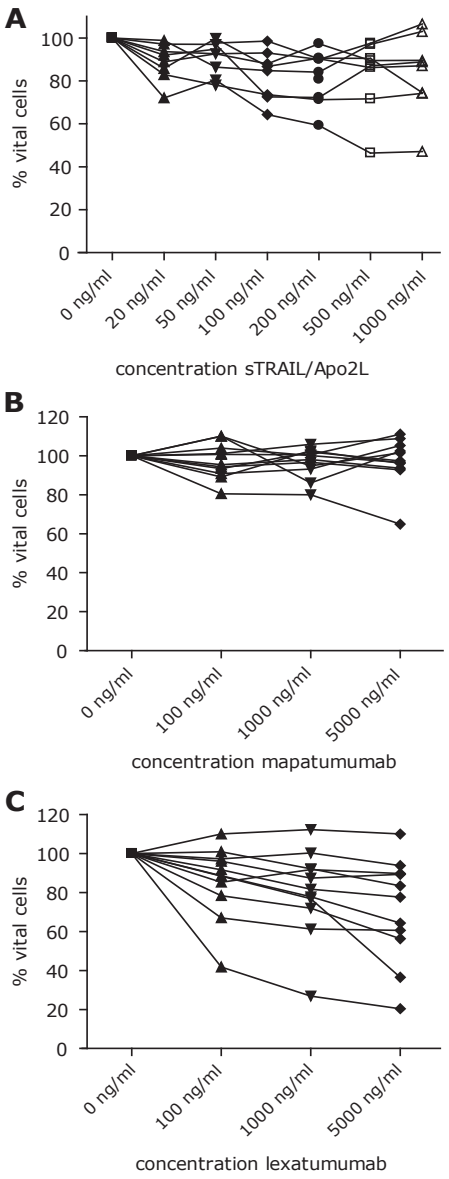

Figure 6A. 10 fresh de novo AML samples were treated with increasing levels of sTRAIL/Apo2L (20-1000 ng/ml) for 18 hours. Spontaneous cell death after 18 hours varied from 0-95%. sTRAIL/Apo2L induced cell death > 20% in 3 out of 7 patients (induced cell death ranged from 0-52 % (mean 19.8%, median 12.9%)).
Figure 6B and 6C. 12 fresh de novo AML samples were treated with increasing levels of monoclonal antibodies that target specifically R1 and R2 (mapatumumab and lexatumumab). Mapatumumab induced cell death > 20% in 2 out of 12 patients (induced cell death ranging from 0 to 39.2% (mean 5.9%, median 2.7%)). Lexatumumab induced cell death > 20% in 7 out of 12 patients (induced cell death ranging from 0 to 79.6% (mean 24.1%, median 16.5%)).

40% cell death had respectively 20% and 14% R1 expression. One patient that had high R1 expression was not sensitive to mapatumumab. Lexatumumab induced cell death > 20% in seven out of twelve patients (induced cell death ranging from 0 to 79.6% (mean 24.1%, median 16.5%)). For lexatumumab there was a correlation between receptor expression of R2 and sensitivity to the antibody; all samples that were sensitive to lexatumumab were also positive for R2 expression (> 20% positive cells), figure 7. High R2 expression did not always result in sensitivity to lexatumumab. Differences in sensitivity to lexatumumab in patients with low (<20%) and high (>20%) are not significant, probably due

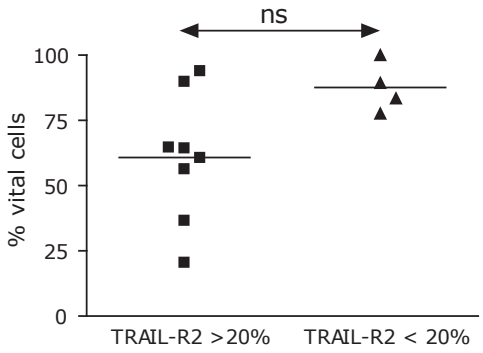

Figure 7. Correlation between receptor expression of R2 on fresh AML samples and sensitivity to lexatumumab. All samples that were sensitive to lexatumumab were also positive for R2 expression (>20% positive cells). High R2 expression did not always result in sensitivity to lexatumumab. Difference between groups is not significant but indicates a trend.

to small groups, but may indicate a trend for a correlation. There is no cross-reactivity of lexatumumab or mapatumumab between the decoy receptors. Therefore the levels of decoy receptors will not affect binding or apoptosis of these antibodies. As expected, sensitivity to lexatumumab was independent of R3 expression (high level of R3 did not impede lexatumumab induced apoptosis).
In conclusion, lexatumumab was more effective at killing primary AML cells than sTRAIL/Apo2L or mapatumumab. Sensitivity to lexatumumab was only observed in samples that were positive for expression of R2.

Discussion

In this study we have evaluated TRAIL receptor expression levels on leukemic blasts of AML patients and compared them to receptor expression levels on myeloid blasts of healthy donors. AML blasts did express significantly more functional receptors (R1 and R2) and significantly lower levels of the decoy receptor R3 when compared to myeloid blasts of healthy donors (R4 expression did not differ). This difference results in a significantly higher pro-apoptotic receptor profile of myeloid leukemic blasts when compared to normal blasts of healthy donors. However, about 20% of patients have high expression levels of the anti-apoptotic receptor R3 on their blasts. These patients (with a relative more anti-apoptotic receptor profile) have a significant shortened OS when compared to the patients with low R3 levels and hence a more pro-apoptotic profile.
As a result of the influence of TRAIL receptor expression on OS (independent of other risk factors like cytogenetics, age and WBC), we hypothesized that myeloid leukemic blasts are principally sensitive to TRAIL mediated killing by mTRAIL and/or sTRAIL expressed and secreted by naturally effector immune cells as a part of immune surveillance. Furthermore, we hypothesized that this physiological anti-tumor activity in vivo is counteracted by high R3 expression in a subset of patients. Upregulation of R3 could be regarded as an expression of immune subversion, ultimately leading to immune escape of leukemic cells.

If proven correct, this would open opportunities for exploitation of the TRAIL pathway in the treatment of AML patients by overruling or bypassing the decoy function of R3.

To provide evidence for our hypothesis we confirmed the assumed anti-apoptotic role of R3 on myeloid cells (which until now was only demonstrated on other cell types). This was clearly demonstrated by enhancing sTRAIL/Apo2L induced cell death after removal of R3 from the cell surface of myeloid cell lines and patients blasts.

Both myeloid leukemic cell lines and fresh primary AML samples were incubated with different concentrations of sTRAIL/Apo2L. In cell lines, we have found a correlation between receptor expression and sensitivity to sTRAIL/Apo2L. However, patient's samples were not very sensitive to sTRAIL/Apo2L, possibly due to high decoy receptor as demonstrated in one sample, in which cell death could be enhanced by removal of R3. In clinical treatment, it will be difficult to modulate R3 expression and we therefore decided to explore the possibility of directly targeting R1 and R2. Myeloid blasts turned out to be most sensitive to lexatumumab, the antibody that selectively targets R2. Moreover, R2 positivity seemed to be requisite for lexatumumab induced cell death. Finally, leukemic stem cells (defined by $CD34^{+}/CD38^{-}$ expression) showed relative highest levels of R3. Targeting of the pro-apoptotic receptors will be necessary to kill these cancer stem cells although harbors the risk of targeting also the normal hematopoietic stem cell. However, no phase I or II study to date using antibodies targeting R1 or R2 has reported hematological toxicity[33].

In conclusion, our results clearly demonstrate that AML blasts can be sensitive to cell death induction via the TRAIL receptor pathway and that R3 can function as a negative regulator of this pathway. However, it is also clear that the majority of myeloid blasts does express pro-apoptotic receptors but fails to undergo apoptosis when only triggering these receptors. It is very likely that activation of only the extrinsic pathway is not effective enough to kill tumor cells that have already been shaped by the immune system. Many studies have now demonstrated synergistic activity of sTRAIL/Apo2L with conventional chemotherapy[33;39-41]. These effects are ascribed to the combined activation of the extrinsic and intrinsic apoptotic pathway. Also, synergistic effects of proteasome inhibitors like bortezomib[29], kinase inhibitors[25] with sTRAIL/Apo2L or TRAIL receptor antibodies have been described. Furthermore, the mechanism of the anti-tumor activity of HDAC inhibitors can also be ascribed to upregulation of TRAIL and its agonistic receptors[42]. All these data clearly demonstrate that the TRAIL pathway is an important player in the complex field of apoptosis induction of cancer cells. We now provide evidence that the TRAIL pathway is involved in immune surveillance of leukemia. Through bypassing the decoy receptors and targeting the pro-apoptotic TRAIL receptors, apoptosis could be induced in primary AML samples. Targeting the pro-apoptotic TRAIL receptors could add a valuable modality to the treatment of AML patients.

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