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Original Article

Activation and crosstalk between TNF family receptors in umbilical cord blood cells is not responsible for loss of engraftment capacity following culture

Keren Mizrahi, Nadir Askenasy

Frankel Laboratory, Center for Stem Cell Research, Department of Pediatric Hematology-Oncology, Schneider Children's Medical Center of Israel, Petach Tikva 49202, Israel

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Abstract: Umbilical cord blood (UCB) is a rich source of hematopoietic progenitors for transplantation. Murine and human progenitors are insensitive to apoptotic signaling mediated by the TNF family receptors, however extension of culture over 48 hours is accompanied by severe deterioration in engraftment and hematopoietic reconstituting capacity. In this study we assessed crosstalk between the Fas, TNF and TRAIL receptors, and questioned whether it contributes to increased mortality and decreased activity of UCB progenitors following extended *ex vivo* culture for 72 hours. The well-characterized TNF-induced expression of Fas is mediated by both TNF receptors, yet the TNF receptors determine survival rather than Fas: superior viability of TNF-R1 progenitors. Additional cross talk includes upregulation of TRAIL-R1 by Fas-ligand, mediated both by fast cycling and inductive crosstalk. These inductive interactions are not accompanied by concomitant sensitization of progenitors to receptor-mediated apoptosis during extended culture, but rather decreased fractional apoptosis in expanded progenitor subsets expressing the receptors. TRAIL upregulates both TRAIL-R1 and TRAIL-R2, accompanied by commensurate susceptibility to spontaneous apoptosis. The current data reveal inductive crosstalk between TNF family receptors, which are largely dissociated from the sensitivity of hematopoietic progenitors to apoptosis. Activation of Fas, TNF and TRAIL receptors and excessive apoptosis are not responsible for loss of engraftment and impaired reconstituting activity of UCB progenitors following extended culture.

Keywords: Umbilical cord blood, receptor crosstalk, Fas, TNF-α, TRAIL, apoptosis, SCID reconstituting activity

Introduction

The tumor necrosis factor (TNF) superfamily includes 19 known pairs of receptor/ligand interactions involved in apoptotic and non-apoptotic signaling [1]. Despite significant structural homology, these cognate receptor/ligand interactions are highly selective, with distinct patterns of activation and signal transduction. Versatile functions have been determined for the TNF family receptors in different tissues, involving survival, apoptosis, proliferation and differentiation [2-5]. In the immune and hematopoietic systems, the TNF family receptors are prime homeostatic factors with negative regulatory activity. The best-characterized function is activation induced cell death (AICD), mediated by Fas cross-linking following T cell activation [6, 7]. The highly selective pattern of cognate receptor/ligand interactions is accompanied by inductive crosstalk, for example upregulation of Fas expression by TNF-α [8-11]. The Fas receptor is considered as a common executioner of apoptosis within the TNF superfamily, which is unique within the family due to obligatory receptor trimerization by membrane-bound Fas-ligand (FasL) [12].

In contrast, the TNF family receptors play different roles in proximal stages of hematopoietic differentiation. In a series of murine cell studies we have characterized the activity of Fas [13], TNF-α [14] and TRAIL [15] receptors in hematopoietic stem and progenitor cells (HSPC). Murine progenitors are largely insensitive to receptor-mediated apoptosis, and the TNF family receptors transduce primarily trophic signals [14-16]. The hematopoietic progeny becomes

gradually sensitive to receptor-mediated apoptosis along the differentiation process, rendering the expanding clones sensitive to negative regulation. Insensitivity of stem and progenitor cells to apoptosis is also characteristic of human umbilical cord blood (UCB) and mobilized peripheral blood (mPB) cells [15, 17, 18], a phenomenon attributed to constitutive upregulation of the NFκB pathway [19]. The physiological involvement of TNF family receptors in the transplant setting includes activation of the stroma [20], immune privilege prioritizes HSPC for engraftment over mature hematopoietic cells [13, 20], and trophic signaling fosters early engraftment [14-17] and durable hematopoietic reconstitution [14, 20]. Polarized susceptibility of hematopoietic cells to apoptosis can be used to enrich and select apoptosis-insensitive progenitors from heterogeneous murine and human hematopoietic grafts *ex vivo* to reduce the threat of graft versus host disease [18, 21].

A standing question is the cause of reduced engraftment of hematopoietic cells after extended *ex vivo* culture, which usually occurs during the third day of incubation and impairs the activity of SCID reconstituting cells (SRC) [22-25]. Loss of engraftment capacity has been attributed to death of hematopoietic progenitors in culture [26-28], mediated by the TNF family receptors [29-33]. In view of our findings of progenitor resistance to apoptotic signaling [13-21], we questioned whether loss of engraftment potential is indeed mediated by receptor-mediated apoptosis. We found that $CD34^+$ UCB progenitors undergo spontaneous apoptosis during extended incubation, without significant impact of receptor-mediated signaling. Subsequently, we questioned the consequences of receptor crosstalk on HSPC sensitivity to apoptosis, particularly in view of the proposed dominant role of Fas suppression of engraftment [8-11, 26, 27]. We found inductive crosstalk between TNF family receptors with variable influences on cell cycling, without affecting the susceptibility to apoptosis.

Materials and methods

Sample collection and cell isolation

UCB units were obtained from healthy donors giving birth at term according to the Institutional guidelines. UCB samples were collected before placental delivery by using syringes containing citrate-phosphate-dextrose with adenine (CP-DA-1) as anti-coagulant for processing within one hour. Blood was diluted with phosphate-buffered saline (PBS; Biological Industries) containing 0.5% HSA and 2 mM EDTA in 1:1 proportion, layered over Lymphocyte Separation Medium (1.07700.1-1.0800 g/ml; MP Biomedical) and centrifuged at 800 *g* for 20 min at 25°C. Mononuclear cells in the interface layer were collected and washed twice with physiological saline [15]. For staining with an intracellular dye, the cells were incubated for 7 minutes with 2.5 μM of 5-(and-6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes), washed and resuspended [17].

Animal preparation and transplantation

In this study we used NOD.CB17-Prkdcscid/J (NOD.SCID, $H2K^d$) mice, purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a barrier facility; the Institutional Animal Care Committee approved all procedures. Prior to transplant, at days -3 and -2 mice received two doses of 25 mg/kg Busulfan (Sigma) [15, 17, 19]. On transplant day (day 0) cells were suspended in 0.2 ml phosphate buffered saline (PBS) and infused into the lateral tail vein.

Flow cytometry

Measurements were performed with a Vantage SE flow cytometer (Becton Dickinson). $CD34^+$ cells were identified using Allophycocyanin (APC)-labeled antibodies (clone 8G12, Pharmingen). Expression of receptors on UCB cells was determined with mAb against: Fas (clone DX2, Miltenyi), TNF-R1 (clone 16803, R&D Systems), TNF-R2 (clone 22235, R&D Systems), TRAIL-R1 (DR4, clone 69036, R&D) and TRAIL-R2 (DR5, clone 71908, R&D). In NOD.SCID, xenochimeras engraftment of human cells was determined in the bone marrow after 12 weeks with mAb against *m*-CD45 (clone 30-F11, eBioscience) and *h*-CD45 (clone ML2, IQP). Cell death and apoptosis was determined in incubated cells with 5 μg/ml 7-aminoactinomycin-D (7-AAD, Sigma) and Annexin-V (IQP), respectively. Proliferation was determined from CFSE dilution and calculated using the ModFit software (Verity Software House).

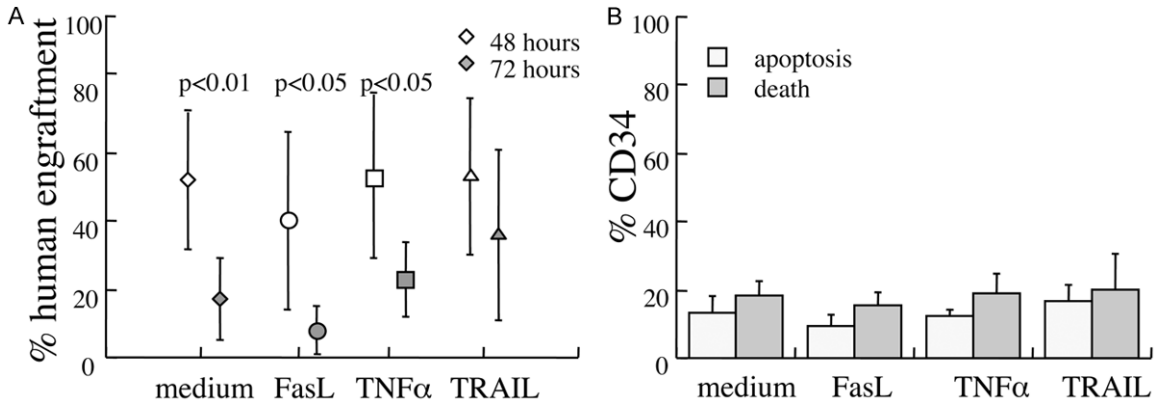

Figure 1. Engraftment and viability of UCB cells after *ex vivo* culture. A: Fresh UCB units were incubated for 48 and 72 hours in medium (n=13) and with 50 ng/ml FasL (n=6), 20 ng/ml TNFα (n=7) and 1.5 μg/ml TRAIL (n=5). Equal numbers of viable cells from the same unit were grafted into NOD.SCID mice conditioned with two doses of 25 μg/g Busulfan, and human chimerism was determined in the bone marrow of xenochimeras after 12 weeks (means ± standard deviations). B: Apoptosis and death of $CD34^+$ progenitors after 72 hours of *ex vivo* culture with and without the ligands, as determined from Annexin-V incorporation and 7-AAD uptake respectively (n=11-15).

Apoptotic challenge

Human MNC UCB cells were incubated ($1x10^6$ cells/ml) for 1-3 days in α-MEM culture medium supplemented with StemPro Nutrient Supplement (Stem Cell Technologies, Vancouver, BC), 2 mM L-glutamine (Biological Industries) and 50 μM 2β-mercaptoethanol (2β-ME) (Sigma). Cells were challenged with 50 ng/ml SuperFasL (Axxora), 20 ng/ml TNF-α and 1500 ng/ml TRAIL (PeproTech) [15, 17, 19]. The apoptotic effect of the ligands was validated in human Jurkat cells as positive controls (E6-1TIB-152, ATCC). Cell death was determined by gating on receptor-positive subsets, to observe their behavior in the context of the bulk UCB population.

Statistical analysis

Data are presented as means ± standard deviations for each experimental protocol. Results in each experimental group were evaluated for reproducibility by linear regression of duplicate measurements. Differences between the experimental protocols were estimated with a post hoc Scheffe *t*-test and significance was considered at $p<0.05$.

Results

TNF family receptor activation does not affect engraftment after extended ex vivo culture

We have previously demonstrated that engraftment of UCB progenitors in NOD.SCID mice is not impaired by cross-linking of Fas and ligation of the TNF and TRAIL receptors for periods of 2 days [15, 17, 19]. To determine the impact of TNF family receptor activation on SRC activity, UCB cells from same units were cultured for 48 and 72 hours with the various ligands (without chemokine supplements). Concentrations of the cognate ligands were determined in preliminary studies to dissociate between apoptosis-insensitive progenitors and apoptosis-sensitive mature cells: 50 ng/ml FasL, 20 ng/ml TNF-α and 1.5 μg/ml TRAIL. Transplantation of equal numbers of live UCB cells from 65 cord units into busulfan-conditioned NOD-SCID mice was evaluated after 12 weeks by human CD45 in the bone marrow of the xenochimeric mice. Summary of these transplant experiments shows significant decline in levels of human chimerism when *ex vivo* incubation in medium was extended from 2 to 3 days (**Figure 1A**). Exposure to FasL and TNF-α had no significant impact on SRC activity, whereas TRAIL showed a trend of improved levels of human xenochimerism. These data negate involvement of TNF family receptor activation on loss of hematopoietic progenitor engraftment capacity during extended *ex vivo* culture.

Overall death of $CD34^+$ progenitors was <20% after 72 hours of liquid culture, with the major mechanism being apoptosis as determined from Annexin-V incorporation (**Figure 1B**). Insignificant impact of the death ligands on overall survival demonstrates insensitivity of the progenitors to activation of the TNF family

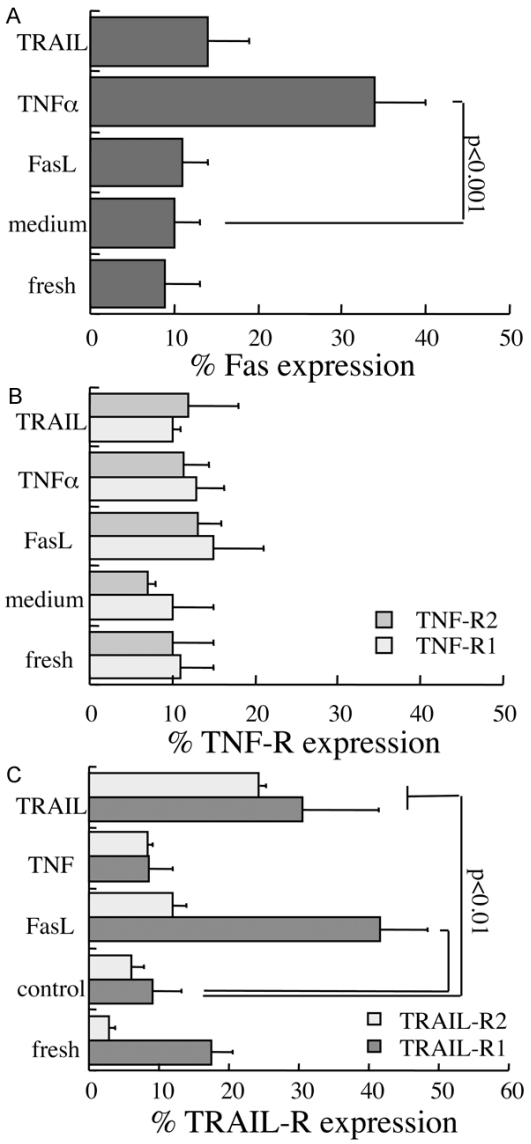

Figure 2. Dynamics of death receptor expression in liquid culture. Mononuclear UCB cells were incubated for 72 hours with 50 ng/ml FasL, 20 ng/ml TNF-α and 1.5 μg/ml TRAIL. Expression of the receptors was determined by gating on $CD34^+$ progenitors in fresh samples and following incubation. A: Fas (n=29 fresh, n=11 incubated); B: TNF-R1 (n=7 fresh, n=5 incubated) and TNF-R2 (n=12 fresh and n=10 incubated); C: TRAIL-R1 (DR4, n=9) and TRAIL-R2 (DR5, n=8).

receptors. Thus, loss of engraftment capacity is unrelated to excessive death of progenitors.

Inductive crosstalk

Insensitivity of hematopoietic progenitors to apoptosis has been attributed to low levels of expression of the TNF family receptors, while TNF-induced upregulation of Fas sensitizes to death [8-11, 26, 27]. To determine the responsiveness to apoptotic signals in culture, expression of the various receptors was determined in gated $CD34^+$ progenitors before and after incubation. The Fas, TNF and TRAIL apoptotic receptors are expressed in ~10% of fresh $CD34^+$ UCB cells, with slightly higher levels of TRAIL-R1 expression (**Figure 2**). TRAIL was the only ligand that induced expression of both cognate receptors in UCB progenitors ($p<0.01$, **Figure 2C**). Cross incubation with the various ligands showed induction of Fas expression by TNF-α in one third of the progenitors ($p<0.001$, **Figure 2A**), and induction of TRAIL-R1 by FasL ($p<0.001$, **Figure 2C**). Thus, receptor induction during extended culture includes cognate interactions of TRAIL and crosstalk mediated by TNF-α and Fas-ligand.

Proliferation rates of receptor-positive progenitors

Fractional increase in expression of a given receptor upon exposure to cognate and non-cognate ligands might be the consequence of expansion of selected subsets within the heterogeneous UCB population. Incubation of UCB cells with the various ligands for 48 hours resulted in insignificant modulation of the proliferation rates [15, 17, 19]. After 72 hours of incubation in medium, significant differences included faster cycling of $CD34^+$ progenitors expressing TNF-R2 as compared to TNF-R1, which were reversed by exposure to the cognate ligand ($p<0.05$, **Figure 3A**). In addition, exposure to FasL increased 2-fold the cycling rates of progenitors expressing TRAIL-R1. The five-fold increase in expression of TRAIL-R1 under the influence of FasL was therefore partially caused by fast proliferation of this subset of cells, in addition to a true inductive effect. Stable proliferation rates of $CD34^+Fas^+$ progenitors demonstrate that upregulation of Fas by TNF-α was a consequence of true inductive crosstalk.

Relative sensitivity to apoptosis triggered by the death ligands

In next stage we questioned whether induced receptor expression and modulation of the cycling rates sensitize UCB progenitors to apoptosis. As seen for shorter incubation periods [15, 17, 19], the main cause of progenitor death was spontaneous apoptosis, with little influence of the death ligands on progenitor viability

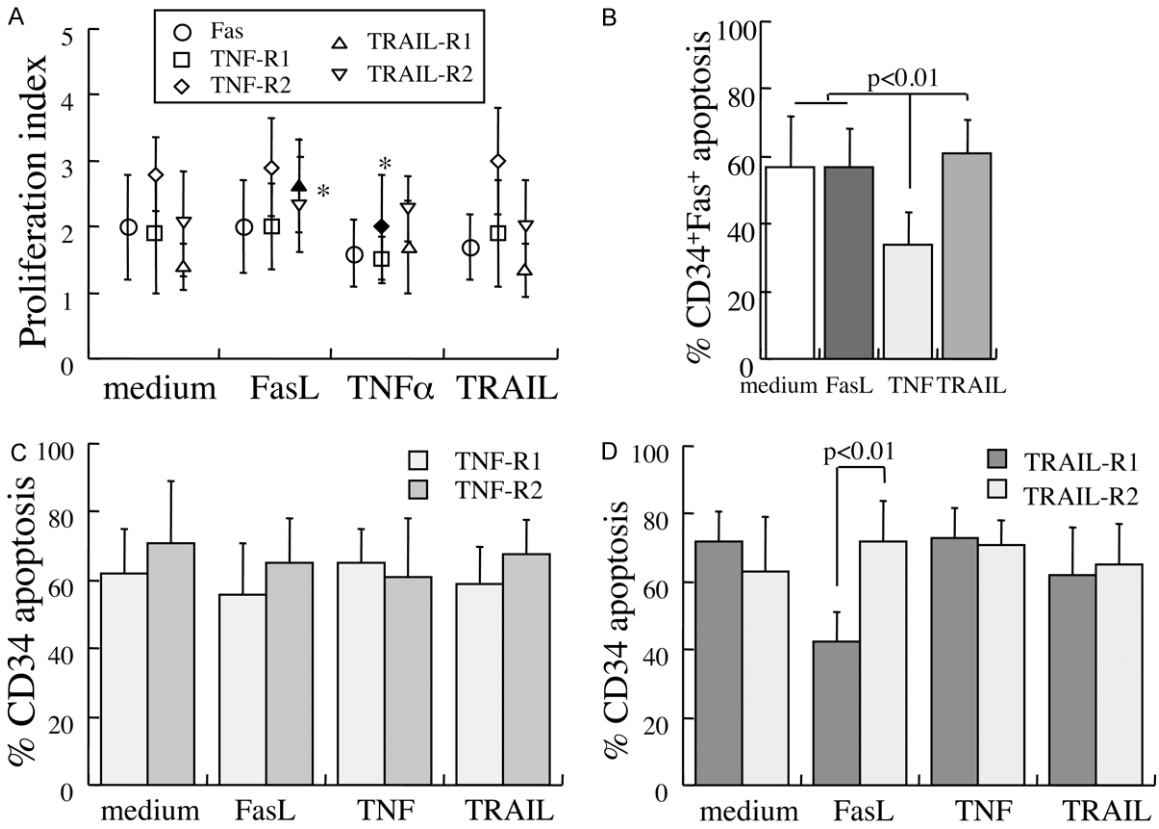

Figure 3. Proliferation and apoptotic death of UCB cells in culture. A: Proliferation rates of CD34$^+$ progenitors expressing the various receptors within 72 hours of culture, as determined from CFSE dilution (n=4-9). Proliferation rates were quantified with the ModFit software. *p<0.05. B-D: Apoptosis was determined according to incorporation of Annexin-V in gated CD34$^+$ progenitors following incubation for 72 hours 50 ng/ml FasL, 20 ng/ml TNF-α and 1.5 μg/ml TRAIL (n=4-7 under each culture condition). Data are presented in reference to expression of: B: Fas, C: TNF-R1 and TNF-R2, C: TRAIL-R1 (DR4) and TRAIL-R2 (DR5).

(**Figure 3B-D**). We found two patterns of attenuation of cell sensitivity to apoptosis under our culture conditions. First, induced TNF-induced Fas expression (p<0.01, **Figure 3B**) and FasL-induced proliferation of TRAIL-R1$^+$ cells (p<0.01, **Figure 3D**) displayed reduced fractional apoptosis, demonstrating dissociation between levels of expression and sensitivity to receptor-mediated apoptosis. Second, the inductive effect of TRAIL on progenitors expressing its cognate receptors was accompanied by proportional sensitization to apoptosis in response to receptor activation (**Figure 3D**).

Co-expression of death receptors

Inductive crosstalk means that ligand-mediated activation of a cognate receptor induces the expression of another receptor, since there is no cross-activation within the TNF superfamily [1]. To determine which TNF receptor is responsible for upregulation of Fas, double positive cells were gated following incubation for 72 hours. Activation of both TNF receptors had equal contribution to upregulation of Fas in >75% of TNF receptor-positive cells (**Figure 4A**). Fas cross-linking during the last day of incubation resulted in significant differences in progenitor viability: progenitors co-expressing Fas and TNF-R1 displayed superior survival following incubation with both ligands as compared to co-expression of Fas and TNF-R2 (p<0.01, **Figure 4B**). The similar differences in survival in CD34$^+$ progenitors devoid of Fas expression indicates that apoptosis was primarily related to the TNF receptors without pro-apoptotic activity of the upregulated Fas.

Discussion

This study demonstrates that resistance to receptor-mediated apoptosis is a conserved

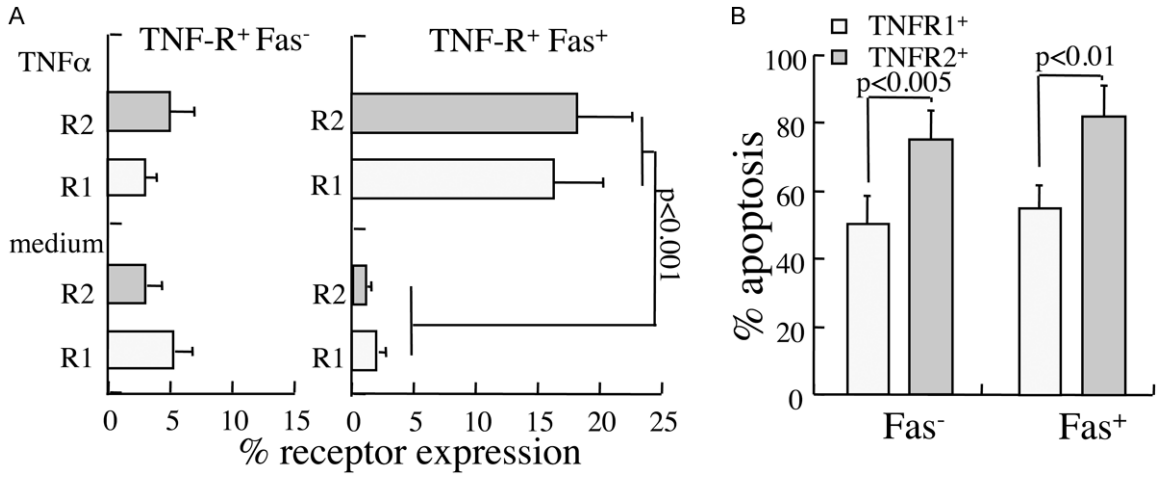

Figure 4. The consequences of inductive cross talk. A: UCB cells were exposed to 20 ng/ml TNF-α for 72 hours and expression of Fas was determined by gating on $CD34^+$ progenitors in reference to expression of the two TNF receptors (n=7) as compared to incubation in medium (n=9). Expression of the two TNF receptors was determined in $CD34^+Fas^-$ (left panel) and $CD34^+Fas^+$ progenitors (right panel). B: Cultures were supplemented with 50 ng/ml FasL during the third day of culture and apoptosis was measured in reference to Fas and TNF receptor expression (n=4-9 under each culture condition).

and consistent characteristic of hematopoietic progenitors, and is not responsible for the decay in progenitor engraftment capacity after extended culture. Similar decrease in progenitor function was observed in the presence and absence of the death ligands, and occurred despite sustained viability by >80% of the progenitors. These findings in culture conditions that simulate graft preparation for transplantation without supporting chemokines differ from prior studies reporting detrimental consequences of TNF receptor activation on progenitor viability and function [8-11, 26, 27, 29-33]. In those studies supporting chemokines and activating cytokines induced cell proliferation and sensitized to receptor-mediated apoptosis *in vitro*. In addition, the presence of dead cells suppresses the clonogenic activities of UCB cells in semisolid cultures [19], and accumulation of apoptotic committed progenitors might have mislead the interpretation of this functional *in vitro* assay. To the extent of confidence in SRC engraftment as a reliable surrogate assay for the activity of human reconstituting cells [34, 35], there is no evidence of detrimental consequences of TNF family receptor ligation.

Crosstalk was identified for the well-known induction of Fas by TNF-α [8-11], which was upregulated in approximately one third of all $CD34^+$ UCB progenitors under our culture conditions (**Figure 5**). Equal contribution was emphasized by co-expression of Fas in ~75% of progenitors expressing either one of the TNF receptors. Induction of Fas is the result of true crosstalk between the receptors at stable proliferation rates, and does not sensitize to apoptosis or reduce the engraftment capacity. Another mechanism of increased fractional expression is stimulation of proliferation of subsets expressing particular receptors. Faster proliferation of viable cells (2-fold) accounted only partially for expansion of $TRAIL\text{-}R1^+$ progenitors mediated by FasL (5-fold), indicating that this receptor was also upregulated through inductive crosstalk. Notably, all mononuclear UCB cells expressing TRAIL-R1 are slow cycling and activation of this receptor transduces trophic signals [15].

The expanded subsets of Fas^+ and $TRAIL\text{-}R1^+$ progenitors induced by TNF-α and FasL respectively, were characterized by reduced fractional death. Spontaneous death of stable absolute numbers of progenitors appeared as reduced fractional apoptosis due to increased levels of expression of the receptors in viable cells, mediated either by inductive crosstalk or fast cycling. These findings are consistent with inherent resistance to apoptosis regulated at the transcriptional level [19], opposing the contention that progenitor insensitivity to apoptosis is a consequence of the low levels of expression of death receptors [8-11]. Notably, the TNF

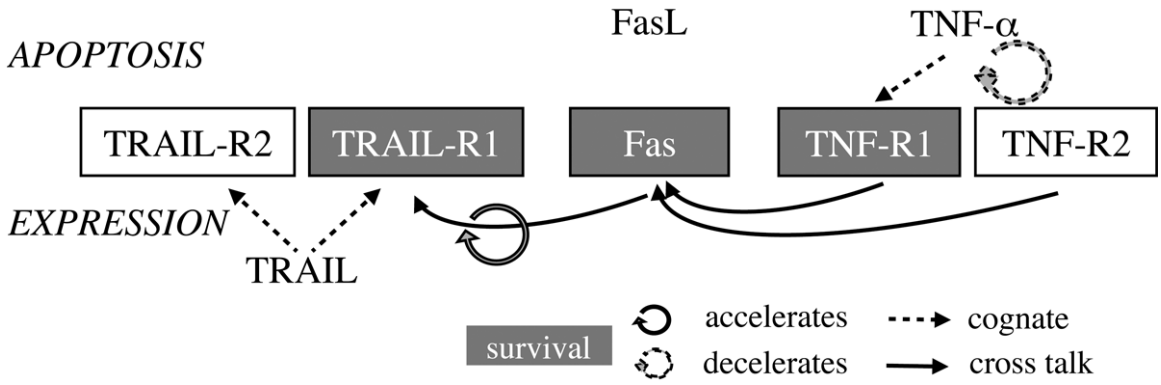

Figure 5. Diagrammatic presentation of the impact of crosstalk on receptor expression, proliferation and sensitivity to apoptosis of hematopoietic progenitors.

family receptors were found in significantly higher fractions of $CD34^+$ progenitors susceptible to spontaneous apoptosis (~60%) as compared to the entire $CD34^+$ progenitor pool (<20%).

The Fas receptor is considered as a common executioner of apoptosis in immunohematopoietic cells, with a particular characteristic requirement of receptor cross-linking mediated only by membrane-bound isoforms of the ligand [12]. In contrast to prior reports showing detrimental consequences of Fas-mediated apoptosis under conditions of TNF receptor activation [8-11, 26, 27, 29-33], we found that the TNF receptors are dominant factors in UCB progenitors co-expressing both receptors. Irrespective of co-expression of Fas, TNF-R2 was associated with increased susceptibility to apoptosis and TNF-R1 was associated with superior survival (**Figure 5**). This observation is consistent with transduction of trophic signals by TNF-R1 that enhances recruitment of progenitors to engage in clonogenic activity in semisolid culture [17]. Although there was no detectable role of TNF-R2 in murine and human progenitors during 48 hours of culture [14, 17], our current data reveal activity of this receptor under extreme conditions of stress. $CD34^+$ progenitors expressing TNF-R2 cycled faster than progenitors expressing TNF-R1 under unstimulated conditions, and displayed similar levels of spontaneous apoptosis. Activation of TNF-R2 by the cognate ligand slowed proliferation of viable UCB progenitors by 50% and joint exposure to FasL and TNF-α increased fractional apoptosis by 50% as compared to progenitors expressing TNF-R1. Both TNF receptors reduce the size of colonies by negative regulation of expanding clones, though their impact is modest relative to Fas cross-linking [17]. Functional involvement of TNF-R2 in hematopoietic progenitor function explains the impaired durable hematopoietic reconstitution of murine bone marrow cells deficient in this receptor [14].

The different consequences of activation of the two TNF receptors can be explained on the basis of the signaling pathways: TNF-R1 recruits complexes composed of TRADD, FADD, RIP-1 and TRAF2, whereas only the latter transduces TNF-R2 signals [36-39]. Despite absence of the complex assembly terminal, TNF-R2 is capable of inducing apoptosis [40, 41] with dual synergistic and antagonistic effects to TNF-R1 activity [42]. The intracellular signaling pathways of these two receptors cooperate [43] and suppress each other under different experimental conditions [44, 45]. It is possible that the dominant activity of TNF-R1 seen in culture originates from activation of this receptor by soluble TNF-α [46], while TNF-R2 is predominantly activated by the membrane-bound ligand [47].

In variance, Fas and TRAIL receptors share a common mechanism of signal transduction through FADD complexing and direct activation of the proteolytic caspase cascade [48-51]. Improved viability following activation of TNF-R1 in Fas^+ UCB progenitors suggests a competitive mode of intracellular signaling: TRADD-mediated aggregation of FADD might reduce the capacity of Fas to initiate the extrinsic apoptotic caspase cascade. In view of high fractional apoptosis upon TNF-R2 activation in both Fas^+ and Fas^- subsets of UCB progenitors,

it is clear that activation of the NFκB pathway by TRAF2 is not the mechanism that dissociates between death and survival. Involvement of caspase-8 in dual apoptotic and trophic signaling [14, 17] suggests further downstream modulation of the pathway activated by TNF-R1 [52-54]. It is possible that Fas-mediated upregulation of TRAIL-R1 is a consequence of common FADD signaling, similar to the inductive effect of TRAIL on its cognate receptors. If this possibility is true, it remains unclear why Fas signaling is not inductive to TRAIL-R2 and selectively reverses the slow cycling of TRAIL-R1$^+$ progenitors.

In summary, *ex vivo* culture exceeding 48 hours is unfavorable to engraftment and reconstituting capacity of UCB progenitors. New insights into the activity of TNF superfamily receptors in hematopoietic progenitors include: a) engraftment deficit is mediated primarily by dysfunction rather than progenitor death; b) UCB-derived hematopoietic progenitors succumb to spontaneous apoptosis in liquid culture *ex vivo* without significant involvement of receptor-mediated apoptotic pathways; c) TNF receptors are dominant in determination of survival of progenitors after TNF-induced upregulation of Fas; d) FasL induces expression of TRAIL-R1. Inductive crosstalk between the receptors is likely to play a role in their coordinated upregulation in bone marrow-homed murine and human progenitors [13-15, 55], with ubiquitous expression in most primitive hematopoietic precursors [13, 15, 20] and failure of durable reconstitution by receptor-deficient cells [16, 17, 20].

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Nadir Askenasy, Frankel Laboratory, Center for Stem Cell Research, Department of Pediatric Hematology-Oncology, Schneider Children's Medical Center of Israel, 14 Kaplan Street, Petach Tikva, Israel 49202. Tel: 972-3921-3954; Fax: 972-3921-4156; E-mail: anadir@012.net.il

References

[1] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003; 3: 745-756.

[2] Locksley RM, Killeen N and Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001; 104: 487-501.

[3] Bhardwaj A and Aggarwal BB. Receptor-mediated choreography of life and death. J Clin Immunol 2003; 23: 317-332.

[4] Gaur U and Aggarwal BB. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochem Pharmacol 2003; 66: 1403-1408.

[5] Aggarwal BB, Gupta SC and Kim JH. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood 2012; 119: 651-665.

[6] Krammer PH, Arnold R and Lavrik IN. Life and death in peripheral T cells. Nat Rev Immunol 2007; 7: 532-542.

[7] Strasser A, Jost PJ and Nagata S. The many roles of FAS receptor signaling in the immune system. Immunity 2009; 30: 180-192.

[8] Maciejewski J, Selleri C, Anderson S and Young NS. Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. Blood 1995; 85: 3183-3190.

[9] Nagafuji K, Shibuya T, Harada M, Mizuno S, Takenaka K, Miyamoto T, Okamura T, Gondo H and Niho Y. Functional expression of Fas antigen (CD95) on hematopoietic progenitor cells. Blood 1995; 86: 883-889.

[10] Takenaka K, Nagafuji K, Harada M, Mizuno S, Miyamoto T, Makino S, Gondo H, Okamura T and Niho Y. In vitro expansion of hematopoietic progenitor cells induces functional expression of Fas antigen (CD95). Blood 1996; 88: 2871-2877.

[11] Sato T, Selleri C, Anderson S, Young NS and Maciejewski JP. Expression and modulation of cellular receptors for interferon-gamma, tumour necrosis factor, and Fas on human bone marrow CD34+ cells. Br J Haematol 1997; 97: 356-365.

[12] Askenasy N, Yolcu ES, Yaniv I and Shirwan H. Fas-ligand as a double-edged immunomodulator to induce transplantation tolerance. Blood 2005; 105: 1396-1404.

[13] Pearl-Yafe M, Yolcu ES, Stein J, Kaplan O, Shirwan H, Yaniv I and Askenasy N. Expression of Fas and Fas-ligand in donor hematopoietic stem and progenitor cells is dissociated from the sensitivity to apoptosis. Exp Hematol 2007; 35: 1601-1612.

[14] Pearl-Yafe M, Mizrahi K, Stein J, Yolcu ES, Kaplan O, Shirwan H, Yaniv I and Askenasy N. Tumor necrosis factor receptors support murine hematopoietic progenitor function in the early stages of engraftment. Stem Cells 2010; 28: 1270-1280.

[15] Mizrahi K, Stein J, Pearl-Yafe M, Kaplan O, Yaniv I and Askenasy N. Regulatory functions of TRAIL in hematopoietic progenitors: human umbilical cord blood and murine bone marrow transplantation. Leukemia 2010; 24: 1325-1334.

[16] Pearl-Yafe M, Stein J, Yolcu ES, Farkas DL, Shirwan H, Yaniv I and Askenasy N. Fas transduces dual apoptotic and trophic signals in hematopoietic progenitors. Stem Cells 2007; 25: 3194-3203.

[17] Mizrahi K, Stein J, Yaniv I, Kaplan O and Askenasy N. TNF-α has tropic rather than apoptotic activity in human hematopoietic progenitors: involvement of TNF receptor-1 and caspase-8. Stem Cells 2013; 31: 156-166.

[18] Mizrahi K, Yolcu ES, Askenasy EM, Stein J, Yaniv I and Askenasy N. Apoptotic signaling through Fas and TNF receptor-1 ameliorates GvHD in mobilized peripheral blood grafts. Bone Marrow Transplant 2013; [Epub ahead of print].

[19] Mizrahi K, Stein J, Kaplan O, Yaniv I, Zipori D and Askenasy N. Resistance of hematopoietic progenitors to Fas-mediated apoptosis is actively sustained by NFkB with a characteristic transcriptional signature. Stem Cell Dev 2013; [Epub ahead of print].

[20] Pearl-Yafe M, Yolcu ES, Stein J, Kaplan O, Yaniv I, Shirwan H and Askenasy N. Fas ligand enhances hematopoietic cell engraftment through abrogation of alloimmune responses and nonimmunogenic interactions. Stem Cells 2007; 25: 1448-1455.

[21] Askenasy N, Mizrahi K, Ash S, Askenasy EM, Yaniv I and Stein J. Depletion of naïve lymphocytes with fas ligand ex vivo prevents graft-versus-host disease without impairing T cell support of engraftment or graft-versus-tumor activity. Biol Blood Marrow Transplant 2013; 19: 185-195.

[22] Gothot A, van der Loo JCM and Srour EF. Cell cycle–related changes in repopulating capacity of human mobilized peripheral blood CD34 cells in non-obese diabetic/severe combined immunedeficient mice. Blood 1998; 92: 2641-2649.

[23] Guenechea G, Segovia JC, Albella B, Lamana M, Ramírez M, Regidor C, Fernández MN and Bueren JA. Delayed engraftment of nonobese diabetic severe combined immunodeficient mice transplanted with ex vivo–expanded human CD34 cord blood cells. Blood 1999; 93: 1097-1105.

[24] Glimm H, Oh IH and Eaves CJ. Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0). Blood 2000; 96: 4185-4193.

[25] Jetmore A, Plett PA, Tong X, Wolber FM, Breese R, Abonour R, Orschell-Traycoff CM and Srour EF. Homing efficiency, cell cycle kinetics, and survival of quiescent and cycling human CD34 cells transplanted into conditioned NOD/SCID recipients. Blood 2002; 99: 1585-1593.

[26] Bryder D, Ramsfjell V, Dybedal I, Theilgaard-Mönch K, Högerkorp CM, Adolfsson J, Borge OJ and Jacobsen SE. Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and tumor necrosis factor receptor activation. J Exp Med 2001; 194: 941-952.

[27] Young JC, Lin K, Travis M, Hansteen G, Abitorabi A, Sirenko O, Murray L and Hill B. Investigation into an engraftment defect induced by culturing primitive hematopoietic cells with cytokines. Cytotherapy 2001; 3: 307-320.

[28] Ratajczak J, Kucia M, Reca R, Zhang J, Machalinski B and Ratajczak MZ. Quiescent CD34+ early erythroid progenitors are resistant to several erythropoietic 'inhibitory' cytokines; role of FLIP. Br J Haematol 2003; 123: 160-169.

[29] Jacobsen FW, Rothe M, Rusten L, Goeddel DV, Smeland EB, Veiby OP, Slørdal L and Jacobsen SE. Role of the 75-kDa tumor necrosis factor receptor: inhibition of early hematopoiesis. Proc Natl Acad Sci U S A 1994; 91: 10695-10699.

[30] Selleri C, Sato T, Anderson S, Young NS and Maciejewski JP. Interferon-gamma and tumor necrosis factor-alpha suppress both early and late stages of hematopoiesis and induce programmed cell death. J Cell Physiol 1995; 165: 538-546.

[31] Zhang Y, Harada A, Bluethmann H, Wang JB, Nakao S, Mukaida N and Matsushima K. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro. Blood 1995; 86: 2930-2937.

[32] Dybedal I, Bryder D, Fossum A, Rusten LS and Jacobsen SE. Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood 2001; 98: 1782-1791.

[33] Dybedal I, Yang L, Bryder D, Aastrand-Grundstrom I, Leandersson K and Jacobsen SE. Human reconstituting hematopoietic stem cells up-regulate Fas expression upon active cell cycling but remain resistant to Fas-induced suppression. Blood 2003; 102: 118-126.

[34] Eaves C, Glimm H, Eisterer W, Audet J, Maguer-Satta V and Piret J. Characterization of human hematopoietic cells with short-lived in vivo re-

populating activity. Ann N Y Acad Sci 2001; 938: 63-70.
[35] Coulombel L. Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene 2004; 23: 7210-7222.
[36] MacEwan DJ. TNF receptor subtype signaling: differences and cellular consequences. Cell Signal 2002; 14: 477-492.
[37] Micheau O and Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003; 114: 181-190.
[38] Schütze S, Tchikov V and Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol 2008; 9: 655-662.
[39] Wilson NS, Dixit V and Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009; 10: 348-355.
[40] Pobezinskaya YL and Liu Z. The role of TRADD in death receptor signaling. Cell Cycle 2012; 11: 871-876.
[41] Bigda J, Beletsky I, Brakebusch C, Varfolomeev Y, Engelmann H, Holtmann H and Wallach D. Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. J Exp Med 1994; 180: 445-460.
[42] Medvedev AE, Sundan A and Espevik T. Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. Eur J Immunol 1994; 24: 2842-2849.
[43] Naudé PJ, den Boer JA, Luiten PG and Eisel UL. Tumor necrosis factor receptor cross-talk. FEBS J 2011; 278: 888-898.
[44] Wajant H, Henkler F and Scheurich P. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. Cell Signal 2001; 13: 389-400.
[45] Roy N, Deveraux QL, Takahashi R, Salvesen GS and Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 1997; 16: 6914-6925.
[46] Wang CY, Mayo MW, Korneluk RG, Goeddel DV and Baldwin AS Jr. NF-jB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998; 281: 1680-1683.
[47] Muller S, Rihs S, Dayer Schneider JM, Paredes BE, Seibold I, Brunner T and Mueller C. Soluble TNF-α but not transmembrane TNF-α sensitizes T cells for enhanced activation-induced cell death. Eur J Immunol 2009; 39: 3171-3180.
[48] Rauert H, Wicovsky A, Müller N, Siegmund D, Spindler V, Waschke J, Kneitz C, Wajant H. Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). J Biol Chem 2010; 285: 7394-7404.
[49] Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998; 17: 1675-1687.
[50] Peter ME and Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003; 10: 26-35.
[51] Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J 2009; 23: 1625-1637.
[52] Lavrik IN and Krammer PH. Regulation of CD95/Fas signaling at the DISC. Cell Death Differ 2012; 19: 36-41.
[53] Sheikh MS and Huang Y. Death receptor activation complexes: it takes two to activate TNF receptor 1. Cell Cycle 2003; 2: 550-552.
[54] Pellegrini M, Bath S, Marsden VS, Huang DC, Metcalf D, Harris AW and Strasser A. FADD and caspase-8 are required for cytokine-induced proliferation of hemopoietic progenitor cells. Blood 2005; 106: 1581-1589.
[55] Wang L, Du F and Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell 2008; 133: 693-703.
[56] Saheki K, Fujimori Y, Takemoto Y and Kakishita E. Increased expression of Fas (APO-1, CD95) on CD34+ haematopoietic progenitor cells after allogeneic bone marrow transplantation. Br J Haematol 2000; 109: 447-452.