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Maturation and apoptosis of primary human acute myeloblastic leukemia cells are determined by TNF-α exclusively through CD120A stimulation

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ABSTRACT

Background and Objective. **Tumor necrosis factor-α plays an important role in hematopoiesis. Its effects are mediated through two membrane-bound receptors: TNF-R I (p55; CD 120a) and TNF-R II (p75; CD 120b). The aim of our study was to investigate the relative roles of these receptors.**

Design and Methods. **We analyzed in 16 acute myeloid leukemia cases whether TNF-α could induce *in vitro* maturation and apoptosis. We then investigated which of the two receptors was provoking monocytic maturation and which was responsible for apoptosis by using the agonistic MoAb HTR-9, directed at CD120a, and the CD120b antagonistic MoAb UTR-1.**

Results. **Monocytic maturation (morphologic and immunologic) was induced in all cases studied, although to different rates, by TNF-α and by HTR-9 incubation. The addition of UTR-1 to TNF-α did not abolish maturation, nor did it affect apoptosis, which was present in primary AML cultures after 4 and 10 days.**

Interpretation and Conclusions. **We present here evidence that the sole stimulation of CD 120a, but not of CD120b, by TNF-α is responsible for both monocytic maturation and apoptosis of primary AML blasts.**

Key words: monocytic maturation, apoptosis, TNF-R I, AML

Tumor necrosis factor-α (TNFα) plays important and various roles in the pathophysiology of conditions such as fever, cachexia and septic shock.[1] On the other hand, TNFα is also relevant as a modulator of normal and leukemic hemopoiesis. In fact, TNFα is able both to stimulate cell growth directly[2] and to induce hematopoietic growth factor production by fibroblasts and endothelial cells[3,4] provoking cell proliferation. At the same time, TNFα blocks erythropoietin (EPO) and granulocyte-colony stimulating factor (G-CSF) induced cell growth.[2] These contrasting activities of TNFα are also maintained in leukemic myelopoiesis.[5,6] Moreover, TNFα exerts cytotoxic and maturation effects on acute myeloid leukemia (AML) blast cells[7] and for these properties TNFα has for some time been considered a potential candidate for application in AML therapy.[8]

The effects of TNFα are mediated through two different receptors, TNF-R type I (CD120a) and TNF-R type II (CD120b), which have molecular masses of approximately 55 and 75kDa, respectively.[9] Both receptors induce gene expression via transcription factor NFkB,[10] most probably through different secondary messengers, as they do not share homology in their cytoplasmic domains, and only CD120a possesses the death homology domain. Although both CD120a and CD120b are found on AML blast cell surfaces, they may be expressed at different densities and either p75 or p55 TNF-R may prevail, irrespective of the FAB subtype of AML.[11] Notwithstanding the fact that CD120b is constantly present on AML cell surfaces, CD120a appears to mediate the majority of positive and negative effects of TNFα: synergistic effects with interleukin (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) proliferation induction via receptor up-regulation, counteraction of G-CSF stimulation via specific receptor down-modulation. The autocrine production of growth factors by AML blasts is mediated by both types of receptors.[11]

It has been observed that TNF-R p55 mediates differentiation and DNA fragmentation in the HL-60 leukemic cell line.[12] We investigated which receptor was involved in TNFα-induced *in vitro* monocytic maturation of 16 cases of primary human AML blasts of various FAB subtypes. To perform such an analysis, we employed the monoclonal antibodies HTR-9, directed at and agonistic to CD120a, and UTR-1, a specific blocker of CD120b.[13] Furthermore, we investigated whether, in AML blasts, TNFα-induced monocytic differentiation and apoptosis were associated events.

Design and Methods

Preparation of cells

Bone marrow and peripheral blood cells were obtained after informed consent from 16 AML patients classified according to the French American British (FAB) group criteria as M1 (2 cases: #2 and 8), M2 (6 cases: #1, 3, 5, 7, 15, 16), M4 (6 cases: #4, 6, 9, 11, 12, 14) and M5a (2 cases: #10 and

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13). AML cells were isolated by Ficoll-Isopaque density gradient centrifugation and subsequently depleted of T lymphocytes by E-rosetting. Cells were than cryopreserved and depleted of adherent cells after thawing.

Tumor necrosis factor and neutralizing antibodies

TNFα was a gift from Boehringer Institute (Vienna, Austria) and was used at a concentration of 1000 U/mL. Anti-TNF-R-(p55) MoAbs HTR-9 (IgG) and anti-TNF-R-(p75) MoAb UTR-1 (IgG) were kindly provided by Dr. Brockhaus, Hoffmann La Roche (Basel, Swizerland)[13] and were both used at a concentration of 10 µg/mL, previously demonstrated to be the optimal stimulating and blocking concentration.[11]

Cell cultures

AML cells were cultured serum free[14] (0.2×10^6/mL) for four and ten days in plastic tubes to prevent cell adherence, in the absence and in the presence of TNFα 1000 U/mL, HTR-9 10 µg/mL, and TNF 1000 U/mL plus UTR-1 10 µg/mL. The serum free medium employed for all experiments was completely deprived of serum, substituted by transferrin, sodium selenite, insulin, cholesterol, β-mercaptoethanol, and linoleic acid.[14] Human leukemic cell line HL60 was maintained in continuous culture in RPMI 1640 medium and 10% fetal calf serum (FCS); stimulation with TNFα and antibodies was performed as described for primary cells.

Morphology

Prior to and after four or ten days of culture, cytospin preparations (Shandon, Cheshire, UK) were stained with May-Grünwald Giemsa (MGG) and the cells were examined by light microscopy using a 100x magnification lens and immersion oil. At least 100 cells per slide were scored twice and the percentage of monoblasts, promonocytes, macrophages, histiocytes and monocytes together with cells of myeloid lineage determined. α-naphtyl acetate esterase (α-NAE) was the cytochemical staining specific for monocytic cells performed prior and after culture. For each slide a score was made on evaluation of 100 cells. At the end of the cultures, nucleated cell number and viability were determined by counting with Türck solution and the Trypan blue dye exclusion test.

Surface antigen analysis

The presence of surface antigens on AML cells cultured with or without TNFα and HTR-9 or UTR-1 monoclonal antibodies was evaluated by indirect immunofluorescence. Flow cytometric analysis was performed using a FACSscan (Beckton Dickinson, San José, CA, USA). The monoclonal antibodies applied were CD11b which binds C3b and is present on the surface of monocytes and granulocytes, and CD16 which reacts with the Fcg receptor III, present on macrophage, granulocytes, and NK cells.

Tritiated thymidine uptake

HL60 cells and primary blast cells were cultured serum free and plated on to 96 microwells plates at the concentration of 1×10^5/mL in the presence of TNFα, HTR-9, and TNF plus UTR-1 at the concentrations above indicated. Control cultures were carried out in the absence of any factor or antibody. After 4 and 10 days of culture in these conditions, cells were exposed for 18 hours to tritiated thymidine ([^{3}H]-TdR, 5 µCi/well). Pulsed cells were harvested by an automated cell harvester (Skatron, Norway) and incorporation of [^{3}H]-TdR evaluated by β scintillation counting.

Cell kinetics and apoptosis

Cells were cultured in 75 cm^2 area flasks (Greiner, Germany) with TNFα or monoclonal antibodies as above mentioned. After incubation, cells were washed thoroughly, resuspended in PBS/ethanol 1:3 vol/vol and kept overnight at 4°C. After fixation, cells were incubated in propidium iodide (PI) 50 mg/mL plus Nonidet 0.01%, RNAse 62 µg/mL. DNA content and cell cycle parameters were measured in a FACScan flow cytometer (Becton Dickinson, San Josè, CA, USA).[15] Analysis of the data was accomplished by the use of the CellFit program, applying the MANL statistic program which quantifies the consistence of the apoptotic peak "pre-G_1", present in the region of channel 100. The presence of cell shrinkage, pyknosis of nuclei and formation of apoptotic bodies was always scored in parallel in MGG stained cytospin preparations to evaluate the percentage of morphologically apoptotic cells.

Annexin V-FITC binding assay

To quantify apoptosis in HL60 cells following incubation with TNFα or monoclonal antibodies (as above mentioned), cells were incubated with 10 µL of Annexin V-FITC and PI (Annexin V Fluos staining kit; Boehringer-Mannheim).Cells positively stained with Annexin V and PI negative were considered apoptotic as analyzed by cytofluorimetry (see above, *Cell kinetics and apoptosis*).

Results

Morphology

In the absence of any stimulation, maturation of AML blasts was scarce. Monocyte-histiocyte maturation is defined as the progressive acquisition by AML blasts of abundant, muddy blue or greyish cytoplasm, containing very fine azurophilic granules and a round, oval, notched or convoluted peripheral nucleus, with a delicate lace like distribution of chromatin, and loss of nucleoli. Histiocytes are often inglobating cell fragments in the cytoplasm. Scarce spontaneous maturation was apparent in 5/16 cases studied (Table 1). The stimulation with TNFα (1000 U/mL) increased the percentage of mature monocytes and/or histiocytes in all cases, to different degrees (i.e. from 44%

Table 1. Morphologic maturation of AML blast cells cultured for 10 days in the presence of TNF-α, HTR-9, UTR-1+TNFα.

Case # & FAB		*None*	*TNFα*	*HTR-9*	*UTR-1+TNFα*
1 M2	Blasts	51	49	12	29
	Myelo*	30	7	4	18
	Mono°	19	44	84	59
2 M1	Blasts	97	27	13	25
	Myelo	3	3	2	6
	Mono	0	70	85	69
3 M2	Blasts	78	40	47	49
	Myelo	20	60	51	50
	Mono	2	0	2	1
4 M4	Blasts	62	43	34	42
	Myelo	8	0	0	0
	Mono	30	57	66	58
5 M2	Blasts	100	10	23	11
	Myelo	0	0	0	5
	Mono	0	90	87	84
6 M4	Blasts	73	19	17	14
	Myelo	2	3	4	10
	Mono	25	78	79	76
7 M2	Blasts	100	65	48	44
	Myelo	0	0	0	0
	Mono	0	45	52	56
8 M1	Blasts	97	46	41	38
	Myelo	0	0	0	0
	Mono	3	54	59	68
9 M4	Blasts	100	48	44	46
	Myelo	0	4	12	12
	Mono	0	48	44	42
10 M5a	Blasts	100	30	42	28
	Myelo	0	4	2	0
	Mono	0	66	56	72
11 M4	Blasts	46	1	2	2
	Myelo	54	5	6	2
	Mono	0	95	92	96
12 M4	Blasts	100	48	46	40
	Myelo	0	4	8	6
	Mono	0	48	46	54
13 M5a	Blasts	100	58	44	
	Myelo	0	2	6	n.v.
	Mono	0	40	50	
14 M4	Blasts	98	54	58	56
	Myelo	0	0	0	0
	Mono	2	46	42	44
15 M2	Blasts	75	32	16	
	Myelo	20	40	36	n.v.
	Mono	5	28	48	
16 M2	Blasts	60	40	38	
	Myelo	20	16	12	n.v.
	Mono	20	44	50	

Myelo: cells showing morphologic maturation (metamyelocytes, band forms, granulocytes); Mono°: cells showing morphologic mono-macrophage maturation (monocytes, histiocytes, macrophages).*

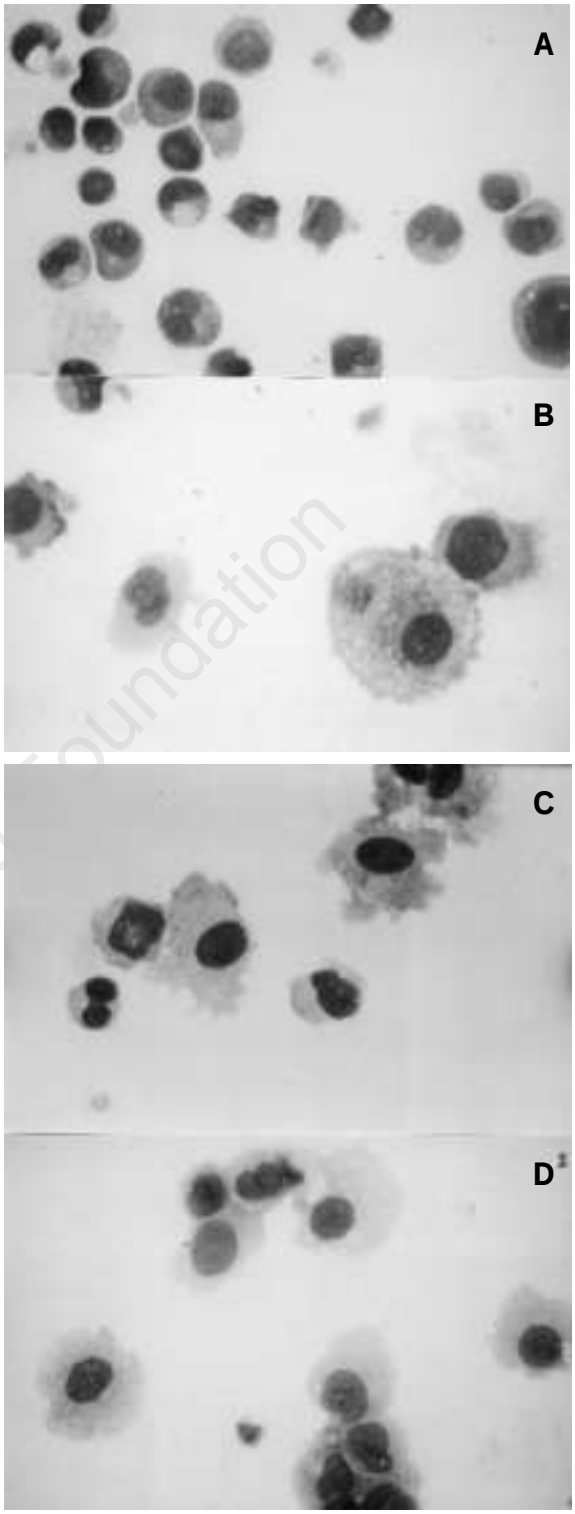

Figure 1. Monocytic maturation after p55 TNF-R (CD120a) stimulation in primary AML blasts (May-Grünwald-Giemsa staining). Cytospins were prepared after 10 days of cultures (case #5): (A) in the absence of stimulation, or (B) in the presence of TNFα 1000 U/mL; (C) monoclonal antibody HTR-9 10 µg/mL; (D) monoclonal antibody UTR-1 10 µg/mL plus TNFα 1000 U/mL. Magnification x100.

Table 2. Percentages of cells positive for α-naphthylacetate esterase (αNAE) after 10 days of culture in the presence of TNF-α, HTR-9, UTR-1+TNF-α.

Case	FAB	None	TNFα	HTR-9	UTR-1+TNFα
1	M2	5	23	21	21
2	M1	0	0	0	0
3	M2	0	5	6	5
4	M4	23	56	33	51
5	M2	0	27	27	50
6	M4	20	72	75	69
7	M2	7	35	31	25
8	M1	1	6	10	7

Table 3. Increase in the expression of surface maturation antigens on AML blasts after 10 days of culture with TNF-α, HTR-9, UTR-1 + TNF-α.

Case	FAB	TNFα		HTR-9		UTR-1+TNFα	
		CD11b	CD16	CD11b	CD16	CD11b	CD16
1	M2	+	–	++	++	+	+
2	M1	+++	++	+++	–	+++	++
3	M2	+	–	+	++	+/–	+/–
4	M4	+/–	nd	++	nd	++	nd
5	M2	+++	nd	++	nd	–	nd
6	M4	+	nd	++	nd	+	nd
7	M2	–	–	–	–	–	–
8	M1	++	nd	+++	nd	++	nd
9	M4	++	+	++	+/–	++	+/–
10	M5a	+/–	+/–	+	+/–	+	+/–
11	M4	+	+/–	+	+/–	+	+/–
12	M4	++	+	++	+/–	+/–	–
13	M5a	+	–	+	–	–	–
14	M4	+++	–	+++	–	++	–
15	M2	+++	+	+++	+	+	+
16	M2	++	+/–	++	+/–	+	–

For treated cells the increase in antigen expression (–, +/–, +, ++, +++) was derived from the shift of specific fluorescence in comparison with the peak channel number of non-stimulated cells. This was according to the following change of specific fluorescence intensity: – 0-4 channels, +/– 5-9 channels, + 10-19 channels, ++ 20-29 channels, +++ 30 or more channels of shift as compared to non-stimulated cells. Cells from case #7 had intense expression of both antigens before stimulation. nd = not done.

to 95% in cases #1 and #11, respectively). Monocytic maturation was detectable in each AML subtype, irrespective of the FAB classification. Stimulation with MoAb HTR-9, activating p55 TNF-R, was able to induce an increase in percentage of monocytes-histiocytes in 15/16 cases studied (Figure 1). The percentage of mature cells ranged from 44% (#9) to 92% (#11) (Table 1). Simultaneous addition of TNFα and MoAb UTR-1 blocking p75 TNF-R did not inhibit monocytic maturation, which was present in 42% (case #9) to 96% (case #11) of the cells. In most cases, cells recovered from unstimulated cultures were negative for α-NAE, which was evaluated in 8/16 cases (Table 2). In only two cases did blasts show positivity before stimulation (#4 and #6, FAB subtype M4). The incubation in the presence of TNFα promoted the appearance of α-NAE positive cells in all negative cases and increased the percentage of positive cells in cases #4 and #6. The significant increase in the score of α-NAE staining was not observed in case #2, despite the morphologic (MGG) appearance of mature monocytic cells in treated cultures. The positivity for the specific esterase was maintained in the cultures exposed to HTR-9, showing the same rate of functional maturation as TNFα treated cultures. The addition of UTR-1 to TNF in cultures did not inhibit the appearance of α-NAE positive cells.

Immunology marker analysis

Surface immunophenotyping of stimulated AML blasts was performed using monoclonal antibodies CD11b and CD16 (Table 3). After 10 days of culture, unstimulated AML blast cells showed faint positivity for CD11b in 6/16 cases (#6, 7, 8, 11, 15, 16). The surface antigen recognized by CD16 was already present before stimulation in 1/4 cases analyzed (#7). After 10 days of culture in the presence of TNFα, CD 11b and CD 16 positive cells increased in 15/16 and 7/12 cases, respectively (Table 3; Figure 2). Stimulation with HTR-9 induced an increase in CD11b and CD16 positivity in 15/16 cases and 8/12 cases, respectively. In cases #1, 4, 6, 8, 10 the shift in the fluorescence channel, indicating intense positivity was more marked after HTR-9 than after TNFα stimulation. The addition of UTR-1 to TNFα-induced cultures partially inhibited the appearance of CD11b positive cells only in case #5, whereas in all other cases cells the same pattern of positivity was shown as in TNFα induced cultures.

Tritiated thymidine uptake

Consistent with that already demonstrated, in 13/16 AML cases evaluated in our study the addition of TNFα or HTR-9 to cultures significantly reduced the rate of thymidine uptake, both at 4 and 10 days. The combination of TNFα and UTR-1 did not prevent the decrease of cell proliferation (data not shown). In HL 60 cultures, the effect of TNFα in inhibiting cell proliferation was mimicked by HTR-9, but not modulated significantly by the contemporary addition of UTR-1 (Table 4).

Cell kinetics and apoptosis

The percentage of apoptotic events and apoptotic bodies was evaluated in 8/16 cases of AML treated with TNFα and monoclonal antibodies (Figure 3a and 3b). In 7/8 cases, the number of apoptotic cells observed at flow cytometric analysis was significantly increased by TNFα and HTR-9 treatment with respect to the number present in control cultures. The addition of UTR-1 to TNFα in culture medium did not modify the rate of cell death, except for case

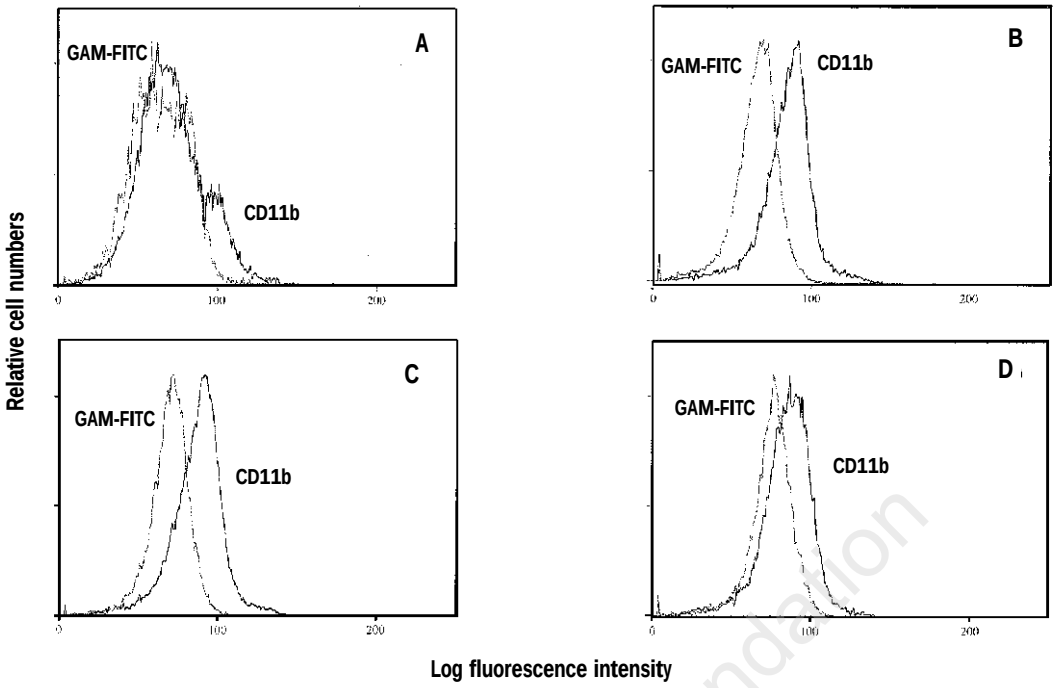

Figure 2. CD11b antigen expression of AML cells after 10 days of culture. The distribution of AML blast cells from case #2 (FAB = M1) is presented as a function of fluorescence intensity after CD11b labeling. Control cells were labeled with GAM-FITC.
A: blasts incubated in the absence of any cytokine or antibody; B: blasts cultured with TNFα 1000 U/mL; C: blasts recovered from culture in the presence of HTR-9 10 µg/mL; D: cells cultured with TNFα plus UTR-1 10 µg/mL.

#10 (Figure 3a). The morphologic evaluation of apoptotic bodies yielded results consistent with those from DNA content analysis (Figure 3b) but it was performed to confirm the presence of programmed cell death features with a more direct parameter, as recommended by Darzynkiewicz.[15]

Table 4. Effects of TNFα and MoAb HTR-9, UTR-1 on HL60 cell proliferation, maturation and apoptosis (on day 4).

	^{3}HTdR uptake (dpm)	*% monocytes histiocytes*	*% Annexin V+ cells*
Control	21,779±232	0	0.79
TNFα	9,750±70	56	34
HTR-9	6,760±120	49	28
TNFα+UTR1	10,130±270	51	26

HL60 cells (1×10^5 cells/mL) were cultured in RPMI 1640 medium, 10% FCS in the absence (control) or in the presence of TNFα and/or MoAbs, at the concentration reported in the text. Tritiated thymidine uptake was measured at day 4; morphologic evaluation of monocytic cells (MGG) and cytometric analysis of Annexin V-FITC positive cells (see Design and Methods for details), was carried out at the same time.

HL60 cells maturation and apoptosis.

Table 4 presents the mean percentage of Annexin V positive-apoptotic cells after incubation with TNFα, HTR-9 or TNFα plus UTR-1. In parallel, the morphology of HL 60 cells was assessed to detect signs of maturation. Both the cytokine and HTR-9 were effective in inducing monocytic maturation as well as specific apoptotic death. UTR-1 monoclonal antibody could not revert such activity. The concentrations used were the same as the ones employed for primary AML cells.

Discussion

We demonstrated that blasts from 16 primary AML cases can be induced to mature towards monocyte-histiocytes by stimulation with the high affinity antibody HTR-9, specific to TNF-R p55 (CD120a) and perfectly mimicking the TNFα maturation effect.[12,16] The incubation of AML blasts with TNFα in combination with the blocking antibody UTR-1 (specific to TNF-R p75; CD120b) did not prevent the appearance of mature monocytoid cells. The maturation effect of TNFα as measured by immunophenotype, specific esterase positivity and morphology was in fact completely reproduced by TNFα-R p55 activa-

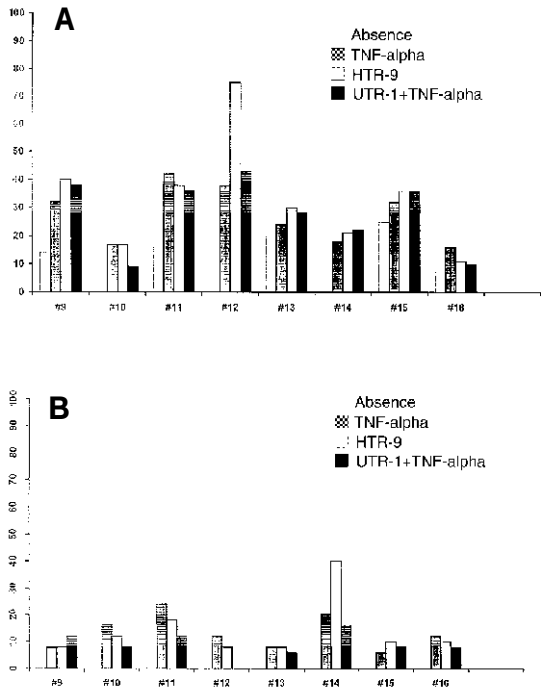

Figure 3. Apoptosis after p55 TNF-R (CD120a) stimulation in primary AML blasts.
A. Percentage of events at FACSscan analysis in pre-G1 peak after propidium iodide incorporation. Cell cultures were performed for 4 days in the absence or in the presence of TNFα, HTR-9 and UTR-1+TNFα.
B. Percentage of apoptotic bodies at morphologic evaluation of AML cell cytospin preparations (May-Grünwald-Giemsa staining). At least 200 cells were scored. Cell cultures were performed for 4 days in the absence or in the presence of TNFα, HTR-9 and UTR-1+TNFα.

tion, and in some cases it was even more marked than after direct stimulation of AML blasts with the cytokine. Anyway, as previously found,[17] the phenotypic maturation inducible in primary AML blast cells is indeed incomplete. The activation of TNF-R p55 (CD120a) in such cells can thus only in part bypass the maturation blockade typical of AML. It is worth noting that monocytic maturation was achievable through CD120a triggering, irrespective of the FAB subtype of the AML cases, similarly to that observed for the induction of granulocytic maturation.[17,18] Although monocytic maturation has been demonstrated to occur spontaneously in *in vitro* culture of AMLs,[19] in this study it was of massive proportion and indeed provoked by specific TNFα stimulation, apparent after 4 and still after 10 days of culture in the presence of monoclonal antibodies and/or TNFα. We also demonstrated that apoptosis is inducible in almost the totality of AML cases, irrespective of FAB subtype, by stimulation of CD120a by specific antibody or by TNFα. Programmed cell death, but not necrosis, was present after 4 days of culture in the presence of the cytokine or of the antibody, indicating an active cellular response. We performed our analyses at two *long term* endpoints, i.e. 4 and 10 days, in order to evaluate the effective induction of terminal monocytic maturation and avoid focusing on the immediate cellular pathways elicited by TNFα. On the other hand, we observed apoptosis after maturation signs had appeared in AML cells in culture. This observation may indicate that programmed cell death followed terminal maturation as a physiologic event.

Both TNF-R p55 and p75 are able to activate the transcription factor NFkB,[20] which is responsible for important gene induction of proteins involved in cell proliferation and maturation. Nevertheless, we confirmed in our system that the functional messages conveyed by the two receptors are distinct and specific. It is known that their cytoplasmic domains share no homology. Only TNF-R p55 (CD120a) possesses a domain of 80 amino acids, designated the death domain, which mediates apoptosis.[21] As CD120a has no enzymatic activity, it associates to TRADD (TNF associated death domain) and through its involvement determines transcriptional activation of NFkB regulated promoters.[21] At the same time CD120a determines activation of neutral sphingomyelinase which catalyzes ceramide production, leading to apoptosis via the JNK/SAPK cascade.[22] Thus, since both these pathways appear to be induced by TRADD but divergently,[23] it seems that TNF-R p55/CD120a can induce maturation and apoptosis in AML by two contemporary but independent phenomena. On the other hand, NFkB activation through TRAF 1 and TRAF 2 (TNF associated factors 1 and 2), complexed with the cytoplasmic domain of TNF-R p75/CD120b[24] did not provoke monocytic maturation in any of the AML cases studied. Recently, it was demonstrated that serum concentrations of the soluble form of TNF-R p55, but not p75, have an important prognostic impact in *de novo* AMLs.[25] Although the correlation may simply reflect the leukemic burden, the fact that TNFα plays its regulatory role in leukemic myelopoiesis predominantly via p55 receptor activation emphasizes the biological significance of the finding and its potential therapeutic value.

The observation that CD120a is specifically responsible for both TNFα-induced maturation and apoptosis of primary AML cells may help to dissect the still enigmatic impairment of maturation signal transduction typical of AML; at the same time it may contribute to evaluation of the general role and the signaling pathways of TNFα during myeloid maturation.

Contributions and Acknowledgments

VS: design of experiments and writing of the manuscript. AG: design and performance of experiments. BS: design and performance of experiments. PRF: peer reviewer, writing manuscript. PRF is the senior author, due to his experience

and his invaluable help in discussions developing the work. VS is the first author, for the essential contribution in ideas and elaboration of the scientific hypothesis, as well as for designing the experiments. AG is second author, due to her important technical and scientific contribution to the realization of the study. BS is third author, she contributed to discussion and to realisation of the work. We thank Fabio Corti for preparing the figures.

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Disclosures

Conflict of interest: none.

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