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Interleukin-1 beta (IL-1β) induces tumor necrosis factor alpha (TNF-α) expression on mouse myeloid multipotent cell line 32D cl3 and inhibits their proliferation

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Abstract

Interleukin-1 alpha (IL-1α) and beta (IL-1β) are well known factors that stimulate hematopoiesis, nevertheless there are reports that show that they can also inhibit this activity. While both IL-1α and IL-1β induce the expression of hematopoietic cytokines, such as growth factors and their receptors on myeloid cells, helping thus to regulate hematopoiesis, it is not known if their inhibitory activity is also mediated through the induction of other specific cytokines. In this work we show that recombinant human IL-1β (rhIL-1β) inhibits the proliferation of a mouse IL-3-dependent myeloid multipotent cell line (32D cl3), without inducing its differentiation. We show that rhIL-1β induces in 32D cl3 cells the expression of the tumor necrosis factor alpha (TNF-α) gene, a well known growth inhibitor, and that the rhIL-1β growth inhibition property on 32D cl3 cells is partially due to this secreted TNF-α, hinting thus that the inhibition of hematopoiesis by IL-1 is mediated through other induced cytokines.

Keywords: Interleukin-1; Hematopoiesis inhibition; TNF-α

1. Introduction

Interleukin-1 alpha (IL-1α) and beta (IL-1β) have been found to be myeloprotectors against either radiation or cytotoxicity [1] and to have a synergistic effect with growth factors to promote the proliferation of myeloid precursors [2]. IL-1β has also been found to promote the proliferation of several types of leukemia and to act as a promoter of malignancy [3]. It has been well documented that IL-1α and IL-1β favour hematopoiesis by inducing the expression of the genes for several growth factors and their receptors, like the granulocyte colony-stimulating factor (G-CSF), the granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-3 and the stem cell factor (SCF) [1].

Even though IL-1 has a well established proliferative role in hematopoiesis, it has been shown that under certain conditions it can also be a potent inhibitor. In fact IL-1 inhibits the growth of M1 a mouse leukemic cell line, of the human myeloid cell line K562 [4–6], and of macrophages immortalized with retroviruses [7]. IL-1 also antagonizes erythropoietin (EPO) blocking the formation of erythroid colonies (E-CFU) [8], and pre-B and pre-T colonies induced by either SCF plus IL-6, SCF plus IL-11 or SCF plus G-CSF of the primitive bone marrow hematopoietic cells Ly-6A/E+ obtained from mice treated with 5-fluorouracil [9,10]. It has also been shown that IL-1 reduces the generation of the

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colony forming unit for erythrocyte–granulocyte–macrophage–megakaryocyte (CFU-GEMM) [11].

It has been shown that IL-1 induces the expression of well known inhibitory factors like the tumor necrosis factor (TNF), the transforming growth factor (TGF), the inflammatory macrophage protein (MIP) and interferon (IFN) [1]. TNF alpha (TNF-α) is a potent hematopoietic inhibitor produced by several cell types, like monocyte-macrophage, CD34+ cells and even by leukemic cells [12–14]. It has been reported that the frequent inoculation of high doses of IL-1 induces the production of TNF-α detected in serum that inhibits the formation of the colonies from CFU-granulocyte–macrophage (CFU-GM) [15]. TNF and IL-1 are responsible for the inhibition of macrophage proliferation [7], and TNF but not IL-1 is also responsible for red cell anemia in rats [16]. In consequence, there is evidence that points to TNF-α to be the hematopoietic growth inhibitor whenever IL-1 is used. In this study we use mouse IL-3-dependent myeloid multipotent cell line (32D cl3) in order to evaluate if these cells are also sensitive to be inhibited by rhIL-1β and to analyse the possible role of TNF-α in this event.

2. Results

2.1. rhIL-1β inhibits the proliferation of 32D cl3 cells

In order to determine the inhibitory capacity of rhIL-1β on the proliferation of 32D cl3 cells, they were cultured for 48 h in the presence of rmIL-3 (0.5 ng/ml) with 0, 0.01, 1, 5 and 50 ng/ml of rhIL-1β. Our results showed a significant inhibition of growth (30%) when 1 ng/ml of this factor was used, arriving to more than 40% at the higher doses (Fig. 1A). The incorporation of tritiated thymidine was also strongly inhibited in 32D cl3 cells by rhIL-1β (5 ng/ml) as compared to rmIFN-γ (25 ng/ml) a well known growth inhibitor (Fig. 1B). Even though both molecules inhibited 32D cl3 proliferation only rmIFN-γ was able to differentiate these cells towards the monocytic lineage, in fact rmIFN-γ induced monocyte-macrophages in more than 80% of the cells measured by alpha-naphthyl acetate esterase (Table 1). Neither rhIL-1β nor rmIFN-γ produced cell death under our culture condition as measured by the exclusion of trypan blue by more than 90% of the treated cells, similar to that obtained in controls with only rmIL-3. In the absence of IL-3 almost all the cells died by apoptosis as measured by the tunnel reaction (Fig. 2).

2.2. rhIL-1β induces the expression of the mRNA for TNF-α and its bioactive secretion by 32D cl3 cells

It is known that IL-1 is capable of inducing the expression of TNF on mature myeloid cells. In order to evaluate if IL-1 can also induce this factor in primitive multipotent cells, 32D cl3 cells were cultured in the presence of rhIL-1β and the expression of the mRNA for TNF-α measured. In fact by RT-PCR we detected the presence of the mRNA for TNF-α only in the cells cultured in the presence of rhIL-1β (Fig. 3).

In order to determine if TNF is induced to be secreted by IL-1β in 32D cl3 cells, they were cultured in the presence of 5 ng/ml of rhIL-1β for 48 h, and the conditioned media (CM) collected to measure the survival of L929 cells sensitive to TNF. When the L929 cells were cultured in the presence of the 32D cl3 CM/rhIL-1β a survival reduction of more than 60% was obtained (Fig. 4A). Cultures with ActD alone, ActD plus CM from non-treated 32D cl3 cells (rhIL-1β free) and ActD plus rhIL-1β were used as negative control and ActD plus rmTNF-α as a positive one. The presence of TNF-α in the CM was corroborated by an ELISA assay, in fact from 0.7 ng/ml of TNF-α in the CM without inducer to 21 ng/ml in the CM when rhIL-1β was used (Fig. 4B).

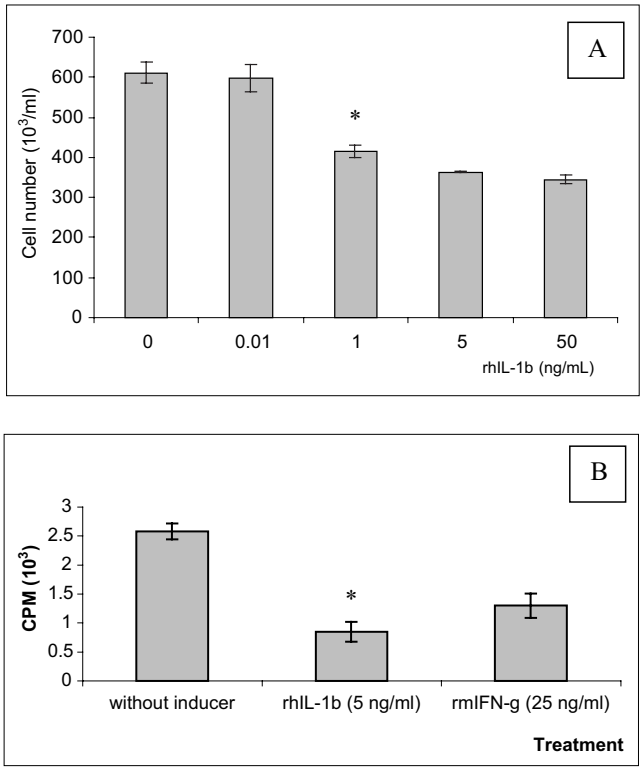


Fig. 1. rhIL-1β inhibits 32D cl3 cell proliferation. (A) 32D cl3 cells were cultured in the presence of rmIL-3 (0.5 ng/ml) and different doses of rhIL-1β. After 48 h of incubation the number of cells was evaluated with Neubauer chamber under the light microscope. (B) 32D cl3 cells in the presence of rmIL-3 (0.5 ng/ml) without (without inducer) or with rhIL-1β (5 ng/ml). After 48 h in culture the incorporation of tritiated thymidine was evaluated. rmIFN-γ (25 ng/ml) was used as a positive control for cell proliferation. Data represent the mean ± SD of three independent experiments and the difference from the without rhIL-1β (*) was significant at $P < 0.05$ by Student's *t*-test.

Table 1
Cell morphology and specific cytochemical stain of 32D cl3 cells treated with rhIL-1β

Treatment	32D cl3 cell differentiation			
	Giemsa (morphology %)		Cytochemical (%)	
	Monocyte-macrophage	Granulocyte-neutrophil	Monocytic lineage	Granulocytic lineage
without/rhIL-1β	5 ± 2	0 ± 0	6 ± 2	25 ± 5
rhIL-1β	4 ± 1	0 ± 0	10 ± 2	25 ± 3
rmTNF-α	5 ± 1	0 ± 0	7 ± 3	25 ± 4
rmIFN-γ	44 ± 11	0 ± 0	80 ± 7	20 ± 7

32D cl3 cells were cultured for 48 h in the presence of rmIL-3 (0.5 ng/ml) without (without rhIL-1β) or with rhIL-1β (5 ng/ml) or with rmTNF-α (10 ng/ml), then transferred on to cover-slips and subsequently stained with either Giemsa or specific cytochemical stains for the monocyte-macrophage lineage (alpha-naphthyl acetate esterase) or the granulocyte-neutrophil one (chloroacetate esterase). The morphology was assessed using an optical microscope. Cells in cultures with rmIFN-γ (25 ng/ml) were used as a positive control. Data represent the mean ± SD of three independent experiments. At least 200 cells were evaluated by assay.

2.3. Anti-TNF-α antibody reduces the inhibition of 32D cl3 cells induced by rhIL-1β

In order to evaluate if the TNF-α secreted by the 32D cl3 cells participates in the inhibition of cell growth mediated by IL-1, we cultured the cells in the presence of rhIL-1β with anti-mouse TNF-α antibody. Our results showed that indeed TNF-α contributes to this inhibition because it was partially reversed when anti-TNF was used (Fig. 5A). This was confirmed by ^{3}H-thymidine incorporation (Fig. 5B). When using 10 ng/ml of rmTNF-α in three independent experiments we did not detect apoptosis by tunnel reaction (mean ± SD; 3 ± 2 positive stained cells without and 5 ± 2 with TNF-α). On the other hand we also did not detect any myeloid morphology differentiation in 32D cl3 cells treated with TNF-α (Table 1).

3. Discussion

In this work we have provided evidence that rhIL-1β inhibits the proliferation, without inducing differentiation and apoptosis, of a mouse IL-3-dependent myeloid multipotent cell line (32D cl3). The inhibitory activity of IL-1 has previously been reported in mouse primitive hematopoietic lineage-negative, Ly-6A/E+, c-kit+ cells [11] and macrophages [6] pointing thus to its inhibitory effect along the myeloid cell lineage. It is interesting to mention that the fact that IL-1 inhibited proliferation of 32D cl3 without inducing differentiation into macrophages as IFN-γ does, as shown in this work, points to an independent mechanism of cell growth inhibition and differentiation mediated by different cytokines.

We also show that the mRNA for TNF-α is induced in 32D cl3 cells by rhIL-1β and that the molecule is secreted in its active form. When the induced cells were cultured in the presence of anti-TNF-α the inhibitory activity was partially reversed, thus hinting that TNF-α is an inhibitory factor in 32D cl3 cells. This observation is confirmed by the fact that, like rhIL-1β, TNF-α by itself also inhibits the proliferation of 32D cl3 cells and that neither of these factors has apoptotic activity nor induction of myeloid differentiation. Our data suggest that rhIL-1β in addition to TNF-α could be inducing other cytokines with inhibitory activity, therefore it would be interesting to evaluate if other inhibitory factors induced by IL-1, like MIP-1α or nitric oxide [1,17] are induced in 32D cl3 cells and their possible effect in the regulation of its proliferation.

When IL-1 is administrated in vivo into primates the level of TNF-α in these animals is significantly elevated

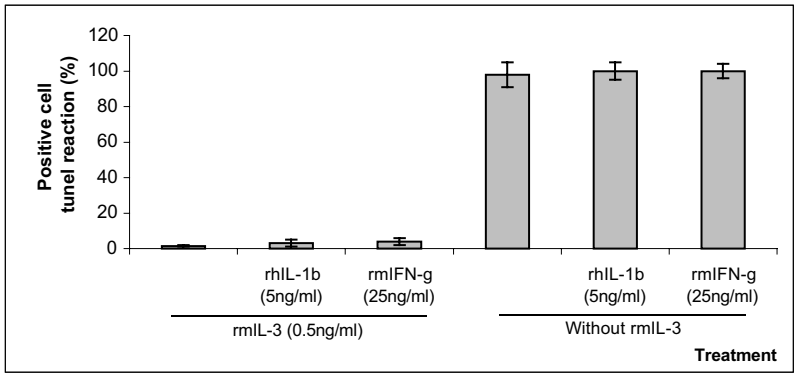


Fig. 2. rhIL-1β does not induce apoptosis in 32D cl3 cells. The 32D cl3 cells were cultured in the presence of rmIL-3 (0.5 ng/ml) with or without rhIL-1β. rmIFN-γ (25 ng/ml) was used as a positive control. Cells were evaluated for a positive tunnel reaction after 48 h in culture. Data represent the mean ± SD of three independent experiments.

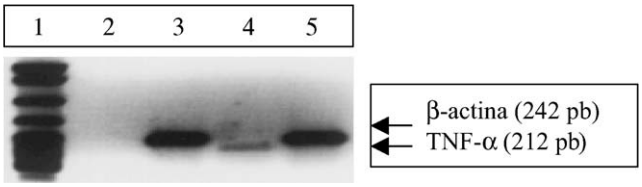
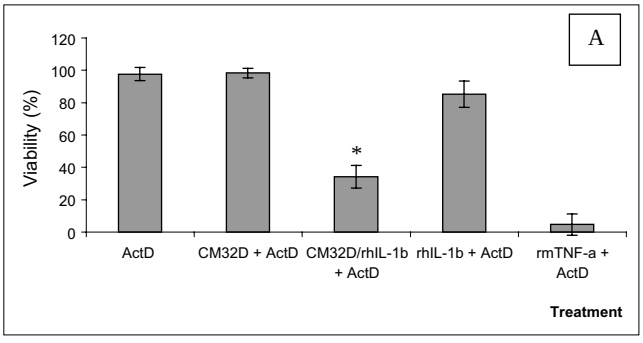


Fig. 3. rhIL-1β induces TNF-α RNA expression in 32D cl3 cells. The 32D cl3 cells were cultured for 24 h in presence of rmIL-3 (0.5 ng/ml) with or without rhIL-1β (5 ng/ml). Total RNA was extracted and RT-PCR was performed. The specificity for the cDNA was verified by the presence of products of the expected size in an agarose gel. Lane 1, molecular weight markers; lanes 2 and 4, TNF-α RNA from 32D cl3 cell without or with rhIL-1β, respectively ; lanes 3 and 5, β-actin RNA without or with rhIL-1β, respectively. The figure is a representative of three different assays.

as well as the inhibition of the proliferation of their GM-CFU [15]. Our data thus suggest that the primitive hematopoietic cells in those primates could be responsible for the increase in serum TNF in response to IL-1 and for the inhibition of hematopoiesis detected in those experiments.

Even though TNF is a well known apoptotic inducer [18] it did not produce apoptotic 32D cl3 cells under our culture conditions, pointing that this factor is capable of inhibiting proliferation of primitive hematopoietic cells without cytotoxicity. In fact TNF has been shown to synergize with IL-1 in the survival and suppression of apoptosis in Lin-Sca-1+ cells [19], and on the other hand primitive CD34+ cells could be induced to a normal proliferation even in the presence of TNF [14].

IL-1 has recently been used in clinical protocols to expand normal hematopoietic cells in conjunction with other growth factors [11]; nevertheless, taking into consideration that IL-1 has also been shown to induce leukemic cell growth [20] and even to favour tumor progression [21], the use of IL-1 should be treated with caution when treating leukemia due to the fact that this factor could be inhibiting the proliferation of the normal hematopoietic cells, as shown in this work and other reports [11], and promoting that of the malignant ones. On the other hand it would be interesting to evaluate the possible role of the



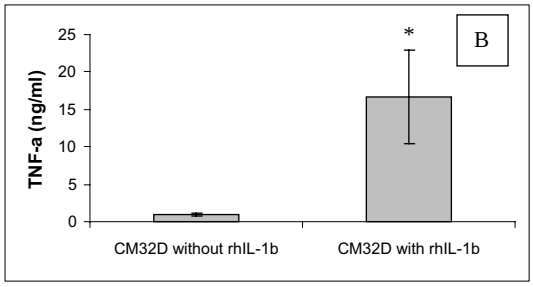
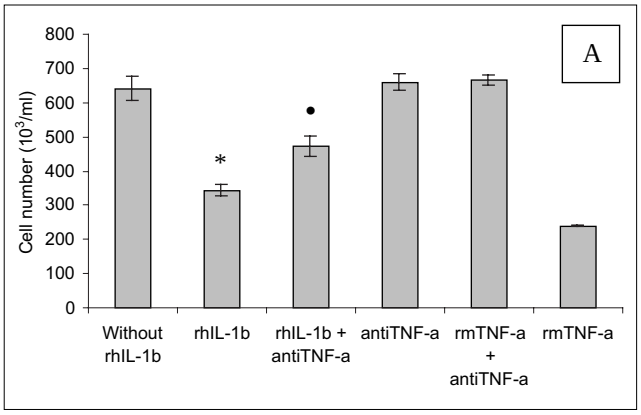


Fig. 4. rhIL-1β induces secretion of bioactive TNF in 32D cl3 cells. (A) The conditioned media (CM) from 32D cl3 cells was collected after 48 h in culture in the presence of rmIL-3 (0.5 ng/ml) without (CM32D cl3) or with rhIL-1β (5 ng/ml) (CM32D cl3/rhIL-1β). This CM was added (20% v/v) to L929 cell cultures in the presence of actinomycin D (ActD) and cell survival was evaluated by trypan blue exclusion. ActD, rhIL-1β + ActD and rmTNF-α + ActD were used as control. (B) Concentration (ng/ml) of TNF-α in CM from 32D cl3 cells treated with or without rhIL-1β detected by ELISA evaluation. Data represent the mean ± SD of three independent experiments and the difference from the without rhIL-1β (*) was significant at $P < 0.05$ by Student's t-test.



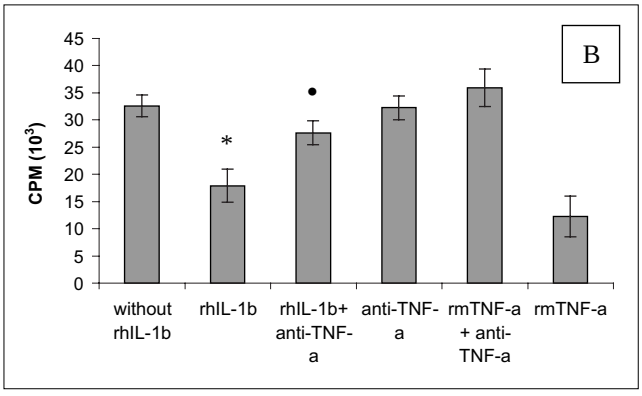


Fig. 5. Anti-mouse TNF-α antibody partially reverses the inhibition of the proliferation of 32D cl3 cells induced by rhIL-1β. (A) 32D cl3 cells were cultured in the presence of rmIL-3 (0.5 ng/ml) with or without rhIL-1β (5 ng/ml) and with or without an anti-mouse TNF-α antibody (anti-TNF-α) (1:100 BSA/PBS). Cell numbers were evaluated after 48 h in culture with Neubauer chamber under the light microscope. A culture with rmTNF-α (10 ng/ml), anti-TNF-α and rmTNF-α + anti-TNF-α was added as a control. (B) 32D cl3 cells were cultured in the presence of rmIL-3 (0.5 ng/ml) with or without rhIL-1β (5 ng/ml) and with or without an anti-mouse TNF-α antibody (anti-TNF-α) (1:100 BSA/PBS). Cell proliferation was evaluated after 48 h by ^{3}H-thymidine incorporation. Data represent the mean ± SD of three independent experiments and the difference with or without rhIL1-β (•) was significant at $P < 0.05$ by Student's t-test.

molecule that inhibits IL-1 activity to treat leukemia because it could suppress the leukemia proliferative effect of IL-1 and its inhibitory effect on normal hematopoiesis.

It is known that mature myeloid cells produce IL-1 and TNF when participating in tumor rejection [22,23], in consequence if as shown in this work IL-1 can induce the secretion of more TNF by immature cells then the increased concentration of this factor in the serum of these patients could increase tumor rejection.

4. Materials and methods

4.1. Cell lines

Mouse IL-3-dependent myeloid multipotent cell line (32D cl3) [24] was a kind gift from Dr. T. Hoang (Hemopoiesis and Leukemia Laboratory, Montreal, Canada). Cells were cultured in Iscove's modified Dulbecco's medium (Gibco BRL, USA) supplemented with 10% foetal bovine serum (FBS, Gibco BRL, USA) and 0.5 ng/ml of rmIL-3 (R&D System, Minneapolis, MN) in a humidified 5% CO_2 atmosphere at 37 °C.

The cell culture was maintained with an initial density of 1×10^5 cells/ml and subsequently re-plated in Petri dishes (Fisher Brand, Denmark) at 48 h when the density reached 1×10^6 cells/ml. The optimal concentration of rmIL-3 was obtained with dose–response curves measuring 32D cl3 cell proliferation. The cell line L929 was maintained under the same culture conditions as mentioned above, except for the addition of IL-3.

4.2. Recombinant cytokines and antibodies

Recombinant human interleukin-1 beta (rhIL-1β), recombinant mouse IL-3 (rmIL-3), recombinant mouse tumor necrosis factor alpha (rmTNF-α), recombinant mouse interferon gamma (rmIFN-γ) and goat anti-mouse TNF-α antibodies (anti-TNF-α) were purchased from R&D Systems, USA.

For the ELISA assays, rmTNF-α and anti-TNF-α were obtained from Pharmingen. The cytokines were reconstituted in PBS containing 0.1% BSA and the antibody was diluted in PBS. Small aliquots were kept at −70 °C until used.

4.3. Cellular assays

To evaluate the effect of rhIL-1β on 32D cl3 cell line proliferation 1×10^5 cells were seeded and kept in culture for 48 h in the presence of rhIL-3 (unless otherwise stated) in a 96-well plate (Nunclon, USA) in the presence or absence of different concentrations of rhIL-1β. In some cases 10 ng/ml of rmTNF-α and 25 ng/ml of rmIFN-γ were used.

4.4. Cell proliferation assays

To evaluate cell proliferation different assays were performed.

4.4.1. Cell number

After the indicated times for each assay, cells were suspended and a small aliquot was evaluated in a Neubauer chamber under the light microscope (Carl Zeiss, Germany) to determine the cell number and to calculate cellular density.

4.4.2. Tritiated thymidine incorporation

Twenty thousand cells per milliliter were cultured in the presence or absence of 5 ng/ml of rhIL-1β and 25 ng/ml of rmIFN-γ for 24 h. After this time 1 μCi/ml of ^{3}H-thymidine (activity 185 mBq, Amersham Pharmacia Biotech, UK) was added and cells were harvested 24 h later, then washed once with PBS and lysate in NaOH (0.4 M). Finally 2 ml of scintillation liquid was added and samples were analysed [25], in a Beckman beta-counter (Beckman LS6500, USA.)

4.5. Cell viability

To determine the presence of necrotic cells in rhIL-1β treated cells, trypan blue dye was used (Sigma Co., USA). A small sample of the cultured cells was mixed in a 1:1 ratio with the dye and incubated for 5 min at room temperature. After this time, cells were counted in the Neubauer chamber to evaluate the difference between non-stained (viable cells) versus stained cells (dead cells) under the light microscope. Apoptosis was evaluated by the tunnel reaction in situ using the TACS.XL apoptosis detection kits (R&D Systems, Minneapolis, MN, USA). Briefly, 1.5×10^5 32D cl3 cells per milliliter were cultured for 48 h in the presence or absence of 0.5 ng/ml of rmIL-3 and 5 ng/ml of rhIL-1β or 25 ng/ml of rmIFN-γ. The cells were then washed with 1% BSA in PBS (PBA) and fixed with 3% formaldehyde, followed by a treatment with a labelling reaction mix, washed in dH_2O and labelled with TdT–BrdU. The sample was covered with antibody anti-BrdU for 1 h, washed and covered with streptavidin–HRP, and finally with TACS blue label and counter-staining with nuclear fast red. The percentage of apoptotic cells was measured by light microscope.

4.6. Cellular differentiation

Morphological differentiation was evaluated on a Giemsa-stained cell smear. Blasts of monocyte-macrophage lineage were differentiated from granulocyte-neutrophils by taking into consideration size, nuclear shape and nuclear-cytoplasm ratio [26].

In order to clearly identify different cellular lineages, specific cytochemical stains for the monocyte-macrophage lineage (alpha-naphthyl acetate esterase) and for the granulocyte-neutrophil (chloroacetate esterase) were used [27].

4.7. Induction of the TNF-α gene expression in 32D cl3 cells treated with rhIL-1β

RNA was extracted using TRIZOL (Invitrogen life technologies CA, USA). Briefly, 2×10^6 32D cl3 cells were cultured in medium containing 5 ng/ml of rhIL-1β or PBS for 24 h in Petri dishes as indicated. Cells were washed once with PBS, lysate with 1 ml of TRIZOL and 200 μl of chloroform; the aqueous phase was precipitated with isopropanol and the pellet was suspended in 15 μl of DEPC-water. RNA was quantified at 260 nm after heating at 65 °C and diluted 1:1000 in DEPC-water. RNA integrity was analysed in a 2% agarose gel stained with ethydium bromide. For RT-PCR, the Perkin–Elmer kit was used (GeneAmp RNA PCR N808-0017, New Jersey USA). Oligonucleotides for PCR were as follows: for β-actin the 5′ primer was 5′-GGG TCA GAA GGA TTC CTA TG-3′ and the 3′ primer was 5′-GGT CTC AAA CAT GAT CTG GG-3′ with an expected product of 242 bp. The primers for the TNF-α reported by Gross et al. [28] were used; the 5′ primer was 5′-TCTCATCAGTTCTATGGCCC-3′ and the 3′ primer was 5′-GGGAGTAGACAAGGTACAAC-3′ with an expected product of 212 bp. RT-PCR conditions were 60 min at 42 °C and 10 min at 90 °C. The 35 PCR cycles were for 15 s for denaturation at 95 °C, 30 s of annealing at 60 °C and 7 min of extension at 72 °C. The specificity of the amplified products was verified by the presence of products of the expected size in a 2% agarose gel stained with ethydium bromide.

4.8. TNF-α biological activity

To determine the biological activity of TNF-α secreted to the culture medium by the 32D cl3 cells when rhIL-1β was present (conditioned medium; MC), the cell line L929 was used since it is known that these cells died in the presence of TNF-α [29]. Briefly, L929 cells were seeded at a cellular density of 3×10^4 cells/0.1 ml in 96-well plates for 12 h. After this time, the medium was changed and the conditioned medium of the 32D cl3 cells was added and 0.5 mg/ml of actinomycin D was added as an adjuvant. Cells were incubated for 16 h and the percentage of cell survival was evaluated by trypan blue exclusion. As negative controls, cells were cultured in the presence of actinomycin D, rhIL-1β or conditioned medium of 32D cl3 cells, and as a positive control 10 ng/ml of rmTNF-α was added to the L929 cells.

4.9. Evaluation of cytokine production in vitro

Supernatants of 32D cl3 cells treated with 0.5 ng/ml of rmIL-3 and cultured for 48 h with or without 0.5 ng/ml of rhIl-1β were used to determine the secretion of TNF-α by a sandwich ELISA assay. Purified primary anti-TNF-α capture antibodies (Pharmingen, San Diego, CA) were diluted at 2 μg/ml in binding solution (0.1 M NaH_2PO_4, pH 9.0) and 100 μl of antibody dilution was added to 96-well ELISA plates (Nunc Maxisorp, Nalge Nunc International Corporation, IL, USA) and incubated overnight at 4 °C. ELISA plates were washed three times with 10% FCS in cold PBS (blocking buffer) and incubated at room temperature for 2 h with blocking solution. Plates were washed twice with PBS/Tween (0.05% Tween 20 in PBS, washing solution). Mouse recombinant proteins and samples were added diluted in blocking buffer containing 0.05% Tween 20, and incubated at 4 °C overnight. Plates were washed four times with washing solution and the anti-TNF-α biotin-conjugated antibody was added at 1 μg/ml in blocking solution/Tween and plates were incubated for 2 h at room temperature. After this time, plates were washed four times with washing solution and avidin–horse radish peroxidase conjugate was added (Pharmingen, 1:2000 in blocking buffer/Tween) to the wells, incubated for 30 min at room temperature and washed five times. Freshly prepared ABTS (Sigma, USA) substrate solution was added (0.15% ABTS in 0.1 M anhydrous citric acid with 0.06% H_2O_2, Sigma) and optical density was measured after 5 min in an ELISA microplate reader (SpectraMax 250, Molecular Devices, USA) at 405 nm.

4.10. Inhibition of proliferation induced by TNF in 32D cl3 cells treated with rhIL-1β

To analyse the role of TNF-α in the inhibition of proliferation of 32D cl3 cell treated with rhIL-1β, these cells were seeded at an initial density of 1×10^5 cells/ml in 96-well plates with 0.5 ng/ml of rmIL-3 in the presence or absence of 5 ng/ml of rhIL-1β and 10 ng/ml of rmTNF-α with or without anti-TNF antibody (1:100). Proliferation was evaluated after 48 h in culture.

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References

[1] Dinarello C. Biologic basis for interleukin-1 in disease. Blood 1996;87:2095–114.

[2] Qi DQ, Perkins SL, Kling SJ, Russell RG. Divergent regulation of 1,25-dihydroxyvitamin D3 on human bone marrow osteoclastogenesis and myelopoiesis. J Cell Biochem 1999;72:387–95.

[3] Beaupre DM, Talpaz M, Marini 3rd FC, Cristiano RJ, Roth JA, Estrov Z, et al. Autocrine interleukin-1beta production in leukemia: evidence for the involvement of mutated RAS. Cancer Res 1999;59:2971–80.

[4] Onozaki K, Matsushima K, Aggarwal B, Oppenheim J. Human interleukin 1 is a cytocidal factor for several tumor cell lines. J Immmunol 1985;135:3962–8.

[5] Onozaki K, Tamatani T, Hashimoto T, Matsushima K. Growth inhibition and augmentation of mouse myeloid leukemic cell line differentiation by interleukin 1. Cancer Res 1987;47:2397–402.

[6] Lovett D, Kozan B, Hadam M, Resch K, Gemsa D. Macrophage cytotoxicity: interleukin 1 is a mediator of tumor cytostasis. J Immnunol 1986;136:340–7.

[7] Ayroldi E, Blasi E, Varesio L, Wiltrout RH. Inhibition of proliferation of retrovirus-immortalized macrophages by LPS and IFN-gamma: possible autocrine down-regulation of cell growth by induction of IL1 and TNF. Biotherapy 1992;4:267–76.

[8] Furmanski P, Johnson CS. Macrophage control of normal and leukemic erythropoiesis: identification of the macrophage-derived erythroid suppressing activity as interleukin-1 and the mediator of its in vivo action as tumor necrosis factor. Blood 1990; 75:2328–34.

[9] Hirayama F, Clark SC, Ogawa M. Negative regulation of early B lymphopoiesis by interleukin 3 and interleukin 1alpha. Proc Natl Acad Sci U S A 1994;91:469–73.

[10] Hirayama F, Ogawa M. Negative regulation of early T lymphopoiesis by interleukin-3 and interleukin-1alpha. Blood 1995;86:4527–31.

[11] Yonemura Y, Ku H, Hirayama F, Souza LM, Ogawa M. Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. Proc Natl Acad Sci U S A 1996; 93:4040–4.

[12] Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. J Immunol 1992; 149:3881–8.

[13] Hsu HC, Tsai WH, Chen PG, Hsu ML, Ho CK, Wang SY. In vitro effect of granulocyte-colony stimulating factor and all-*trans* retinoic acid on the expression of inflammatory cytokines and adhesion molecules in acute promyelocytic leukemic cells. Eur J Haematol 1999;63:11–8.

[14] Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, et al. Numerous growth factors, cytokines, and chemokines are secreted by human $CD34^+$ cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 2001;97:3075–85.

[15] Gasparetto C, Laver J, Abboud M, Gillio A, Smith C, O'Reilly RJ, et al. Effects of interleukin-1 on hematopoietic progenitors: evidence of stimulatory and inhibitory activities in a primate model. Blood 1989;74:547–50.

[16] Moldawer LL, Marano MA, Wei H, Fong Y, Silen ML, Kuo G, et al. Cachectin/tumor necrosis factor-alpha alters red blood cell kinetics and induces anemia in vivo. FASEB J 1989;3:1637–43.

[17] Reykdal S, Abboud C, Liesveld J. Effect of nitric oxide production and oxygen tension on progenitor preservation in ex vivo culture. Exp Hematol 1999;3:441–50.

[18] Galloway CJ, Madanat MS, Mitra G. Monoclonal anti-tumor necrosis factor (TNF) antibodies protect mouse and human cells from TNF cytotoxicity. J Immunol Methods 1991;140:37–43.

[19] Jacobsen SE, Veiby OP, Myklebust J, Okkenhaug C, Lyman SD. Ability of flt3 ligand to stimulate the in vitro growth of primitive murine hematopoietic progenitors is potently and directly inhibited by transforming growth factor-beta and tumor necrosis factor-alfa. Blood 1996;87:5016–26.

[20] Attias D, Grunberger T, Vanek W, Estrov Z, Cohen A, Lau R, et al. B-lineage lymphoid blast crisis in juvenile chronic myelogenous leukemia: interleukin-1 mediated autocrine growth regulation of the lymphoblast. Leukemia 1995;9:884–8.

[21] Estrov Z, Kurzrock R, Talpaz M. Interleukin-1 and its inhibitors: implications for disease biology and therapy. Cancer Treat Res 1995;80:51–82.

[22] Urban JL, Shepard HM, Rothstein JL, Sugarman BJ, Schreiber H. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. Proc Natl Acad Sci U S A 1986;83:5233–7.

[23] Onozaki K, Matsushima K, Kleinerrman ES, Saito T, Oppenheim JJ. Role of interleukin 1 in promoting human monocyte-mediated tumor cytotoxicity. J Immunol 1985;135:314–20.

[24] Greenberger J, Sakakeeny M, Humphries R, Eaves C, Eckner R. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc Natl Acad Sci U S A 1983;80:2931–5.

[25] Zentella A, Weis FMB, Ralph DA, Lahio M, Massague J. Early responses to TGF-b in cells lacking growth suppressive RB function. Mol Cell Biol 1991;11:4952–8.

[26] Johnston RB, Zucker-Franklin D. The mononuclear phagocyte system; monocytes and macrophages. In: Zucker-Franklin D, Greaves MF, Grossi CE, Marmont AM, editors. Atlas of blood cells; function and pathology. 2nd ed. Italy: Edi-Ermes; 1988. p. 321–57.

[27] Li C, Lam K, Yam L. Esterases in human leukocytes. J Histochem Cytochem 1973;21:1–12.

[28] Gross A, Spiesser S, Terraza A, Rouot B, Caron E, Dornand J. Expression and bactericidal activity of nitric oxide synthase in *Brucella suis*-infected murine macrophages. Infect Immun 1998;4:1309–16.

[29] Aggarwal BB, Gutterman JU. Tumor necrosis factor. In: Aggarwal BB, Gutterman JU, editors. Human cytokines; handbook for basic and clinical research. Boston, USA: Blackwell Scientific Publication; 1992. p. 270–86.