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In vitro effects of IL-12 and IL-2 on NK cells, cytokine release and clonogenic activity in myelodysplastic syndromes (MDS)

B Sarina[1], A Cortelezzi[1], C Cattaneo[1], M Pomati[1], I Silvestris[1], M Di Stefano[1], D Lambertenghi-Deliliers[1], C Hu[2], M Monza[2] and AT Maiolo[1]

[1]*Servizio di Ematologia,* [2]*Ist Med Int, Università di Milano, IRCCS, Ospedale Maggiore, Milano, Italy*

We evaluated the *in vitro* effects of IL-12, alone and in association with IL-2 on MDS bone marrow and peripheral blood cells. Thirty-six patients and 14 healthy subjects were studied. Natural killer-activity (NK-a) levels and lymphocyte immunophenotypes were determined in fresh bone marrow (BMMNC) and peripheral blood mononuclear cells (PBMNC), which then were resuspended in medium containing IL-2, IL-12 or IL-2 + IL-12 for 7 days. Re-evaluation of NK-a levels, lymphocyte immunophenotypes, clonogenic activity and cytokine release showed that, unlike IL-2, IL-12 did not significantly increase NK-a or $CD3^-/56^+$ cell levels in either bone marrow or peripheral blood; IL-2 + 12 led to a significant increase that fell between the values reached by each cytokine alone. IL-2 + 12 and, although to a lesser extent, also IL-12 alone induced the release of large amounts of γ-IFN and α-TNF. In addition, the number of clusters particularly decreased in the samples treated with IL-2 + 12 and IL-12 alone. Clonogenic activity was not modified after stimulation with any of the treatment. These data suggest that IL-12 induces the release of inhibitory cytokines in normal as well as MDS cells and that it could be used in patients with elevated bone marrow blastosis.

Keywords: myelodysplastic syndromes; interleukin-12; interleukin-2; NK activity; clonogenic activity

Introduction

Severe peripheral blood cytopenia, together with bone marrow hypercellularity and dysplasia are frequently the hallmark of myelodysplastic syndromes (MDS).[1–5] Although it has been established that these hematological disorders arise from the clonal expansion of the progeny of an aberrant pluripotential stem cell,[6–8] little it is known about their etiology and pathogenesis.

Morphological involvement of the myeloid lineage is always observed in MDS, whereas there is evidence of peripheral lymphopenia and functional of B and T cell abnormalities only in some cases. Attention has recently been directed to the role of NK cells and immunoregulatory cytokines in the genesis of MDS. A number of studies have shown that peripheral blood natural killer-activity (NK-a) was decreased in MDS patients,[9–11] even though these observations do not necessarily imply the clonal involvement of NK cells.[12,13]

IL-12 is a heterodimeric cytokine physiologically produced by phagocytes, B lymphocytes and other antigen-presenting cells (APC).[14–16] It has been shown to cause the proliferation of activated T and NK cells, to enhance the lytic activity of NK cells and to induce the production of antileukemic cytokines, particularly γ-IFN.[16–20] In a previous study, we demonstrated that the defective bone marrow (BM) and peripheral blood (PB) NK-activity in MDS patients can be restored *in vitro* by the addition of another immunoregulatory cytokine, IL-2.[21]

Correspondence: A Cortelezzi, Servizio di Ematologia, Via Francesco Sforza, 35, Ospedale Maggiore, 20122 Milano, Italy
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In this study, we investigated the effect of IL-12, alone and in association with IL-2, on PB and BM NK cells, lymphocyte phenotype, cytokine release and BM clonogenic activity in MDS patients, with the aim of exploring further the impact of the cytokine network on the proliferation and differentiation of MDS cells.

Materials and methods

Patients

BM and PB cells were obtained from 36 patients (M/F = 19/17) with *de novo* MDS and 14 normal bone marrow and peripheral blood donors, after they had given their informed consent. The median age of the patients was 73.5 years (range 21–88), and they were classified according to FAB criteria as being affected by refractory anemia (RA n = 10), refractory anemia with ring sideroblasts (RARS n = 11), refractory anemia with excess of blasts (RAEB n = 14) and refractory anemia with excess of blasts in transformation (RAEB-t n = 1). Their clinical characteristics are summarized in Table 1. For the purposes of this study, we decided to define RA + RARS as low-risk (LR) MDS and RAEB + RAEB-t as high-risk (HR) MDS. Patients with CMML were excluded from the study because of the small number who had been under our observation.

Cell preparation

The PB and BM samples were collected in preservative-free heparin. The mononuclear cells (MNC) were separated by gradient centrifugation using Ficoll Lymphoprep (Nicomed Pharma, Oslo, Norway) and then washed twice with phosphate-buffered saline.

BM and PB liquid cultures

The PBMNC were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) with 10% human AB serum (Flow ICN Biomedicals, Costa Mesa, CA, USA), whereas the BMMNC were resuspended at the same concentration in IMDM (Life Technologies) with 10% FCS (Hyclone Laboratories, Logan, UT, USA). RhIL-12 (Sigma-Aldrich, Milan, Italy) at a concentration of 0.1 ng/ml with or without RhIL-2 (Proleukin; Cetus Corporation, Emeryville, CA, USA) 500 U/ml was added to the liquid cultures and then incubated at 37°C in 5% CO_2 for a week. The cells were then washed twice in PBS and the viability (always more than 90%) of the BM and PB cells was evaluated by the Trypan blue exclusion test at the end of the culture.

Table 1 Characteristics of the patient group

Patient	*Sex/Age*	*FAB*	*Blasts*[a]	*Karyotype*
1	F/81	AR	2.75	46, XX
2	M/76	AR	3	46, XY
3	M/58	AR	1.75	47, XY, +8
4	M/59	AR	3.75	46, XY
5	M/89	AR	4.75	46, XY
6	F/57	AR	3	46, XX
7	F/70	AR	2	46, XX
8	F/63	AR	4.25	complex karyotype
9	F/88	AR	2	46, XX, inv 9
10	M/21	AR	1.5	46, XY
11	F/62	ARS	3.5	46, XX
12	M/59	ARS	4.75	46, XY
13	F/64	ARS	3.5	46, XX
14	M/69	ARS	4.5	46, XY
15	F/33	ARS	1.25	46, XX
16	M/72	ARS	2.5	46, XY
17	M/77	ARS	2	46, XY
18	M/84	ARS	3	46, XY
19	M/61	ARS	4.25	46, XY
20	F/57	ARS	1	46, XX
21	M/80	ARS	4.75	46, XY
22	F/82	AREB	7	45, XX, –7
23	M/69	AREB	17.75	46, XY
24	F/74	AREB	5.75	47, XX, +8, 5q–, 12q–
25	F/64	AREB	19.75	46, XX, 3q–
26	F/75	AREB	5.75	46, XX/ 47, XX, +8
27	M/60	AREB	9	NE
28	F/75	AREB	8	46, XX, 5q–
29	M/72	AREB	9	NE
30	F/70	AREB	5.75	47, XX, +13
31	M/57	AREB	6	46, XY
32	M/67	AREB	6.5	48, XY, +8, +6
33	F/54	AREB	10	46, XX
34	M/45	AREB	8.75	46, XY, 5q–
35	F/53	AREB	10.25	46, XX, 5q–
36	M/66	AREB-t	25.5	46, XY

[a]BM blasts at the time of the study.
NE, not evaluated.

NK-activated cell assay

Before testing NK activity, we determined the percentage of lymphocytes in the PBMNC and BMMNC in order to confirm the effector/target ratio. A 4 h ^{51}Cr release assay was then used with K562 cells as the target. The target cells were labelled with 100 μCi of $Na_2\ ^{51}CrO_4$ (Amersham, Arlington Heights, IL, USA) per 1×10^6 cells for 1 h at 37°C, and used at a final concentration of 5×10^3/well. The effector cells were added to 96-well microculture plates (Cel-Cult, Sterilin Limited, Feltham, UK) and incubated at different effector/target cell ratios (50:1, 25:1, 12.5:1, 6.25:1) for 4 h at 37°C in 5% CO_2. Radioactivity was measured in a gamma-counter (Cobra Auto-Gamma, PACKARD, Canberra Company, IL, USA), and specific cytotoxicity was calculated from the mean counts per minute (c.p.m.) of three replicate samples using the following formula:

$$\%\ ^{51}\text{Cr release} = \frac{\text{exp. c.p.m.} - \text{spont. rel. c.p.m.}}{\text{max c.p.m.} - \text{spont. rel. c.p.m.}}$$

The spontaneous release value was always less than 10% of total radioactivity.

Lymphocyte phenotype analysis

The PB and BM mononuclear cells were labeled with anti-CD3Fitc and CD56PE (Leu-4, Leu-3a+3b, Leu-2a, Leu-12; Becton Dickinson Immunocytometry System, San Jose, CA, USA), and analyzed using a flow cytometer (FACSCAN, Becton Dickinson).

Release of GM-CSF, γIFN and αTNF in the supernatant of BM liquid cultures

The supernatants obtained by spinning the contents of the BM liquid cultures at the end of the culture time were stored at –20°C until used, and then investigated for their GM-CSF, γ-IFN and α-TNF content by means of commercially available immunoenzymatic assay kits (Medgenix Diagnostic, Fleurus, Belgium). Minimum detectable concentratium (MDC) was 3 pg/ml for α-TNF and GM-CSF, and 0.03 IU/ml for γ-IFN.

Agar colony assay (CFU-GM)

Clonogenic potential after incubation with IL-12 ± IL-2 was evaluated by means of agar colony assay (CFU-GM). 1×10^6 cells/ml in IMDM were cultured in 1 ml of a mixture containing 20% FCS, 3% agar, rhGM-CSF 200 U/ml (Genzyme, Cambridge, MA, USA) and IL-3 100 U/ml (Genzyme). The plates were incubated in humidified air with 5% CO_2 at 37°C for 2 weeks. The aggregates containing >50 cells were scored as colonies, whereas those containing <50 cells were scored as clusters. All of the cultures were set up in triplicate.

Statistical analysis

The Wilcoxon-matched-pairs-signed-rank's test was used to evaluate the within-group differences, and the Mann–Whitney test for the differences between groups. A P value of <0.05 was considered to be statistically significant.

Results

In a preliminary phase of the study, we evaluated the effect of IL-12 on NK-a in PB mononuclear cells taken from healthy donors at different times of incubation and at various concentrations. The dose–response curve is shown in Figure 1. We can assume that IL-12 0.1 U/ml for 1 week better stimulates NK-a, although not significantly so in comparison with the basal value. As far as IL-2, GM-CSF and IL-3 are concerned, we used the same dosages as those used in our previous study.[21] Once again, we also evaluated the sequential stimulus IL-2 → IL-12 (IL-2 was added at $t = 0$, IL-12 after 24 h), but we did not observe any increase in NK-a with respect to the sample treated with a simultaneous stimulus (data not shown).

NK activity

As already observed in a previous study,[21] baseline peripheral and bone marrow NK-a in the MDS patients was lower than in the normal subjects: the average specific lysis in peripheral blood was 14.6% in MDS patients and 38.6% in normal controls ($P < 0.01$), whereas bone marrow NK-a was 5.3% in MDS patients and 7.2% in donors (NS). IL-12 stimulation did not induce any increase in NK-a in peripheral and bone marrow MDS cells with respect to pre-cultured controls (average specific peripheral and bone marrow lysis was 13.3 and 8%, respectively), although a significant increase was observed between the cultured controls and the IL-12-stimulated BM and PB cells. After incubation with IL-2, a significant ($P < 0.01$) increase in NK-a was observed (average specific peripheral and bone marrow lysis was 51.7 and 36.9%, respectively), which was only slightly lower than that seen in the IL-2-stimulated normal controls. The association of the two cytokines led to an increase in NK-a that fell between the values reached by each of them alone (average percent specific peripheral and bone marrow lysis was 40.2 and 22.7%, respectively). No statistically significant differences were observed between LR and HR groups (Figure 2a and b).

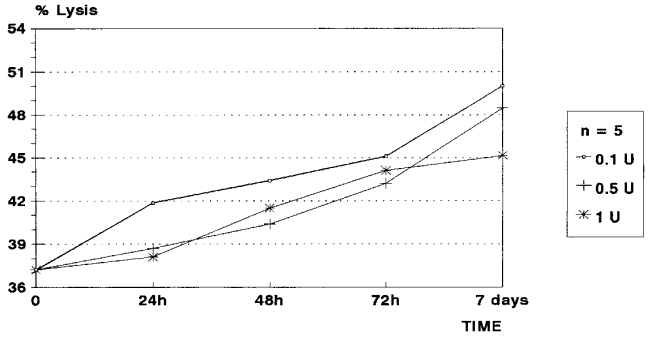

Figure 1 Effect of IL-12 on NK-a of normal PB cells at time 0 and after 1, 2, 3 and 7 days.

Lymphocyte immunophenotype

No difference was observed in the immunophenotypes of the MDS samples with and without the addition of IL-12 after 7 days of culture. As already observed,[16] in comparison with cultured controls, a significant increase in peripheral blood ($P < 0.05$) and bone marrow ($P < 0.01$) $CD3^-/56^+$ cells occurred after the addition of IL-2 alone (Figure 3a and b). Moreover, the association of IL-2 + IL-12 increased the percentage of $CD3^-/CD56^+$ cells to values falling between those reached by each of them alone. No statistically significant differences were observed between LR and HR groups.

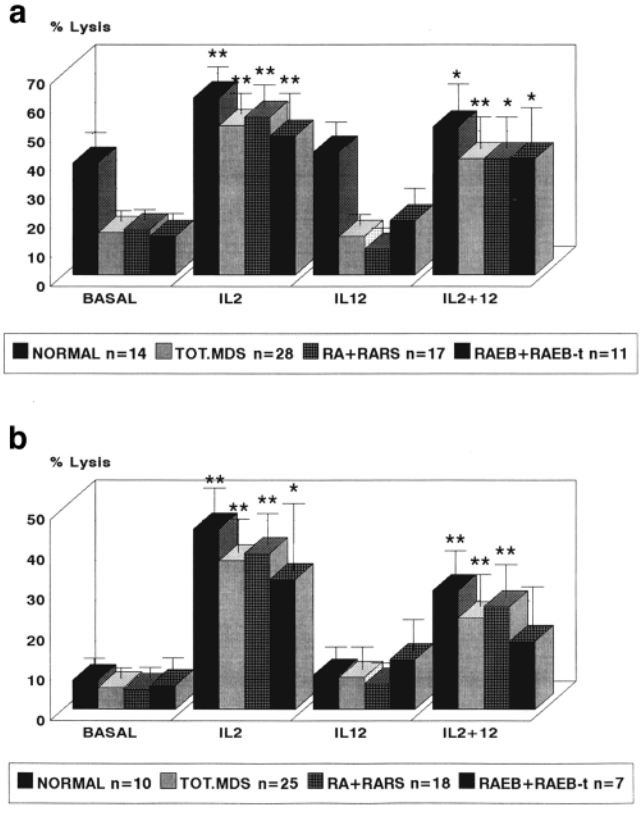

Figure 2 Pre- and post-IL-12 and/or IL-2 stimulation on PB (a) and BM (b) NK-activity (± s.e.) in normals and MDS patients. Percentage of specific lysis of K562 cells at an E:T ratio of 50:1 for PB and of 25:1 for BM. *$P < 0.05$; **$P < 0.01$.

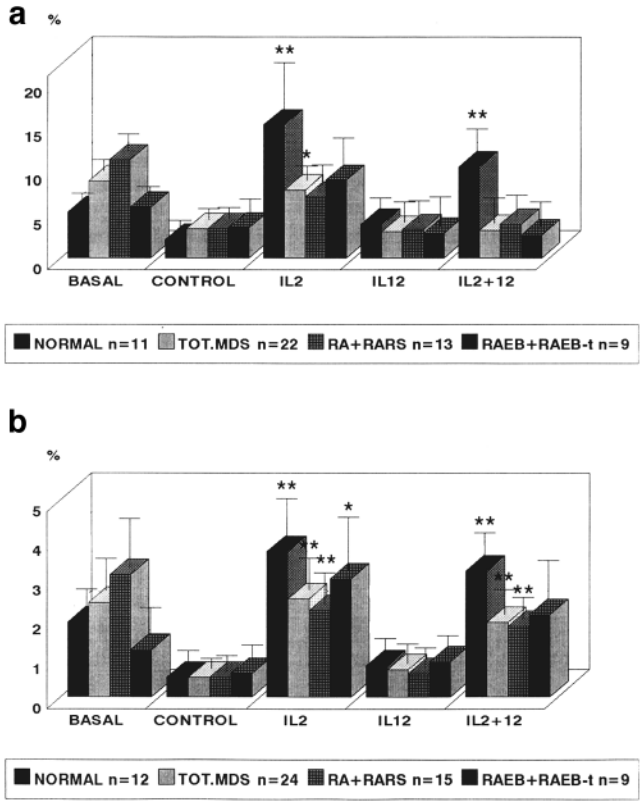

Figure 3 $CD3^-/56^+$ percentage (± s.e.) of stimulated MDS peripheral blood (a) and bone marrow (b) cells in comparison with basal and untreated samples (control). Statistical comparison carried out between controls and stimulated samples. $^*P < 0.05$; $^{**}P < 0.01$.

Cytokine release

GM-CSF, γ-IFN and α-TNF release in the supernatant of the MDS bone marrow liquid cultures significantly increased after incubation with IL-12, IL-2 and IL-2 + 12. IL-12 alone increased γ-IFN and α-TNF, but not GM-CSF values to a greater extent than IL-2 (mean values: 48.8 *vs* 10.5 IU/ml for γ-IFN, 1883.1 *vs* 655.3 pg/ml for α-TNF, 142.8 *vs* 168.9 pg/ml for GM-CSF) (Figure 4a, b and c). In particular, the IL-2 + 12 association increased GM-CSF and α-TNF values in an additive manner, whereas the effect of IL-2 + 12 on γ-IFN was synergistic (290-fold with respect to the basal).

Clonogenic assay

The 7-days preincubation with IL-12 in the liquid culture did not induce any modification in the number of CFU-GM (mean baseline CFU-GM/ml = 33; mean IL-12 CFU-GM/ml = 25.8), but the number of clusters decreased (although not significantly) after the addition of the cytokines to the medium with respect to the untreated control (IL-2 + 12>12>2) (Figure 5). Unlike in our previous study,[16] we did not observe any significant increase in the number of CFU-GM after incubation with IL-2.

Discussion

It is well known that IL-12 is capable of stimulating the cytotoxic activity of both T and NK cells, although, in the latter

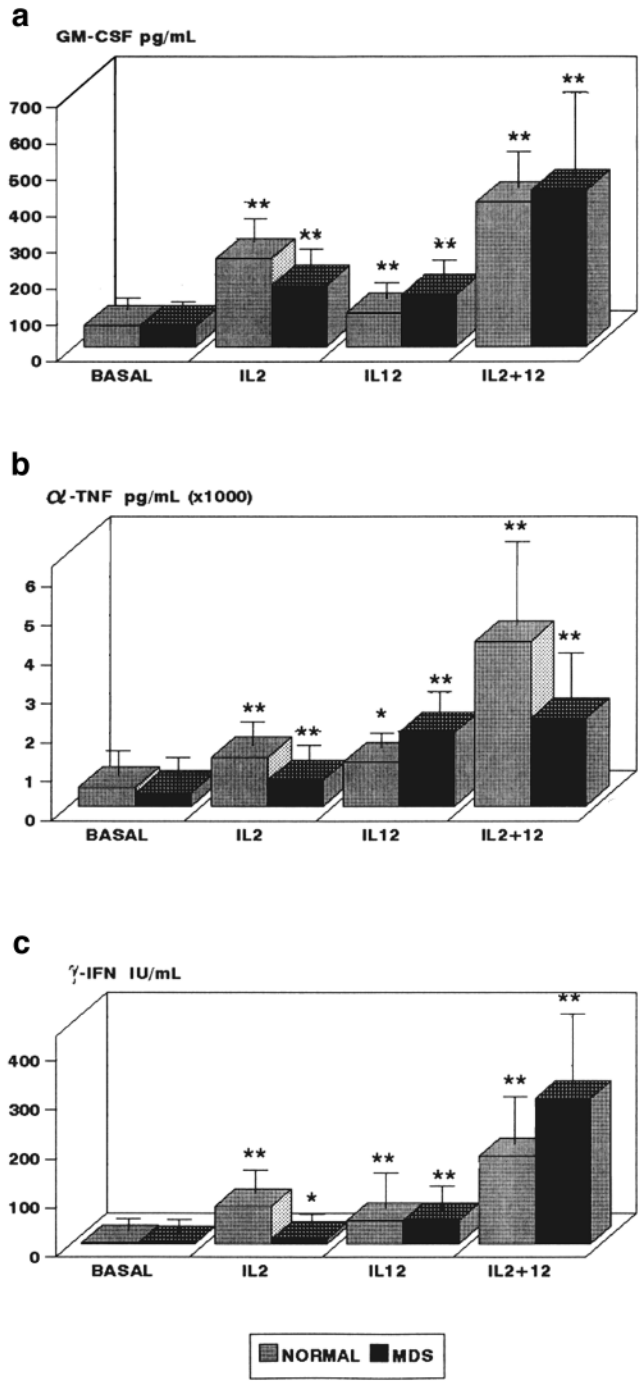

Figure 4 GM-CSF (a), α-TNF (b) and γ-IFN (c) in supernatants (± s.e.) of MDS bone marrow cultures after incubation with IL-12 and/or IL-2. $^*P < 0.05$; $^{**}P < 0.01$.

case, to a lesser extent than optimal doses of IL-2.[14] It is also known that IL-12 releases into the culture medium large amounts of γ-IFN,[16–20] a cytokine with potent antiproliferative activity. These characteristics, together with the fact that NK-a is reduced in MDS patients, prompted us to evaluate the *in vitro* effects of IL-12 on the bone marrow and peripheral blood cells of such patients.

In our *in vitro* system, IL-12 induced a modest but non-significant increase in NK-a in both normal and myelodysplastic cells in comparison with basal values. In contrast, the use

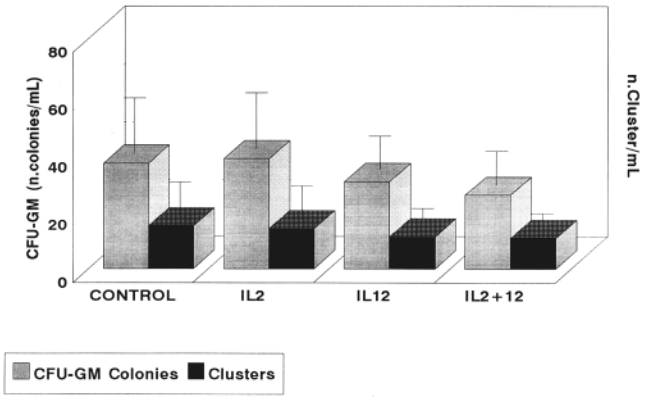

Figure 5 MDS bone marrow clonogenic activity (± s.e.) after incubation with IL-12 and/or IL-2 in comparison with untreated control.

of IL-2 led to a marked increase in BM and PB cell NK-a in the MDS patients that was only slightly lower than that observed in normal subjects (average specific lysis of 36.9 *vs* 44.8% and 51.7 *vs* 61.4%, respectively). Unlike Ogata *et al*,[22] we did not observe any synergistic activity between IL-2 and IL-12 in the enhancement of NK-a in either normal or MDS subjects; however, this may be due to our different IL-2 and IL-12 doses and culture times. Gately *et al*[23] observed that the association of IL-12 and IL-2 in culture for 4 days may lead to increases or decreases in LAK activity, depending on the quantity of IL-12. Furthermore, other studies[24] have shown that the association of IL-12 + IL-2 in culture for 18 h increases cytolytic activity only marginally in comparison with optimal doses of IL-2.

We did not observe any increase in the number of $CD3^-/CD56^+$ cells after IL-12 stimulation in either the MDS patients or controls, which was not the case after stimulation with IL-2; as for NK-a, the increase after double stimulation with IL-12 + IL-2 fell between the values obtained using the two cytokines individually. This result also confirms the data published in the literature concerning normal control subjects in whom IL-2 induces $CD3^-/CD56^+$ proliferation, whereas IL-12 appears to have no effect on the proliferation of the non-activated NK compartment. Furthermore, although IL-12 is capable of inducing activated T cell proliferation, it mainly inhibits the IL-2-induced proliferation of NK cells.[25] This effect is at least partially indirect, and depends on the α-TNF production induced by IL-12, as can be seen by the fact that anti-TNF antibodies are capable of restoring approximately 70% of the IL-2-induced proliferation.[23,25–27]

However, in our *in vitro* system, IL-12 led to a dramatic increase in γ-INF and α-TNF in the cell culture medium of both normal subjects and MDS patients. In particular, in the MDS samples, IL-12 proved to be a more potent inducer of cytokines with antitumoral activity than IL-2, whereas the increase in GM-CSF was less after culture with IL-12 than after IL-2. In the MDS samples, double stimulation with IL-12 + IL-2 led to an additive increase in cytokine release in the case of GM-CSF and α-TNF, and a synergistic increase in the case of γ-INF. Once again, these findings are in line with the data in the literature concerning normal subjects; it is well known that, when IL-2 and IL-12 are added to cultured T lymphocyte and NK cells, they act at the post-transcriptional level of the γ-INF gene and lead to a marked increase in the half-life of specific mRNA.[28] The MDS cells therefore preserve their ability to respond to immunological stimuli by secreting cytokines in amounts similar to those secreted by normal control cells.

In our *in vitro* system, we did not observe any increase in the number of CFU-GM prepared after 7 days of incubation with IL-2, IL-12 and IL-2 + 12 in comparison with control values. The non-significant increase in clonogenic activity after the addition of IL-2 appears to contradict our previous results and may be related to the longer time of incubation: the amount of inhibitory cytokines released in the supernatant after 7 days was much higher than that observed after the 24- and 72-h incubation periods used in our previous study. Furthermore, we did not observe any increase in the number of immature myeloid cells after incubation with IL-2, IL-12 and IL-2 + 12 when the centrifuged cells were morphologically analyzed (data not shown). Finally, we observed a progressive, although not significant, decrease in the absolute number of clusters after incubation with IL-2, IL-12 and IL-2 + 12 in comparison with control samples (respectively: 13.9 clusters/ml, 11 clusters/ml, 10.6 clusters/ml and 14.8 clusters/ml). The colony/cluster ratio was 2.5 in the controls, which increased to 2.8 after stimulation with IL-2, and to 2.7 after stimulation with IL-12. After double stimulation (IL-2 + IL-12), the ratio was 2.4 which may be explained by the inhibitory action of γ-IFN on normal myelopoiesis,[29] as its levels were considerably higher in the supernatant. We can therefore conclude that neither IL-2 nor IL-12 stimulate the proliferation of the leukemic component. It is known that IL-12, together with other cytokines such as IL-3 and SCF,[30–32] belongs to the group of early-acting cytokines that acts on hemopoietic progenitors. Further studies are necessary to evaluate whether IL-12 and other early-acting cytokines may stimulate clonal and non-clonal stem cells in MDS.

Our study shows that IL-12 does not induce a significant increase in NK activity or in the number of $CD3^-/CD56^+$ cells in MDS subjects, but it is particularly active in the release of antitumoral cytokines, especially γ-IFN. This last finding makes it possible to hypothesize the use of IL-12 particularly in clinical studies involving patients with a high bone marrow blastic component.[33] In addition to having a potent antitumoral effect,[34] γ-IFN may also play a protective role against intracellular pathogen infections[35–37] as a result of the known γ-IFN-driven macrophage activation. Finally, IL-12 may play a further and important role in resistance to infection by increasing the cell-mediated response that depends on the Th1 cell development that is regulated by IL-12 itself.[23,38] The double stimulus of IL-2 + IL-12 may potentiate not only the release of antileukemic cytokines but also the release of GM-CSF, which could enhance the proliferation of residual normal hemopoietic progenitors. The potential importance of these possible effects would justify further and more specific investigations.

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