

Granulocyte-macrophage colony-stimulating factor (GM-CSF) counteracts the inhibiting effect of monocytes on natural killer (NK) cells

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SUMMARY

GM-CSF is known to accelerate haematopoietic recovery following allogeneic bone marrow transplantation (BMT). In addition, it may restore and enhance both granulocyte and monocyte functions. Stimulation of monocyte functions may induce a direct or an indirect anti-leukaemic activity due to an increase of cellular cytotoxicity and production of cytokines which may result in a reduction of the relapse rate after BMT. NK cells may play a crucial role in this activity. Therefore we studied the influence of monocytes on NK activity in combination with GM-CSF. Lymphocytes and monocytes were isolated from buffy coats of healthy individuals by counterflow centrifugation elutriation (CCE). NK activity was exerted by CD3⁻CD56⁺ cell populations and could be enhanced by IL-2 incubation overnight. Incubation of CD3⁻CD56⁺ cells with GM-CSF in the presence or absence of IL-2 hardly influenced NK activity of the lymphocyte population. Low amounts of monocytes enhanced NK activity. NK activity in lymphocyte population in the presence of equivalent numbers of monocytes with or without IL-2 was strongly decreased irrespective of the effector:target ratio (ETR). This appeared not to result from sterical hindrance effects of the present number of cells. However, addition of GM-CSF abrogated the inhibition of NK activity by monocytes in the presence of IL-2. In monocyte fractions neither IL-2 nor GM-CSF yielded NK activity. Our findings indicate that GM-CSF can affect NK activity by counteracting the suppressing effects of monocytes, and hence may improve the outcome after BMT.

Keywords GM-CSF NK activity monocytes cytotoxicity IL-2

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is at present the most effective treatment of patients with leukaemia, though long-term survival is still not optimal [1,2]. The major complications influencing treatment outcome, such as graft-versus-host disease (GVHD), opportunistic infections due to a prolonged neutropenic phase, and relapse of the underlying malignancy after BMT, still account for significant morbidity and mortality. GVHD can be successfully prevented by partial T cell depletion from the transplant [3] or immunoprophylaxis with cyclosporin-A and methotrexate [4].

Haematopoietic growth factors have been shown to shorten the neutropenic period [5]. GM-CSF also stimulates particular effector functions of mature granulocytes and macrophages [6].

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In vitro studies have shown that GM-CSF can increase antibody-dependent and independent cellular cytotoxicity (ADCC) [7-10] mediated by monocytes directed against tumour cell lines [11]. In a prospective randomized study among BMT recipients [12], GM-CSF administration was associated with a reduced relapse rate suggesting an improvement of anti-leukaemic activity.

Several reports have shown that GM-CSF can affect particular T lymphocyte cytotoxic functions *in vitro* [13,14]. In addition, a synergistic effect of GM-CSF with IL-2 resulting in a more pronounced cytotoxicity of T lymphocytes and NK cells has been described [15]. These data suggested that GM-CSF may also enhance anti-leukaemic activity mediated by lymphocytes. However, monocytes appeared to up- or down-regulate NK activity depending on their functional state and concentration [16,17].

We studied the effect of GM-CSF on the anti-leukaemic activity mediated by NK cells and monocytes. We found that GM-CSF did not enhance anti-leukaemic activity in monocytes directly, but counteracted the inhibiting effect of monocytes on NK cells, resulting in an enhanced anti-leukaemic activity.

MATERIALS AND METHODS

Isolation of monocytes and lymphocytes

Mononuclear cells were separated from peripheral blood buffy coats of five healthy volunteers by Percoll 1.075 g/ml (Pharmacia, Uppsala, Sweden) and centrifugated at 500 g/min for 20 min.

Separation of monocytes and lymphocytes from mononuclear cells was performed by cell-scatter-monitored counterflow centrifugation elutriation (CCE), using a Curamé-3000, four-chamber-rotor (Dijkstra bv, Alkmaar, The Netherlands) as described previously [18,19]. Different cell fractions were collected by reducing the rotor speed from 3000 to 2000 rev/min with a constant flow rate of 12 ml/min (Fig. 1). Purification was assessed by morphological analysis of May-Grünwald-Giemsa (MGG)-stained cytospin samples. Viability was determined by trypan blue dye exclusion.

Monoclonal antibodies and complement

The following IgG2a isotype-expressing and rabbit complement (RC)-fixing murine MoAbs were used: CD3 (WT32); CD7 (WT1); CD8 (WT 82) kindly provided by Dr W. Tax (Department of Nephrology, University Hospital Nijmegen, The Netherlands); CD4 (OKT4), purchased from American Type Culture Collection (ATCC, Rockville, MD) cell bank; CD2 (Leu-5b); CD16 (Leu-11c) and CD56 (Leu-19) purchased from Becton Dickinson (Mountain View, CA). MoAbs were purified by using *Staphylococcus aureus* protein A coupled to Sepharose (Pharmacia Biosystems BV, Woerden, The Netherlands). Sera of 4–6-week-old New Zealand white rabbits were used as RC source. Sera of all rabbits were tested separately for specific and non-specific lysis of mouse spleen cells and human T cell lines. Sera with optimal cytotoxicity were pooled, snap frozen and stored at -70°C until use.

Adherence of monocytes

Cells from lymphocyte fractions that were contaminated with monocytes (2200–2150 rev/min fractions; Fig. 1) were washed twice with glucose PBS (GPBS) and resuspended (2×10^6 cells/ml) in culture medium containing RPMI 1640 medium (Flow Labs, Irvine, UK) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (Flow Labs), 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin (Flow Labs) in plastic tissue culture flasks and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 1 h in order to allow monocytes to adhere.

Afterwards, non-adherent cells were harvested by gently pipetting. The non-adherent cell population contained > 99% lymphocytes as determined in cytospin centrifuge preparations after MGG staining, with a viability > 98%. The remaining adherent cells were incubated at 4°C during 30 min and washed three times with GPBS. The adherent cell population contained > 85% monocytes, with a viability > 95%. The adherent and non-adherent cells were resuspended (5×10^6 cells/ml) in culture medium and directly used as effector populations in ^{51}Cr -release assay.

Phenotypic analysis of lymphocyte subpopulations

Lymphocyte subpopulations were characterized by flow cytometric immunofluorescence analysis (Coulter Epics Elite; Coulter Corporation, Hialeah, FL) using FITC-conjugated CD3 (UCHT1; Dakopatts, Glostrup, Denmark) and PE-conjugated

CD56 (Leu-19; Becton Dickinson). $\text{CD}3^{-}\text{CD}56^{+}$ was considered as NK population. Staining was performed using a standard method. Briefly, 100 μl of different CCE cell fractions were incubated at 4°C for 15 min with the appropriate volume of PE- and FITC-conjugated MoAbs. Remaining erythrocytes were subsequently lysed by FACS lysing reagent (Becton Dickinson) for 10 min at room temperature. After lysis, cells were washed once with PBS and flow cytometrically analysed.

Cytotoxicity

Lymphocytes, monocytes or combinations of both were incubated with or without 500 U/ml recombinant IL-2 (Glaxo, Geneva, Switzerland) and with or without 250 ng/ml recombinant GM-CSF (GM-CSF; Sandoz Pharma Ltd, Basel, Switzerland) in a final volume of 1.0 ml culture medium in a humidified incubator with 5% CO_2 in air at 37°C . In control experiments beads with a diameter of 10 μm (DNA Check; Coulter) were mixed with lymphocytes. After 18 h of incubation, supernatants were removed. Cells were resuspended in culture medium and used as effector cells in a ^{51}Cr -release assay.

In order to determine the NK activity and IL-2-induced cytotoxicity a ^{51}Cr -release assay was used with cell line K562 as target. K562 was maintained in logarithmic growth phase in culture medium in a humidified incubator with 5% CO_2 in air at 37°C . Briefly, 2×10^6 target cells were labelled with 100 μCi ^{51}Cr (NaCrO_4 ; Amersham, Aylesbury, UK) at 37°C for 1 h. After incubation, cells were washed three times with GPBS and suspended in culture medium. One hundred microlitres ^{51}Cr -labelled target cells (1×10^4) were incubated with 100 μl effector cells in different ratios (6:25, 12:5, 25 and 50) in 96-well U-bottomed plates (Costar, Cambridge, MA) at 37°C for 3.5 h. Subsequently, plates were centrifuged and 100 μl supernatant from each well were collected. Radioactivity was determined in a LKB gamma counter. Percentage of specific ^{51}Cr release was calculated using the following formula: Percentage ^{51}Cr release = (experimental ^{51}Cr release – spontaneous ^{51}Cr release) / (total ^{51}Cr release – spontaneous ^{51}Cr release), where the spontaneous ^{51}Cr release represents the release of target cells as such. The total ^{51}Cr release was determined in cell suspensions of target cells treated with 10 μl zap-oglobin (Coulter Electronics Ltd, Luton, UK).

Immunophenotyping of NK effectors

Immunophenotypic expression of the cytotoxic effector cell population was assessed by determination of the cytotoxicity of the MoAb in combination with RC. Cells ($5 \times 10^6/\text{ml}$) were incubated in culture medium with 10 $\mu\text{g}/\text{ml}$ MoAb in the presence of 1:4 v/v RC for 1 h. Subsequently, cells were washed, resuspended in culture medium and incubated in the presence of 500 U/ml IL-2 for 18 h to enhance NK activity. Thereafter cytotoxicity was determined in a ^{51}Cr -release assay as described, using the initial cell count in order to test the original number of cells that were responsible for NK activity.

Statistical analysis

Due to biological variation between different donor samples, percentage of specific ^{51}Cr release was log-transformed in order to equalize the variances of the measurement error at different levels of the percentage of ^{51}Cr release (i.e. to improve homoscedasticity).

To improve the fit of this linear model against the effector:

target ratio (ETR), the regression of the log (^{51}Cr release) versus redoubling of the ETR ($\log_2(\text{ETR}/6.25)$) was studied. The influences of monocytes and GM-CSF in presence or absence of IL-2, separately, on the increase of log (^{51}Cr release) were determined in an analysis of covariance. Therefore the ETR of 12.5 was used as representative value to compare the other respective ETRs. The addition of monocytes and GM-CSF was tested as independent class variables. Using these models the log-transformed ^{51}Cr -release was related to the ETR as follows:

$$\log(^{51}\text{Cr release})_{mg} = e^{\mu_{mg} + \beta_{mg} \log_2(\text{ETR}/6.25) - \beta_{mg}}$$

where ETR ranges from 6.25 to 50, m and g indicate monocyte and GM-CSF, respectively, β indicates the increase of the log (^{51}Cr release) per doubling of the ETR, and μ indicates the log (^{51}Cr release) at ETR of 12.5.

RESULTS

Evaluation of elutriation fractions

Mononuclear cells of five donors were isolated by CCE. The evaluation of the cell number of each fraction (2850–2000 rev/min) and the percentage of T cells and NK cells determined, using flow cytometric immunofluorescence analysis, is shown in Fig. 1. Almost all lymphocytes were recovered in the rotor speed fractions down to 2150 rev/min, whereas most monocytes were eluted thereafter. Fractions that contained >95% monocytes (2100 and 2000 rev/min) were collected and used as

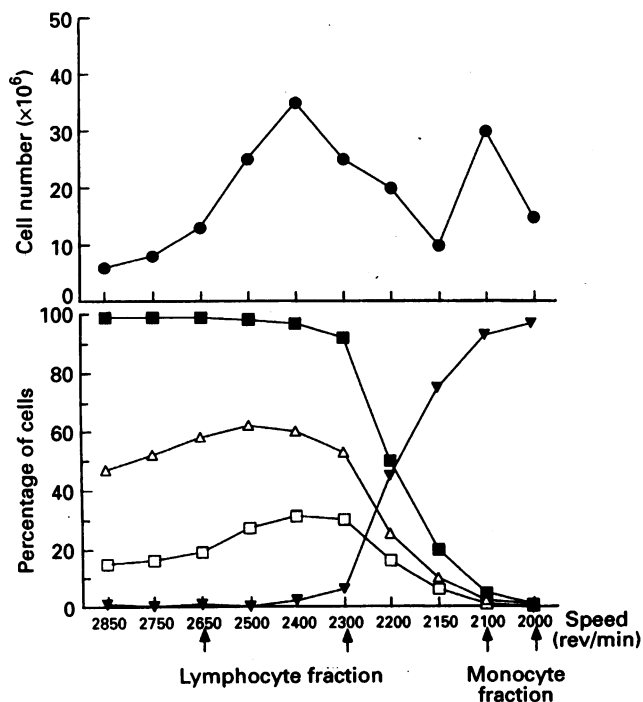


Fig. 1. The absolute number of nucleated cells (\bullet), determined by cell counting, and the percentage of lymphocytes (\blacksquare) and monocytes (\blacktriangledown) by morphological analysis, in the different fractions of counterflow centrifugation elutriation (CCE) of five donors. The lymphocyte subpopulations of T cells (CD3^+ expression; Δ) and NK cells ($\text{CD3}^-\text{CD56}^+$ expression; \square) in each fraction were analysed by flow cytometric immunofluorescence. Fraction 2650 to 2300 rev/min were collected as lymphocyte fractions and fractions 2100 and 2000 rev/min as monocyte fractions.

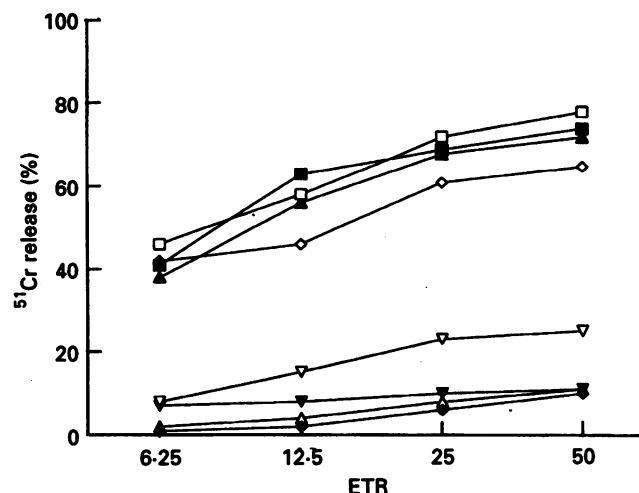


Fig. 2. ^{51}Cr release by the effector cell population after incubation with MoAbs CD3 (\blacktriangle), CD4 (\square), CD8 (\diamond), CD2 (∇), CD7 (\blacktriangledown), CD16 (Δ) and CD56 (\blacklozenge) in combination with rabbit complement (RC) or without any addition (\blacksquare) for 1 h is plotted for different effector:target ratios (ETRs). Cell line K562 was used as target. One out of three representative experiments is shown.

monocyte fraction. Fractions that consisted of >95% lymphocytes (2650–2300 rev/min) were collected. This lymphocyte fraction contained 58% CD3^+ cells and 26% NK cells.

Immunophenotypic analysis of the cytotoxic effector population

The remaining NK activity after elimination of varying subpopulations was determined in a ^{51}Cr -release assay (Fig. 2). Pre-incubation with RC only did not influence NK activity (data not shown). Elimination of cells by CD3, CD4 and CD8 did not influence NK activity. Elimination of CD7, CD16 and CD56 resulted in a strong reduction, whereas CD2 partly inhibited NK activity. These results showed that the effector cells consisted of $\text{CD3}^-\text{CD56}^+$ cells, which also express CD7, CD16 and partly CD2.

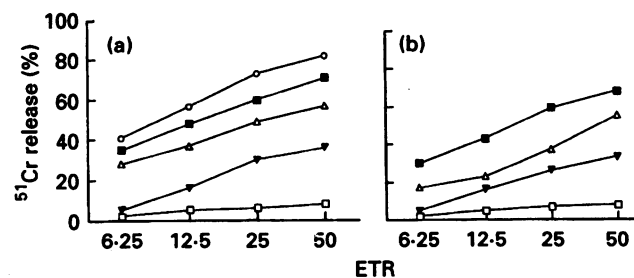


Fig. 3. (a) NK activity was determined in the lymphocyte fraction in the absence (\blacksquare) or presence of equal (\blacktriangledown) or 1:10 (\circ) amounts of monocytes. Inert beads were added to the lymphocyte fraction as control (Δ). NK activity in the added monocyte population was negligible (\square). (b) NK activity in counterflow centrifugation elutriation (CCE) fraction 2200–2150 rev/min (Δ) was compared with the activity of monocyte depletion (\blacksquare) and after adding back monocytes to the remaining lymphocytes (lymphocytes:monocytes = 1:1; \blacktriangledown). Monocytes as such were used as control (\square). Cells were incubated in the presence of 500 U/ml IL-2 to enhance NK activity and determined in a ^{51}Cr -release assay. One out of three representative experiments is shown. ETR, Effector:target ratio.

Monocyte: lymphocyte ratio effect on NK activity

NK activity was determined in lymphocytes from the lymphocyte fraction which are described in Fig. 1. Results of one representative experiment out of three are shown in Fig. 3a. Addition of equivalent amounts of monocytes resulted in a strong inhibition of NK activity to K562. Addition of lower numbers of monocytes (1:10) did not suppress but rather stimulated NK activity. To show that the inhibiting effect was not induced by sterical hindrance by the presence of the high number of cells, equivalent amounts of inert beads of the same size as monocytes were mixed with lymphocytes. NK activity was slightly inhibited. These experiments were also repeated in the presence of four-fold excess of erythrocytes from the lymphocyte donor. No inhibition of NK activity was found (data not shown).

In order to study whether this suppressing effect was artificially induced by adding monocytes, NK activity was determined in CCE fraction 2200–2150 rev/min that contained substantial amounts of monocytes (monocyte:lymphocyte ratio of 1:1) before and after removing the monocytes by adherence to plastic (Fig. 3b). Adherence resulted in a five-fold reduction of monocytes. The remaining fraction showed a significant increase of NK activity which was comparable to the NK activity of lymphocyte fractions without detectable levels of monocytes (Fig. 3a). Addition of equal amounts of

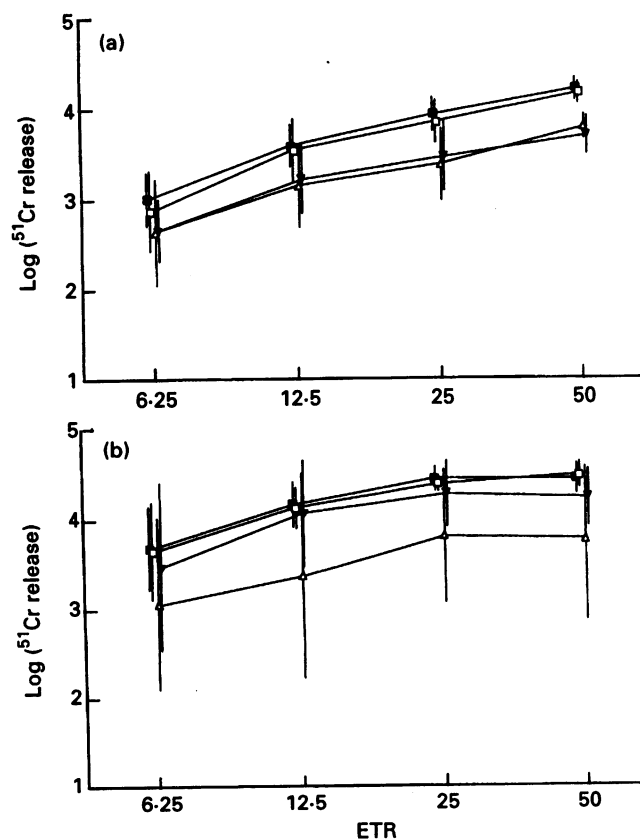


Fig. 4. NK activity was determined in the absence (a) and presence (b) of IL-2 ($n = 5$). Lymphocytes from fractions 2650–2300 rev/min were incubated without GM-CSF (\square, \triangle) or with GM-CSF ($\blacksquare, \blacktriangledown$) in the absence (\square, \blacksquare) or presence ($\triangle, \blacktriangledown$) of equal amounts of monocytes for 18 h. ETR, Effector:target ratio.

Table 1. Results of the linear model of the log-transformed ^{51}Cr release

- IL-2			
Monocytes	GM-CSF	Log ^{51}Cr release* (s.e.m.)	β (s.e.m.)
-	-	3.404 ^a (0.082)	0.451 (0.074)
-	+	3.488 ^a (0.041)	0.408 (0.070)
+	-	3.054 ^b (0.082)	0.373 (0.070)
+	+	3.090 ^b (0.082)	0.348 (0.070)
+ IL-2			
-	-	4.022 ^a (0.144)	0.280 (0.123)
-	+	4.054 ^a (0.144)	0.262 (0.123)
+	-	3.370 ^b (0.144)	0.267 (0.123)
+	+	3.884 ^a (0.144)	0.260 (0.123)

-, +, Without or with addition of monocytes and GM-CSF, respectively.

* Log-transformed percentage of ^{51}Cr release at an effector:target ratio of 12.5.

β , Regression coefficient of the increase of the log-transformed ^{51}Cr release.

^{a,b} Different letter indicates significant difference within the group in the absence or presence of IL-2.

monocytes to the depleted lymphocyte population reintroduced the inhibiting effect.

Influence of GM-CSF on inhibitory effect of monocytes on NK activity

As an extension of the results of the previous experiments we studied the influence of GM-CSF on suppression of NK activity in the lymphocyte fraction in the presence as well as the absence of monocytes and IL-2. The mean and standard error of log-transformed data of five donors for the different ETR levels are depicted in Fig. 4.

Overall analysis of NK activity showed that the effect of GM-CSF was significantly different in the presence and absence of IL-2. Therefore the influence of GM-CSF on log (^{51}Cr release) under different conditions of monocytes was studied with or without IL-2 separately. Changes in ETR affected log-transformed values less in the presence than in the absence of IL-2 which was expressed by lower regression coefficients (β s; Table 1). The differences in these regression coefficients were not statistically significant in the presence or absence of IL-2, indicating that increase in NK activity with increasing ETR is comparable for each combination of additions.

Addition of monocytes with or without IL-2 inhibited NK activity significantly ($P < 0.05$) irrespective of the ETR (Fig. 4). Addition of GM-CSF in the absence of monocytes did not significantly influence NK activity. Without IL-2 the inhibiting effect of NK activity by monocytes was also not affected. However, in the presence of IL-2, GM-CSF abrogated the inhibiting effect of monocytes significantly ($P < 0.05$), resulting in a NK activity level as achieved with the lymphocyte fraction alone. Only one donor did not completely restore this suppressive activity. Purified monocytes showed $< 10\%$ NK activity in all combinations (data not shown).

DISCUSSION

Treatment of bone marrow allograft recipients with GM-CSF has been shown to shorten the neutropenic phase, resulting in a lower incidence of infections after BMT [3]. Moreover, GM-CSF appeared to exert a graft *versus* leukaemia activity (GVL), since the relapse rate was lower in GM-CSF-treated recipients [12]. The mechanism behind this enhancement of GVL activity is at present not completely understood.

In allograft recipients GM-CSF induces a strong increase in the number of monocytes which may possess anti-leukaemic activity [20]. Previous studies have shown the influence of GM-CSF in regulating monocyte activity against tumour cell lines [21] and against leukaemic target cells [22]. Moreover, *in vitro* experiments revealed that GM-CSF enhances ADCC by granulocytes [8], monocytes [7,9,10] and lymphocytes [33,35]. Although T lymphocytes appeared not to express receptors for GM-CSF, some studies suggested that GM-CSF also potentiates proliferation of lymphocytes upon IL-2 stimulation [34] and their cytotoxic activity [35].

Okamura *et al.* [13] showed that NK activity was significantly enhanced by GM-CSF in the presence of IL-2. This enhancement could be an indirect effect by interferon-gamma (IFN- γ), which was produced as a result of monocyte stimulation, that activates the cytotoxic activity of NK cells.

In the present study we examined *in vitro* the influence of GM-CSF on monocytes and NK cells with regard to the induction of NK activity. NK cells are tumoricidal, and their cancer-related cytotoxic potential is augmented after activation with IL-2 [23,24]. Monocytes are able to down-regulate these IL-2-stimulated cells, depending on their functional state [25,26]. Monocytes were obtained by Percoll gradient centrifugation followed by CCE [19]. This method allows one to obtain highly purified monocytes that are not stimulated by the separation technique as such. These monocytes did not show cytotoxicity in the added concentrations against the NK-sensitive cell line K562, even in the presence of GM-CSF. This may be due to the resting stage of these monocytes, in contrast to monocytes obtained by the usually applied Ficoll gradient centrifugation and adherence to plastic.

Lymphocyte fractions of CCE were used as the source of NK cells. Characterization of these fractions showed that the cells responsible for NK activity expressed CD2, CD7, CD56, and were CD3⁻. We found that addition of high amounts of monocytes to lymphocytes inhibited NK activity in the presence and absence of IL-2. Addition of comparable amounts of inert beads showed only a slight inhibition. Depletion of T cells from the lymphocyte population did not decrease NK activity. These results suggested that the inhibiting effect was induced by a direct functional effect of monocytes, in the used concentration.

This inhibition by monocytes was observed previously by other groups [27,28] using lower amounts of monocytes. However, these monocyte populations were stimulated with lipopolysaccharide (LPS) before addition to the target population. The discrepancy with our findings can be explained by the high amounts of monocytes we used in our experiments. These monocyte fractions are expected to contain a small number of cells already stimulated *in vivo* before isolation. In our hands small numbers of monocytes did not inhibit but rather stimulated NK activity. Although present data are controversial,

prostaglandin E₂ (PGE₂) appears to be involved in the inhibiting effect of monocytes [29–31], and the functional balance between IL-1 [31] and PGE₂ determines whether suppression or augmentation of NK activity is involved.

GM-CSF hardly influenced NK activity both in the presence and absence of monocytes. However, addition of GM-CSF in the presence of IL-2 abrogated completely the suppressive effect of monocytes on NK activity in almost all donors. In one donor this abrogation appeared to be partial. This may be explained by a higher number of monocytes that could be stimulated *in vivo* before isolation. These results suggest that the anti-leukaemic activity in BMT recipients who are treated with GM-CSF may be enhanced by the abrogation of the suppressive activity of monocytes rather than by direct stimulation of the monocytes.

In conclusion, GM-CSF does not directly influence NK cells, but indirectly affects NK activity by reversing the suppressive activity of monocytes on NK activity against (or killing of) the NK-sensitive cell line K562.

These data suggest that NK activity and anti-leukaemic effects may be impaired *in vivo* by the recovery of monocytes following BMT. Preliminary data in an ongoing clinical trial demonstrated that patients treated with GM-CSF for 3 months showed no significant difference in monocyte:lymphocyte ratios [32]. These ratios were higher than 1 up to 2 months after BMT. Thus, it may be speculated that GM-CSF, in addition to accelerating monocyte recovery, potentially enhancing cellular cytotoxicity and cytokine release, restores NK activity and hence improves the outcome after BMT. A large clinical trial is required, however, to prove this hypothesis. The precise mechanism by which GM-CSF affects NK activity remains to be elucidated.

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