Differential Cytokine Effects on Primitive ($CD34^+CD38^-$) Human Hematopoietic Cells: Novel Responses to Flt3-Ligand and Thrombopoietin

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Summary

A high proportion of the $CD34^+CD38^-$ cells in normal human marrow are defined as long-term culture–initiating cells (LTC-IC) because they can proliferate and differentiate when co-cultured with cytokine-producing stromal feeder layers. In contrast, very few $CD34^+CD38^-$ cells will divide in cytokine-containing methylcellulose and thus are not classifiable as direct colony-forming cells (CFC), although most can proliferate in serum-free liquid cultures containing certain soluble cytokines. Analysis of the effects of 16 cytokines on $CD34^+CD38^-$ cells in the latter type of culture showed that Flt3-ligand (FL), Steel factor (SF), and interleukin (IL)-3 were both necessary and sufficient to obtain an ~30-fold amplification of the input LTC-IC population within 10 d. As single factors, only FL and thrombopoietin (TPO) stimulated a net increase in LTC-IC within 10 d. Interestingly, a significantly increased proportion of the CFC produced from the TPO-amplified LTC-IC were erythroid. Increases in the number of directly detectable CFC of >500-fold were also obtainable within 10 d in serum-free cultures of $CD34^+CD38^-$ cells. However, this required the presence of IL-6 and/or granulocyte/colony-stimulating factor and/or nerve growth factor β in addition to FL, SF, and IL-3. Also, for this response, the most potent single-acting factor tested was IL-3, not FL. Identification of cytokine combinations that differentially stimulate primitive human hematopoietic cell self-renewal and lineage determination should facilitate analysis of the intracellular pathways that regulate these decisions as well as the development of improved ex vivo expansion and gene transfer protocols.

The most primitive cells of the hematopoietic system are characterized by an ability to generate very large numbers of all known mature blood cell types. For example, in humans, multilineage clonal blood cell populations of $>10^{13}$ cells derived from putatively normal stem cells have been reported (1, 2). However, variability in the number of divisions that may precede terminal hematopoietic differentiation has been noted (3), and evidence of cells with extensive in vivo repopulating ability but at least partially restricted differentiation potential has been reported (4, 5). Such findings have focused attention on the identification of other potentially unique functional properties of human hematopoietic stem cells with self-sustaining multilineage repopulating ability that might serve as surrogate markers for the specific quantitation of these cells. One approach has relied on the recognized ability of stromal cells to support the production in vitro for several weeks of clonogenic cells (colony-forming cells [CFC][1]) from a very small and phenotypically distinct subpopulation of hematopoietic cells that, in the mouse, have not been separable from cells with long-term in vivo repopulating ability (6–8). In humans, a subset of very primitive hematopoietic cells has also been identified by virtue of its similar ability to generate CFC progeny for at least 5 wk on suitable fibroblast-containing feeder layers of human or murine origin (9, 10). During this process, some of the input long-term culture–initiating cells (LTC-IC) thus detected also undergo self-renewal divisions (11), although this does not result in a net amplification of their numbers (12).

The first two authors contributed equally to this work.

[1] *Abbreviations used in this paper:* BFU, burst-forming unit; CFC, colony-forming cell; Ep, erythropoietin; FL, Flt3-ligand; GEMM, granulocyte-erythroid-megakaryocyte-monocyte; HC, hydrocortisone; HFN, Hank's Hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide; HLTM, long-term culture medium; LTC-IC, long-term culture–initiating cells; NGF-β, nerve growth factor β; SF, Steel factor; TPO, thrombopoietin.

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Recently we (11, 13) and others (14, 15) have shown that a significant expansion of input LTC-IC populations can be obtained in vitro when various cytokine supplements are added and other alterations to the culture conditions are made. For example, in cultures initiated with single $CD34^+CD38^-$ cells isolated from normal adult human marrow, an amplification of LTC-IC of 30-fold was seen within 10 d with evidence of a further 2-fold expansion of this primitive progenitor population after an additional 2–3 wk (11). In these cultures, the cells were maintained in the absence of stroma in a serum-free medium containing 100 ng/ml each of Flt3-ligand (FL) and Steel factor (SF), 20 ng/ml each of IL-3, IL-6, and G-CSF, and 5 ng/ml of nerve growth factor β (NGF-β). This combination of cytokines was originally chosen on the basis of previous evidence that each component included might contribute to the proliferation or maintenance of various types of primitive hematopoietic cells (16–19) or was produced by fibroblast feeders that had these properties (20, and Coulombel, L., personal communication). The purpose of the studies described here was to identify the specific factors or combination(s) of factors that can stimulate an expansion of LTC-IC in serum-free cultures initiated with $CD34^+CD38^-$ cells. In addition, experiments were undertaken to determine how these factor requirements might compare with those needed to maximize CFC production from the same starting population.

Materials and Methods

Purification of Bone Marrow Cells. Light-density cells (<1.077 g/cm^3) were isolated from previously frozen human bone marrow obtained from cadaveric organ donors using Ficoll-Paque (Pharmacia, Uppsala, Sweden). After washing these cells once in PBS containing 2% FCS (StemCell Technologies, Vancouver, BC, Canada), we resuspended them in Hank's Hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN). The cells were then incubated simultaneously at $\leq 10^7$ cells/ml for 30 min at 4°C with mAbs specific for CD34 (8G12-fluorescein) and CD38 (Leu 17-PE) at 10 and 2.5 μg/ml, respectively. Cells were then washed once in HFN and once in HFN containing 2 μg/ml propidium iodide (PI, p-5264; Sigma Chemical Co., St. Louis, MO) before resuspension in Hank's Hepes-buffered salt solution containing 2% FCS for isolation of the $CD34^+CD38^-$ population. Throughout this staining procedure, the cells were kept at 4°C. Cells were sorted on a FACStar Plus® (Becton Dickinson & Co., San Jose, CA) equipped with a 5-W argon laser and a 30-mW helium laser. Viable (PI^-) cells with low-medium forward scattering, low side scattering characteristics, and a $CD34^+$ $CD38^-$ phenotype were collected in Eppendorf tubes containing serum-free Iscove's medium. Positive and negative staining with antibodies specific for CD34 and CD38, respectively, was defined as the emission of a level of fluorescence that exceeded (CD34) or did not exceed (CD38) levels obtained by 99% of the cells from the same starting population when these were stained with control IgG1 antibodies labeled with the corresponding fluorochrome.

Colony Assays. Cell suspensions to be assayed for erythropoietic (CFU-E and burst-forming unit [BFU-E]), granulopoietic (CFU-GM), and multilineage (CFU–granulocyte-erythroid-megakaryocyte-monocyte [GEMM]) CFC were plated at suitable concentrations (to give <100 colonies per 1 ml culture) in Iscove's medium containing 0.9% methylcellulose, 30% FCS, 1% BSA, 10^{-4} M 2-ME (Methocult H4430; StemCell Technologies) supplemented with 3 U/ml of highly purified human erythropoietin (Ep; StemCell Technologies), 50 ng/ml of SF, and 20 ng/ml each of IL-3 (Sandoz International, Basel, Switzerland), IL-6, G-CSF (Amgen, Thousand Oaks, CA), and GM-CSF (Sandoz International). SF and IL-6 were purified from the supernatants of COS cells transiently transfected in the Terry Fox Laboratory with corresponding human cDNAs. Methylcellulose cultures were aliquotted in 1.1-ml volumes in 35-mm petri dishes and were then incubated at 37°C for 2–3 wk. At the end of this time, all colonies present were scored according to standard criteria.

LTC-IC Assays. For enumeration of LTC-IC, cells to be tested were placed in 35-mm tissue culture dishes that already contained a feeder layer of irradiated (8,000 cGy) mouse fibroblasts engineered to express 4 ng/ml of human IL-3, 190 ng/ml of human G-CSF, and 4 ng/ml of human SF (21), plus 2.5 ml of human long-term culture medium (HLTM). HLTM contains 12.5% horse serum, 12.5% FCS, and 10^{-4} M 2-ME (Myelocult; StemCell Technologies), and 10^{-6} M hydrocortisone sodium hemisuccinate (HC; Sigma Chemical Co.) added from a freshly prepared solution just before use. The cultures were then incubated for 6 wk at 37°C with weekly replacement of half of the medium and removal of half of the nonadherent cells (21). After 6 wk, all of the remaining nonadherent cells from each assay culture were removed and added to the corresponding trypsinized adherent cells; the combined pool was then washed and assayed for CFC as described above. The counts obtained were used to calculate the total yield of CFC (BFU-E plus CFU-GM plus CFU-GEMM) at the end of 6 wk from each input inoculum tested as this value provides a relative, but nevertheless, quantitative measure of the number of LTC-IC initially present (9).

Stroma-free Liquid Suspension Cultures. For most experiments, $CD34^+CD38^-$ cells were incubated in 100 μl of Iscove's medium supplemented with 20 ng/ml of BSA, 10 μg/ml of human insulin, and 200 μg/ml of human transferrin (BSA insulin transferrin; StemCell Technologies) plus 40 μg/ml low density lipoproteins (LDL; Sigma Chemical Co.), 10^{-4} M 2-ME, and various growth factors plus or minus 10^{-6} M HC (as indicated) in round-bottomed wells of 96-well plates as previously described (11). In one case (see Fig. 4) HLTM was used instead of the BSA insulin transferrin plus LDL serum substitute. The growth factors tested, either alone or in various combinations, were: FL and IL-7 (Immunex Corp, Seattle, WA); SF and G-CSF (Amgen); IL-1 (Biogen, Cambridge, MA), IL-3 and GM-CSF (Sandoz International); IL-11 and IL-12 (Genetics Institute, Cambridge, MA); IL-6, macrophage inflammatory protein 1α (MIP-1α), and NGF-β (R&D Systems, Inc., Minneapolis, MN); leukemia inhibitory factor (LIF) and Ep (StemCell Technologies); TNF-α (Genentech, San Francisco, CA); and thrombopoietin (TPO; ZymoGenetics, Seattle, WA). The suspension cultures were incubated unperturbed for 10 d at 37°C (except as indicated to the contrary), and then all cells in each type of culture were harvested and assayed separately for LTC-IC and/or CFC.

Factorial Design Analysis Experiments. A 2^5 factorial analysis design was used to investigate the relative roles of six growth factors (two of which, NGF-β and G-CSF, were considered as a fixed pair) in stimulating LTC-IC amplification. Each of the 32 possible combinations of growth factors was tested in duplicate (each with a different source of $CD34^+CD38^-$ cells). The actual testing of each of these sets was broken into two subexperiments that were performed on different occasions (16 combinations each time) with the same cells. Each of these subexperiments also included

four replicate cultures containing all six factors at half the concentrations used in the other combinations. The LTC-IC expansion obtained with each growth factor or combination of growth factors in individual subexperiments was normalized as follows. In each subexperiment, the difference between the logarithm (log) of each LTC-IC expansion measured and the mean of all the log LTC-IC expansions was first determined. This difference was then divided by the standard deviation of all the log LTC-IC expansions. This transformation allowed all the results from the four subexperiments to be analyzed as a single data set, using the Jass Software program (version 2.0; Joiner Associates, Madison, WI).

Results

Multifactorial Analysis of the Relative Contributions of Six Growth Factors on LTC-IC Amplification. In a first series of experiments with four separately isolated $CD34^+CD38^-$ cell populations (obtained from two marrow sources), aliquots of 200 $CD34^+CD38^-$ cells were incubated for 10 d in 100-μl liquid suspension cultures containing different combinations of six growth factors. LTC-IC assays were performed both on the initial $CD34^+CD38^-$ cells and on the cells harvested from the 10-d-old cultures to determine the extent of the expansion obtained with each condition. The six factors evaluated in these experiments were FL (100 ng/ml), SF (100 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), and G-CSF (20 ng/ml) plus NGF-β (5 ng/ml), since previous experiments had shown that a cocktail of all of these factors would stimulate a marked amplification of LTC-IC in cultures of single $CD34^+CD38^-$ cells (11). G-CSF and NGF-β were evaluated together because initial results of our own (data not shown) and others (Coulombel, L., personal communication) had already suggested that NGF-β would not stimulate the proliferation of $CD34^+CD38^-$ cells; this strategy also considerably reduced the magnitude of the experiments.

A measure of the LTC-IC content of each of the four input $CD34^+CD38^-$ cell suspensions as well as of the cells harvested from each of the 80 different 10-d-old liquid suspension cultures (16 different combinations of growth factors plus the four replicates in each of four subexperiments) was obtained by assessing the total CFC output for a fixed number of original $CD34^+CD38^-$ cells. The validity of this approach (rather than the use of limiting dilution analyses for quantitating LTC-IC in each suspension) is based on previous data showing that the numbers and types of CFC generated from individual LTC-IC, although highly variable, remain on average the same when freshly isolated and cultured LTC-IC are compared (10, 11). More recently, these relationships have been confirmed by formal limiting dilution analysis experiments (our manuscript in preparation), although as noted below, one circumstance where at least the type of CFC produced can be altered was also discovered in the present studies. However, this change appeared to be uniquely related to the exposure of LTC-IC to a single factor. The extent of LTC-IC amplification when all six growth factors were present was 24 ± 5-fold (n = 7, Fig. 1). This magnitude of LTC-IC expansion is

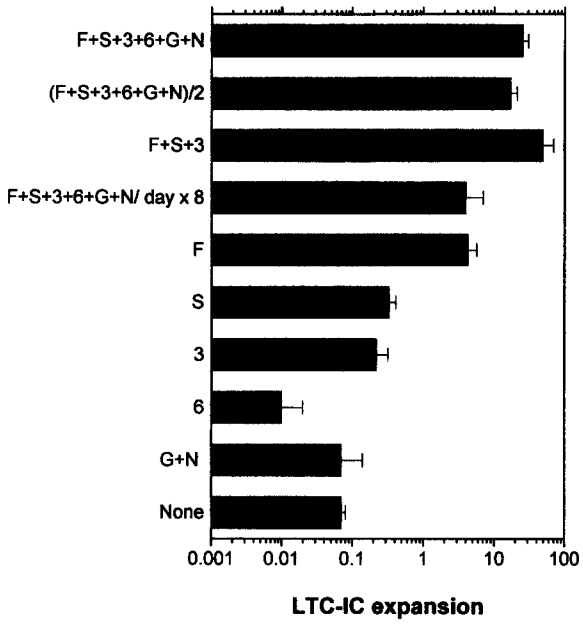

Figure 1. Variable LTC-IC expansion from $CD34^+CD38^-$ cells incubated for 10 d in serum-free cultures containing different cytokine combinations as shown. Flt3-ligand (*F*), Steel factor (*S*), IL-3 (*3*), IL-6 (*6*), G-CSF (*G*), and NGF-β (*N*). 168 ± 48 CFC per 100 input cells were generated in the 6-wk LTC-IC assays of the original $CD34^+CD38^-$ cells used to initiate the serum-free expansion cultures. (*Bars*) Mean ± SEM of the increases measured relative to the starting LTC-IC–derived CFC output value of the same experiment for data pooled from two to seven experiments.

similar to that measured previously when single $CD34^+CD38^-$ cells were cultured in the same cocktail (11), suggesting that the extent of LTC-IC amplification obtained is relatively independent of the initial concentration of $CD34^+CD38^-$ cells up to at least 200 per 100 μl (i.e., 2×10^3 $CD34^+CD38^-$ cells per ml).

The ability of the six factors tested (four individually and G-CSF and NGF-β as a pair) to support the amplification of LTC-IC was then evaluated by multiparameter analysis of variance of the normalized data from all four experiments. Such an analysis identifies individual or interactive effects as significant when the result of any particular factor or combination of factors is not explained by the variation accounted for by the normal probability distribution resulting from the combined data from all groups (22). A comparison of the standard deviation of the four sets of replicate center points (i.e., data from cultures containing all factors at half the concentration used in all other groups) established that the interexperimental variability was not significant (block effect in Fig. 2). Fig. 2 shows that the results of all of the possible combinations of growth factors tested, with the exception of FL, SF, and IL-3 alone, lie within the variance of the normal probability distribution. An indication of the unique and relative activities of these three factors to support the amplification of LTC-IC from $CD34^+CD38^-$ cells can also be seen in Fig. 1. FL alone was able to increase the number of LTC-IC significantly ($P < 0.05$) above the input value. SF and IL-3, alone, each maintained

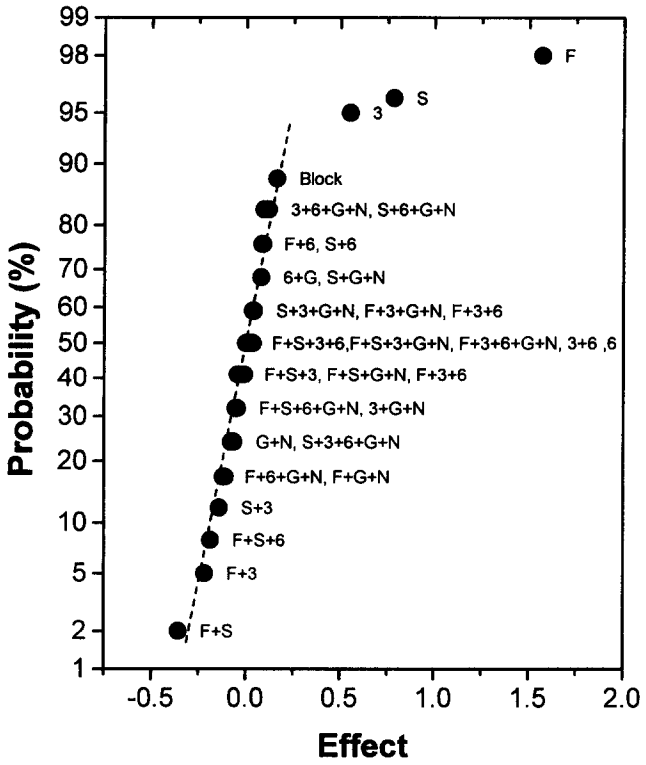

Figure 2. Linearized normal probability distribution of the normalized effect of different combinations of cytokines on LTC-IC expansion in 10-d serum-free cultures of $CD34^+CD38^-$ cells. Significant effects do not fall on the dashed line representing the normal probability distribution. In this plot, an effect is defined as the average change in LTC-IC expansion that occurred in cultures containing the growth factor(s) shown relative to cultures not containing these factor(s). Abbreviations are as indicated in the legend to Fig. 1.

LTC-IC numbers at a significantly higher level ($P < 0.05$) than was seen when no factors were added, although neither SF nor IL-3 alone was able to stimulate an overall net increase in LTC-IC numbers. Interestingly, even in a completely defined medium that contained insulin but no other growth factors, the rate of LTC-IC decline was relatively slow. Accordingly, under these conditions many LTC-IC (7 ± 1% of input values) could still be detected after 10 d (Fig. 1). This is similar to results obtained previously for a similar starting population of $CD34^+HLA\text{-}DR^\pm$ cells incubated without growth factors but in the presence of serum (18). Also evident in both Figs. 1 and 2 is the finding that the minimum combination of FL plus SF plus IL-3 was sufficient to obtain the greatest LTC-IC amplification observed for any combination of the six factors tested. Moreover, there were no significant effects attributable to interactions between multiple factors (Fig. 2) (22). When the concentration of each of the six growth factors in the cocktail was reduced twofold, the extent of LTC-IC amplification obtained was also reduced (by 25%, Fig. 1). On the other hand, repeated daily addition of all six growth factors (in the same amounts as when added only at the beginning of the culture at full strength) resulted in a decrease in LTC-IC yields (by 84%, Fig. 1). Taken together, these results provide some indication of the existence of a dose–effect relationship for the stimulation of LTC-IC amplification by the combination of FL, SF, and IL-3.

Effects of Different Growth Factors on CFC Production by $CD34^+CD38^-$ Cells. In earlier experiments, we showed that LTC-IC maintenance in serum-containing cultures was not affected by several variations in the growth factor composition of the medium that, nevertheless, had marked effects on the number of CFC detectable after 5 wk (18). It was, therefore, of interest to determine whether LTC-IC amplification and the production of CFC from $CD34^+$ $CD38^-$ cells would show a similar or different growth factor dependence. The CFC outputs measured for the various growth factor combinations tested in at least three experiments are shown in Fig. 3. As noted previously (11, 23), the frequency of CFC in the initial $CD34^+CD38^-$ populations was low (7 ± 3%). Of the single factors tested, IL-3 was the most potent stimulator of CFC production over the 10-d period (~10-fold increase), and neither FL nor SF alone supported a net expansion of the CFC population. On the other hand, when combined, these three factors stimulated an ~80-fold increase in CFC numbers, and this was further increased (another four- to eightfold) when IL-6, G-CSF, NGF-β, or all three of these factors were also present.

Ability of Other Factors to Influence LTC-IC Amplification. A final set of experiments was undertaken to determine whether any of a number of other cytokines previously shown to have direct effects on primitive hematopoietic cells could further enhance LTC-IC yields from $CD34^+$

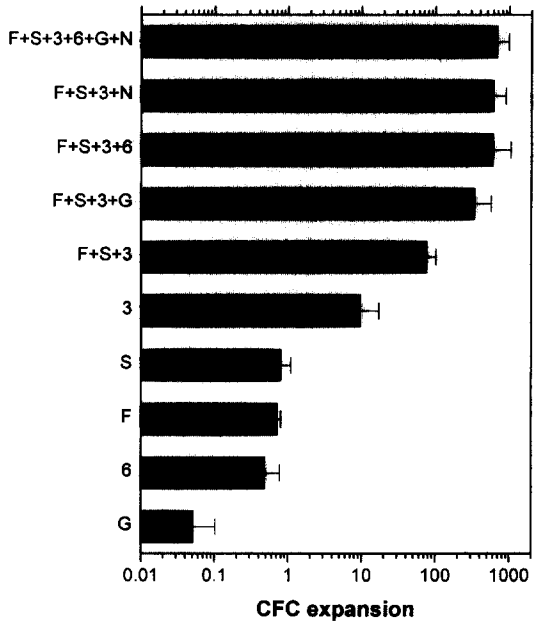

Figure 3. Variable CFC expansion from $CD34^+CD38^-$ cells incubated for 10 d in serum-free cultures containing different cytokines as shown. 7 ± 3 colonies per 100 $CD34^+CD38^-$ cells were generated in CFC assays performed on the original cells used to initiate the serum-free expansion cultures. (*Bars*) Mean ± SEM of the increases measured relative to the starting CFC value in the same experiment ($n \geq 3$).

$CD38^-$ cells cultured under serum-free conditions in the absence of stroma. In addition, the effects of HC and HLTM (a medium containing both horse and fetal calf serum) on LTC-IC amplification were examined. Each condition was tested either alone or in combination with the original six-factor cocktail analyzed above. The results of these experiments are shown in Fig. 4. Of the 10 additional cytokines tested, only TPO and IL-1 on their own (at 40 and 1,700 U/ml, respectively) were able to significantly increase ($P < 0.05$) the number of LTC-IC detectable after 10 d in comparison to the numbers measured in the absence of any factor supplement. Moreover, aside from FL (Fig. 1), TPO was also the only factor that, on its own, appeared able to stimulate LTC-IC numbers to increase above the input value ($P < 0.18$; see also Table 1). In addition, none of these 10 cytokines (including TPO), when added as a supplement (at the same concentration) to the original six-factor combination, was able to further enhance the LTC-IC expansion already stimulated (Fig. 4). Many, (e.g., IL-12 at 50 ng/ml, IL-7 at 100 ng/ml, MIP-1α at 100 ng/ml, HC at 10^{-6} M, and TPO at 40 U/ml) in fact, appeared to be neutral in terms of having no modifying effect on LTC-IC output (like IL-6, G-CSF, and NGF-β). Others clearly had a significant negative effect (e.g., TNF-α at 500 U/ml, $P < 0.005$; IL-11 at 100 ng/ml, $P < 0.05$; LIF at 100 ng/ml, $P < 0.05$, and Ep at 3 U/ml, $P < 0.10$). TNF-α in combination with the six-factor combination was particularly potent in this regard, resulting in a decrease in LTC-IC numbers after 10 d to below both the input value and the number of LTC-IC detectable in cultures to which TNF-α alone was added. The dramatic decline in LTC-IC numbers that occurred when TNF-α was present together with the six-factor cocktail was not, however, associated with a general loss of $CD34^+CD38^-$ cell viability. In fact, these conditions stimulated a large amplification (~350-fold) in the total number of cells present after 10 d, which was also accompanied by the generation of a large population of progenitors of small macrophage colonies (data not shown).

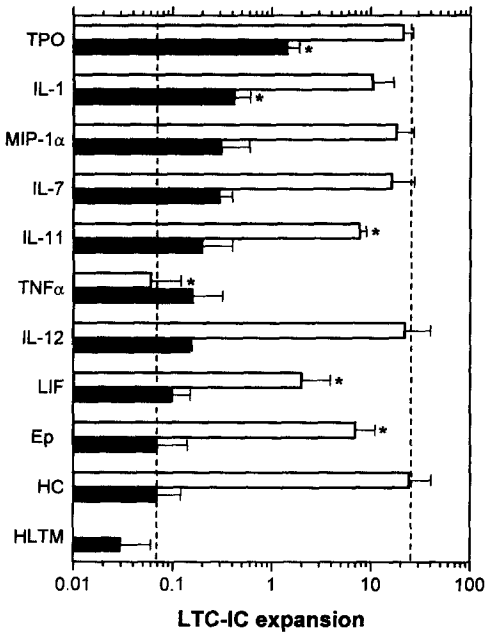

Figure 4. Effect of 10 specific cytokines and HC on LTC-IC expansion from $CD34^+CD38^-$ cells incubated for 10 d in serum-free cultures either alone (*solid bars*) or in combination with the six growth factor cocktail analyzed in Fig. 1 (*open bars*). The effect of culture in HLTM alone was also tested. (*Dotted lines*) Fold change in numbers of LTC-IC detectable in the absence of factor addition or in the presence of the six-factor combination alone (as indicated in Fig. 1). (*) Significant differences versus no growth factor addition (*TPO* and *IL-1*, $P < 0.05$) or versus the six-factor combination (*TNF-α*, $P < 0.005$; *LIF* and *IL-11*, $P < 0.05$; *Ep*, $P < 0.10$) as determined by a two-tailed *t* test. (*Bars*) Mean ± SEM for data pooled from two to seven experiments.

On evaluating the LTC-IC produced in cultures that contained TPO alone or TPO plus the six-growth factor cocktail, a change in the type of CFC present in the 6-wk-old LTC-IC assays became apparent. In both of these instances, a significantly higher proportion of the progeny CFC were erythroid progenitors ($P < 0.05$) in comparison to those generated either from the initial ($CD34^+CD38^-$) LTC-IC, or from the LTC-IC produced in parallel cultures containing the six-factor cocktail but no TPO (Fig. 5). It is particularly interesting to note that although this effect of TPO was most pronounced for the LTC-IC produced in the presence of TPO alone, a significant shift in favor of erythropoietic progenitors was also evident in the assays of LTC-IC obtained from cultures where TPO was added to the six-factor cocktail, in which case there was no apparent effect of the TPO on LTC-IC amplification. To examine whether an alteration of LTC-IC differentiation behavior had occurred in cultures containing any of the other cytokines tested, the ratios of CFU-GM to BFU-E to CFU-GEMM from all LTC-IC assays were compared. As is illustrated by the examples shown in Table 1, these values were not found to be altered by any other factor or factor combination tested.

Discussion

This study presents a comprehensive analysis of the effects of various cytokines on the generation within a 10-d period of two functionally distinguished but closely related populations of primitive human hematopoietic cells. To minimize potentially confounding effects of serum or accessory cells (or their products), the cultures were initiated at a low cell density with a highly purified population of $CD34^+CD38^-$ cells isolated from normal marrow and incubated in a completely defined medium. The results (summarized in Table 1) show that the number of cells detectable as LTC-IC could be increased in 10-d cultures that contained only FL or TPO, indicating unique roles for these factors either in triggering the activation of properties required for cells to be detected as LTC-IC (within 6 wk) or for their self-renewal, as has been recently confirmed for FL by LTC-IC [^{3}H]thymidine suicide experiments (24). To our knowledge, the action of TPO on LTC-IC amplification has not been reported previously. It has been re-

Table 1. *Effects of Selected Culture Conditions on the LTC-IC and CFC Detected in 10-d-old Serum-free Cultures Initiated with 200 $CD34^+CD38^-$ Cells*

Cytokine(s)	LTC-IC expansion		LTC-IC–derived CFC GM/B/GEMM	CFC expansion	
TPO	1.3 ± 0.4	(5)*	86:12:2	ND	
FL	4.3 ± 1.4	(3)	100:0:0	0.7 ± 0.1	(4)
SF	0.3 ± 0.1	(2)	100:0:0	0.8 ± 0.3	(4)
IL-3	0.2 ± 0.1	(2)	99:1:0	9.6 ± 2.5	(4)
IL-6	0.01 ± 0.01	(2)	98:2:0	0.5 ± 0.3	(4)
G-CSF/NGF-β	0.07 ± 0.07	(2)	100:0:0	0.05 ± 0.05‡	(3)
None	0.07 ± 0.01	(6)	100:0:0	ND	
FL/SF/IL-3/IL-6/ G-CSF/NGF-β	24 ± 5	(7)	97:2:1	670 ± 300	(4)
FL/SF/IL-3	49 ± 20	(2)	97:3:0	76 ± 25	(3)
Input $CD34^+CD38^-$ cells§		(7)	95:3:2		

*Values shown represent the mean ± SEM (No. of experiments).
‡CFC expansion determined for G-CSF alone; i.e., without the addition of NGF-β.
§168 ± 48 CFC were measured in the 6-wk harvest of the LTC-IC assays from 100 input $CD34^+CD38^-$ cells. The same input cells contained 7 ± 3 CFC per 100 cells.

ported that FL in combination with SF, Pixy-123, IL-3, GM-CSF, and Ep can enhance the plating efficiency of high-proliferative potential CFC (25–27); a possible role of FL in LTC-IC stimulation by marrow feeder layers has been inferred from the results of antisense Flt3 experiments (28) and experiments in which FL has been added repeatedly to LTC-IC assay cultures (29). More recently, we reported that FL in combination with SF, IL-3, G-CSF, and NGF-β could amplify LTC-IC in cultures of single $CD34^+CD38^-$ cells, although the specific role of FL in eliciting this response was not examined in those studies (11).

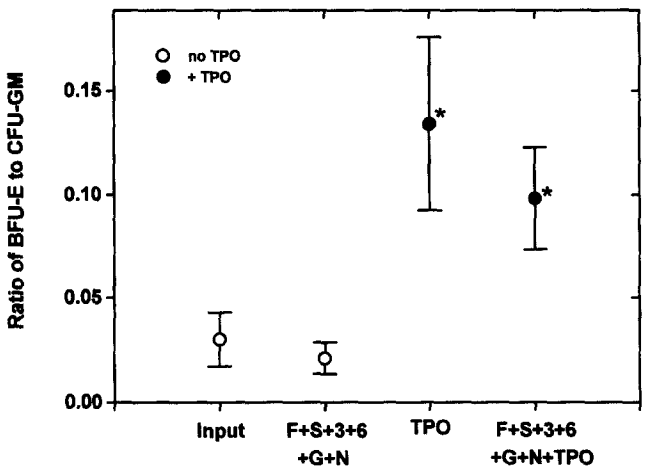

Figure 5. Effect of TPO alone or together with a six-growth factor cocktail on the relative proportions (*ratio*) of primitive erythroid (*BFU-E*) and granulopoietic progenitors (*CFU-GM*) produced after 6 wk from the LTC-IC generated in 10-d cultures containing these factors. Abbreviations are as indicated in the legend to Fig. 1. Values shown are the mean ± SEM of ratios calculated from five separate experiments. (*) Significant difference ($P < 0.05$) relative to the ratios measured both for the starting LTC-IC and the LTC-IC present after 10 d in cultures containing the six-factor cocktail only.

The results of the multifactorial analysis studies presented here show that within 10 d, FL alone can stimulate a significant net increase in the number of LTC-IC present in cultures initiated with $CD34^+CD38^-$ cells, and that the addition of SF and IL-3 to FL further enhances this increase. The multifactorial analysis also showed that there were no other significant interactions between any of the six factors tested in terms of their effects on LTC-IC amplification. In contrast, IL-3 was the only factor that alone was able to expand the CFC population. Nevertheless, the combination of FL, SF, and IL-3 showed evidence of strong synergy in stimulating the production of CFC; this could be further enhanced by the addition of factors like G-CSF or IL-6 that had no effect on LTC-IC amplification. These latter findings are consistent with those reported for the effects of FL on primitive murine lymphomyeloid, myeloid, and lymphoid CFC (30). Taken together, these results extend previous evidence of differences in the mechanisms that allow LTC-IC function to be maintained (with or without cell division) as compared with those that may stimulate loss of this function by the acquisition of properties that allow direct colony formation in semi-solid medium (18).

Of the additional factors tested in combination with FL, SF, IL-3, IL-6, G-CSF, and NGF-β, the negative effect of TNF-α on LTC-IC numbers was the most striking, particularly in view of the rapid cell proliferation and concomitant acquisition of restricted macrophage differentia-

tion potential observed. This latter response could reflect the selective stimulation of a previously undefined unique cell type also present in the $CD34^+CD38^-$ population, as has been suggested for less purified populations responding to SF, GM-CSF, and TNF-α (31). Alternatively, TNF-α may exert a direct deterministic action on $CD34^+CD38^-$ LTC-IC (or CFC) when these are simultaneously stimulated by certain other growth factors. Single cell experiments will be required to resolve between these possibilities. Additionally, the observation that LTC-IC generated in the presence of TPO subsequently showed a uniquely increased production of erythropoietic progenitors was also unanticipated. Occasional examples of apparently deterministic effects on lineage restriction or switching have been previously reported in various immortalized hematopoietic cell lines (32–34); however, the ability of such lines to yield further information about mechanisms of normal hematopoietic differentiation processes are questionable because of their transformed state. The apparent ability of TPO to reprogram the differentiation behavior of primary LTC-IC, as suggested by the present findings, would circumvent this type of criticism. This experimental model may therefore offer new opportunities for future studies of how commitment events are normally regulated.

Characterization of culture conditions that allow human hematopoietic stem cell expansion is an important requirement for the successful implementation of many clinical transplantation and gene therapy protocols currently under development. The present studies may represent a significant step towards the practical realization of such approaches by beginning to define the growth factor conditions that most effectively stimulate LTC-IC and CFC amplification. This information is also likely to be crucial for the design of larger scale systems for culturing human hematopoietic cells. Finally, the present findings provide new distinctions between the biological effects of specific growth factors on a phenotypically defined ($CD34^+$ $CD38^-$) human hematopoietic cell target population. Further analysis of the cellular and molecular basis of these different responses should provide additional clues about early events in hematopoietic cell differentiation and how they are activated by interactions with the extracellular milieu.

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