

# Production of T Lymphocyte Colony-Forming Units From Precursors in Human Long-Term Bone Marrow Cultures

By Ivo Touw and Bob Löwenberg

T cell differentiation in human marrow was studied in Dexter type long-term bone marrow cultures. In these cultures, T lymphocyte colony-forming units (TL-CFU), E rosette-forming cells ( $E^+$ ), and  $T3^+$ ,  $T4^+$ , and  $T8^+$  cells (assayed by indirect immunofluorescence) were found to be present for at least 7 weeks. It was investigated whether the existence of T cells in long-term culture resulted from the persistence of inoculated T lymphocytes or from the production by immature progenitors. No significant numbers of  $E^+$ ,  $T3^+$ ,  $T4^+$ , or  $T8^+$  cells were detected in cultures that were established from  $E^+$  lymphocyte-depleted bone marrow, indicating little or no production of T lymphocytes from E-negative precursors. On the other hand, bone marrow cells purged of  $E^+$  lymphocytes did not contain TL-CFU, but appeared to regain high numbers of TL-CFU during Dexter culture; this suggested that an

earlier step in T cell differentiation may take place in this culture system. The generation of TL-CFU in the E-negative long-term marrow cultures only occurred when an adherent stroma layer had been established in the culture flask; it did not require added mitogens or detectable interleukin 2 in the culture medium. TL-CFU in fresh marrow (TL-CFU II) are mature ( $E^+$ ,  $T3^+$ ) T cells and are capable of producing helper ( $T4^+$ ) and suppressor/cytotoxic ( $T8^+$ ) phenotype cells in colonies. The TL-CFU newly formed in E-depleted Dexter cultures (TL-CFU I) are distinct from this population, as they are E-negative and give rise to colonies of the helper type only. T3 cell depletion of the marrow inoculum prior to culture did not prevent the appearance of TL-CFU I in long-term culture; this suggests that TL-CFU I are derived from an  $E^-$  and  $T3^-$  precursor (pre-TL-CFU).

**D**URING THE PAST FEW YEARS, factors regulating the proliferation and activation of mature T lymphocytes have been identified, and it has now become possible to culture these cells under well-defined in vitro conditions.<sup>1</sup> Still, very little is known about the regulation of earlier steps of T cell differentiation. The thymus plays a major role in this process during ontogeny. However, recent studies in congenitally athymic (nude) mice have shown that functionally active T lymphocytes may also be produced in the absence of this organ.<sup>2,3</sup> Dexter type long-term bone marrow cultures (LTBMC) have been employed to study in vitro T cell differentiation in murine bone marrow.

Schrader and Schrader<sup>4</sup> were the first to demonstrate the potential of LTBMC to induce T cell commitment in marrow-derived hematopoietic precursors. They showed that LTBMC gave rise to cells capable of differentiation into mature T lymphocytes when injected into irradiated mice. This finding was further substantiated by Jones-Villeneuve et al,<sup>5</sup> who reported the maintenance in LTBMC of immature, Thy-1-negative, progenitor cells in LTBMC, which could give rise to colonies of Thy-1-positive cells. These cells,

developing in vitro in the absence of a thymic micro-environment, showed helper activity for cytolytic T lymphocyte precursors, a characteristic of a mature T lymphocyte subpopulation.<sup>6</sup> To what extent T cell differentiation can occur in murine LTBM has not been established. In one series of experiments,<sup>7</sup> no Thy-1-positive cells were detected in the cultures. This suggested that terminal differentiation to T lymphocytes was prohibited. By contrast, in investigations by Dorshkind and Phillips,<sup>8</sup> under appropriate culture conditions, Thy-1-positive functional lymphocytes were maintained in LTBMC, although it was suggested that this was due to contamination of already mature T cells rather than the result of differentiation of immature (Thy-1-negative) precursors.

The LTBMC system has been recently adapted for cells from other than the murine species.<sup>9-11</sup> In this article, we describe experiments directed towards T cell kinetics and differentiation in long-term cultures of human bone marrow. Our data indicate that terminal differentiation from precursor cells does not take place, but that E-negative progenitors are produced in culture. These progenitors (TL-CFU I) give rise to mature helper phenotype progeny (ie,  $T3^+$ ,  $T4^+$ ,  $T8^-$ ) upon transfer to a T cell colony assay system. In this respect, they differ from the  $E^+$ ,  $T3^+$  TL-CFU subset (TL-CFU II) in fresh marrow, which is capable of the production of colonies of both the helper ( $T4^+$ ) and the suppressor/cytotoxic ( $T8^+$ ) phenotypes.

## MATERIALS AND METHODS

### Bone Marrow

Bone marrow cells obtained from posterior iliac spine aspirates from hematologically normal donors were collected in Hanks' bal-

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anced salt solution (HBSS) containing preservative-free heparin. Excess red blood cells were removed by sedimentation (20 minutes) at unit gravity in 0.1% methylcellulose. Light density ( $\leq 1.077$  g/mL) marrow fractions were obtained by Ficoll-Isopaque separation.<sup>12</sup>

### *E Rosetting and E Depletion*

E rosetting of lymphocytes was achieved by using the method of Madsen and Johnsen,<sup>13</sup> with 2-aminoethylisothiuronium bromide (AET)-treated sheep red blood cells (AET-SRBC). E rosette-negative ( $E^-$ ) cell fractions were obtained after centrifugation of the rosetted cells through Ficoll-Isopaque. According to evaluations following a second rosetting procedure, the  $E^-$  fractions contained less than 2% E-positive ( $E^+$ ) lymphocytes.

### *Antibody- and Complement-Mediated Cytotoxicity*

Some of the marrow specimens were treated with cytotoxic monoclonal antibody (MoAb) OKT3 and rabbit complement (C') to remove T3-positive ( $T3^+$ ) lymphocytes. First,  $2 \times 10^6$  marrow cells in 100  $\mu$ L were incubated on ice with 5  $\mu$ L OKT3 for 30 minutes. When more than  $2 \times 10^6$  cells were treated, the incubation volume and the amount of OKT3 were increased proportionally. Subsequently, cells were washed with HBSS (1 $\times$ ), resuspended in 1 mL C' (30% vol/vol in HBSS), and incubated for 30 minutes at 37 °C. After adding a few grains of deoxyribonuclease (bovine pancreas DNA-se I, B-grade; Calbiochem, San Diego) to avoid cell clumping and washing with HBSS (2 $\times$ ), cells were prepared for both viability (trypan blue exclusion) and indirect immunofluorescence (after a second OKT3 labeling) assays to evaluate the efficiency of the lysis and for tissue culture experiments. Control incubations with OKT3 alone and with C' alone were included in each experiment. These incubations did not show a cytoreductive effect. In one experiment, a T3 depletion was applied to an E-depleted marrow suspension that contained less than 2%  $T3^+$  cells. In this case, the efficiency of the in vitro treatment was assessed in a parallel incubation of peripheral blood mononuclear cells with OKT3 and C'.

### *Indirect Immunofluorescence Studies*

Cells positive for T3, T4, T6, and T8 cell surface antigens were assessed with indirect immunofluorescence assays using MoAbs of the OKT series (Ortho Pharmaceutical Corporation, Raritan, NJ) as the first layer and purified goat anti-mouse Ig immunoglobulin G coupled to fluorescein isothiocyanate (GAM/FITC) as the second layer. After fixation with paraformaldehyde,<sup>14</sup> cells were treated on ice with MoAb (in titers of 1:20) and subsequently with 1:40 diluted GAM/FITC (Nordic Immunological Reagents, Tilburg, The Netherlands), according to standard procedures.<sup>15</sup> Using a Zeiss fluorescence microscope with plan-neofluar 25 $\times$  and 63 $\times$  lenses, 200 cells per slide were studied. Control incubations with GAM/FITC alone, included in each test to check for nonspecific binding of the reagent to the cell surface, were negative in all experiments. Normal granulocyte macrophage (CFU-GM) and acute and chronic myeloid leukemia (AML and CML) colony cells were found to lack reactivity with the OKT MoAbs used, indicating that these reagents were valid for studying T cell differentiation in colony culture.

### *T Cell Colony Assay*

T lymphocyte colonies were grown in the PHA-leukocyte feeder system, as previously described.<sup>16</sup> In brief,  $0.5 \times 10^5$  cells were plated in a 0.4-mL liquid upper layer, supplemented with 0.01 mL PHA (Wellcome Diagnostics, Dartford, England) upon a 1-mL 0.5% agar layer containing  $2 \times 10^6$  irradiated (2,500 rad) peripheral blood

nucleated cells in 35-mm Petri dishes (Costar, Cambridge, Mass). After seven days of incubation (37 °C, 5% CO<sub>2</sub> in air) in a humidified atmosphere, colonies of more than 50 cells were counted with a Zeiss inverted microscope. Colony cells were harvested with a Pasteur pipette, washed twice with HBSS, and prepared for E rosette and indirect immunofluorescence assays.

### *Long-Term Bone Marrow Culture (LTBMC)*

Dexter-type cultures for human bone marrow were set up in two phases. In the first two to four weeks of culture, an adherent stroma cell layer, consisting of fibroblast-like cells, adipocytes, and macrophages, was allowed to form. A second portion of autologous marrow cells, cryopreserved<sup>17</sup> on the day of aspiration, was then added. The time of addition of this second inoculation (or recharge) was considered as the initiation or time zero of LTBMC. Culture conditions have been described in detail elsewhere.<sup>18</sup> However, slight modifications were introduced to adapt the culture system for the studies reported here. Second inoculates contained 3 to  $10 \times 10^6$  Ficoll-Isopaque isolated marrow cells (either nondepleted, E depleted, or E depleted plus T3 depleted). Prior to the reinoculation, all the spent medium, including the remaining nonadherent cells of the first inoculate, was discarded, the adherent layer washed twice with 5 mL HBSS, and fresh medium added. LTBMC were then monitored weekly, ie, the nonadherent cells in the cultures were counted and used for E rosetting, immunofluorescence, and T cell colony formation.

### *Detection of Interleukin 2 (IL-2) Activity*

IL-2 activities of LTBMC conditioned media were determined as described by Gillis et al<sup>19</sup> for murine IL-2. The media were kept at -20 °C until use. After thawing, they were added in a 10% dilution in culture medium (Iscove's with 10% fetal calf serum and  $10^{-4}$  mol/L beta-mercaptoethanol) in a vol of 0.2 mL to microtiter wells containing  $5 \times 10^3$  cells of a murine IL-2-dependent cytotoxic lymphocyte line (Dr L. Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Significant proliferation of these cells was obtained with samples containing 0.1 U/mL of partially purified (mitogen-free) human IL-2.

## RESULTS

### *Kinetics of T Lymphocytes in LTBMC*

Marrow cells in LTBMC were checked for the presence of E rosette (nine experiments) and T3, T4, T6, and T8 positive cells (three experiments). Cultures inoculated with nondepleted and with E rosette-depleted marrow cells were compared. In the nondepleted LTBMC, cells showing mature T cell characteristics (ie,  $E^+$ ,  $T3^+$ , and  $T6^-$ ) were maintained in relatively constant concentrations (20% to 40% of the cells in suspension). Both T lymphocytes with the helper ( $T4^+$ ) and with the suppressor/cytotoxic ( $T8^+$ ) phenotype were present. On the other hand, in the cultures established from E rosette-depleted marrow, numbers of cells expressing these T cell markers remained low, indicating that production of mature T cells from precursors did not occur. Results of the weekly determinations of T cell markers on LTBMC suspension cells in a complete experiment are given in Fig 1.

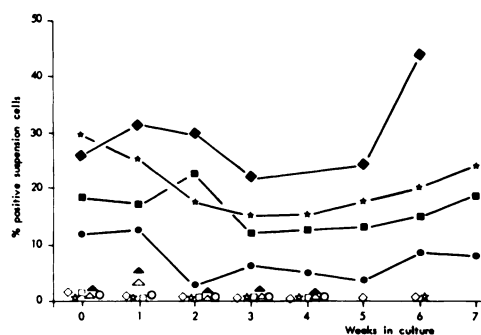


Fig 1. Kinetics of suspension cells carrying T cell surface antigens in LTBMCM derived from nondepleted (closed symbols) and from E rosette-depleted marrow (open symbols). (◆) E; (★) T3; (■) T4; (▲) T6; (●) T8.

### TL-CFU in the Marrow at the Time of Inoculation in LTBMCM (TL-CFU II)

Some characteristics of the TL-CFU present in the bone marrow prior to inoculation in long-term culture are presented in Table 1. In all cases, TL-CFU were removed from the marrow following E rosette depletion. Most likely, this was the result of separation of the TL-CFU and not of removal of factors essential for colony growth, as colony formation was not restored in E-negative marrow samples by the reconstitution of the plated marrow suspension with irradiated E lymphocytes or by the addition of human leukocyte-derived IL-2 to the colony culture. These results indicate that the TL-CFU in the marrow are all E-positive cells. In separate experiments, it was found that TL-CFU were completely eliminated from the bone marrow following *in vitro* treatment with MoAb OKT3 and complement, but not with complement or MoAb alone. Thus, marrow TL-CFU are also positive for the T3 antigen and apparently represent a population of mature T lymphocytes. We have designated this colony former as TL-CFU II.

### Kinetics of TL-CFU in LTBMCM

At first, TL-CFU numbers in nondepleted LTBMCM were estimated in six experiments. In five experiments, it was found that TL-CFU numbers had increased during culture. Data corrected for the weekly culture medium replacements<sup>20</sup> reveal a mean production of 323% (range, 158% to 760%) as compared with TL-CFU numbers in culture at time zero. An example of the TL-CFU kinetics in a nondepleted LTBMCM (Fig 2) indicates a constant increase of TL-CFU during six weeks of incubation. It was subsequently investigated as to whether E-negative cells were responsible for this TL-CFU production. LTBMCM were established from E-depleted bone marrow, so that no TL-CFU II were inoculated in culture. The results of three separate experiments are given in Fig 3. It appeared that new TL-CFU were produced in these LTBMCM and remained detectable in culture during five to six weeks (upper panel of the figure).

Meanwhile, the consistent E-negative nature of these LTBMCM throughout this period was evident in each experiment (lower panel). These results suggest that the new TL-CFU were generated in LTBMCM from immature (E<sup>-</sup>) cells. To exclude the possibility of production of TL-CFU due to the incomplete elimination of mature T lymphocytes from the marrow suspension at the onset, LTBMCM were set up with marrow cells that had been subjected to a double procedure of T lymphocyte elimination. After the E rosette separation, the cells underwent antibody- and complement-mediated lysis with MoAb OKT3 (E<sup>-</sup> cells treated with C' alone serving as a control). Table 2 shows that the numbers of TL-CFU generated in LTBMCM after inoculation with one step (E) and double step (E as well as T3) depleted bone marrow were comparable. Thus, the additional T3 depletion did not significantly affect the TL-CFU production in culture; this makes it unlikely that the newly formed TL-CFU originated

Table 1. T Lymphocyte Colony Growth From Human Bone Marrow (TL-CFU II)

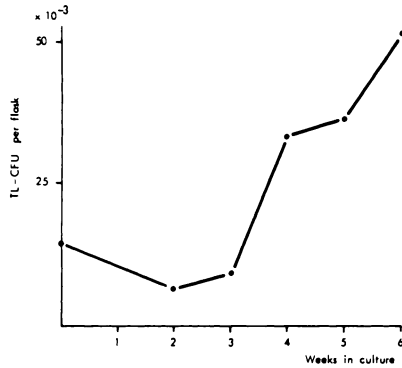
	Percent E <sup>+</sup> Lymphocytes Before Colony Culture	T Lymphocyte Colonies†
Nondepleted marrow (n = 9) (low-density Ficoll-Isopaque fraction)	33.8 ± 8.5*	301 ± 183*
E rosette-depleted marrow (n = 9)	1.2 ± 0.8*	11 ± 14*
+ 25 U IL-2‡ (n = 2)	0.25	2
+ 50 U IL-2‡ (n = 1)	1.5	28
+ 100 U IL-2‡ (n = 1)	1.5	8
+ irradiated (2,500 rad) E <sup>+</sup> lymphocytes‡§ (n = 2)		0
OKT3 + complement-treated marrow (n = 2)		0

\*Mean ± standard deviation.

†Numbers per 10<sup>5</sup> plated cells; E positivity of the colony cells was always verified.

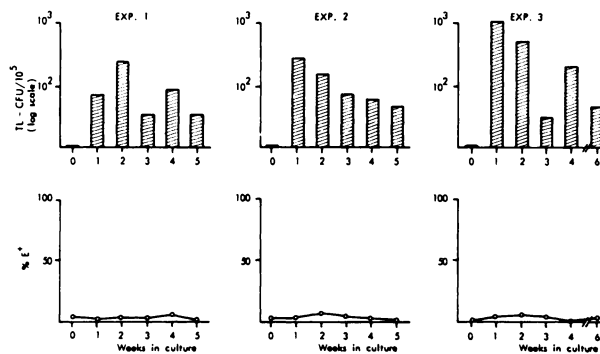
‡Added to the upper layer of the cultures.

§Added numbers reconstituted the concentrations present in nondepleted marrow.



**Fig 2. Kinetics of TL-CFU in a nondepleted LTBMC.** Total TL-CFU numbers per culture flask are expressed as values corrected for weekly medium replacements, according to Gregory and Eaves.<sup>20</sup>

from a residual population of mature phenotype T cells insufficiently removed from the marrow inoculates. In addition, it was excluded that TL-CFU production was the result of detachment of TL-CFU present in the preformed adherent stroma layer. In stroma preestablished control cultures to which an irradiated (2,500 rad) marrow portion had been added or in cultures without a second marrow charge, no TL-CFU were detected in suspension (data not shown). This provided evidence to indicate that E<sup>-</sup>, T3<sup>-</sup> precursor cells (without colony-forming capacities) gave rise to TL-CFU in LTBMC. We considered the possibility that these newly formed TL-CFU in consistently T cell-devoid cultures (Fig 1) lacked T cell surface characteristics and were different in this respect from the common TL-CFU (TL-CFU II) in fresh bone marrow. E depletion experiments, performed on LTBMC suspension cells after one week of culture, were carried out to test this possibility. It was found that two LTBMC suspensions still showed high concentrations



**Fig 3. T lymphocyte colony-forming cells (TL-CFU II) per 10<sup>5</sup> suspension cells measured at weekly intervals in LTBMC (upper panel) in comparison with the kinetics of E<sup>+</sup> lymphocytes (lower panel).** LTBMC were established from E rosette-depleted bone marrow. Results are from three separate experiments.

**Table 2. Comparison of TL-CFU Production (TL-CFU I) in One-Step (E) and Two-Step (E as well as T3) T Lymphocyte-Depleted LTBMC**

Marrow*	Numbers of TL-CFU per Culture Flask †				
	Week 0	Week 1	Week 2	Week 3	Week 4
E depletion ‡	0	4,108	6,044	1,240	4,935
E as well as T3 depletion §	0	3,267	4,290	1,862	3,115

\*In vitro treatment of the bone marrow was done at the time of the inoculation onto the preestablished stroma layer (time zero). TL-CFU were then assessed at weekly intervals.

†Data are not corrected for the weekly removal of nonadherent cells due to medium replacements.

‡E rosette-depleted bone marrow was incubated with C' only.

§E rosette-depleted bone marrow was incubated with OKT3 and C'. Since E-negative bone marrow contained less than 2% OKT3-positive cells (Fig 1), the efficacy of the procedure was assessed in a parallel incubation on peripheral blood mononuclear cells using the same OKT3 and C' batches in identical concentrations. The blood cells contained 54% OKT3-positive cells and were all viable (on the basis of trypan blue exclusion); after the MoAb and C' treatment, OKT3 staining was reduced to 5%, and 54% of the cells had ingested trypan blue.

of TL-CFU (ie, 543 and 207/10<sup>5</sup> cells) after E depletion. This indicated that the TL-CFU generated in LTBMC, in contrast to TL-CFU II, do not express the E rosette receptor. We have designated this E-negative TL-CFU population as TL-CFU I.

**Phenotyping of Colony Cells**

The immunologic phenotypes of the colony cells grown from (E<sup>-</sup>) TL-CFU I harvested at week 2 and week 4 of E<sup>-</sup> LTBMC and from unfractionated fresh marrow-derived (E<sup>+</sup>, T3<sup>+</sup>) TL-CFU II are given in Table 3. The data indicate that both TL-CFU I and TL-CFU II produced mature T lymphocyte, ie, E<sup>+</sup>, T3<sup>+</sup>, and T6<sup>-</sup> progeny. A marked difference, however, was evident with respect to the helper and suppressor/cytotoxic phenotype of the colony cells. Colonies derived from the TL-CFU I were almost entirely of the

**Table 3. T Lymphocyte Surface Markers on Marrow Cells Before and After T Cell Colony Formation (Percent Positive Cells)**

Markers	Normal Marrow (TL-CFU II)		E <sup>-</sup> LTBMC Suspension † (TL-CFU I)			
	Before Culture*	Colonies	Week 2		Week 4	
			Before Culture	Colonies	Before Culture	Colonies
T3	46	90	<1	72	<1	72
T4	30	60	<1	56	<1	75
T6	<1	<1	<1	<1	<1	<1
T8	16	26	<1	1	<1	8
E	39	86	1	91	0	97

\*Ficoll-Isopaque-separated light density (≤ 1.077 g/mL) marrow cells.

†Nonadherent cells from E rosette-depleted marrow inoculated cultures, assayed at week 2 and at week 4 of culture.

helper (T4<sup>+</sup>, T8<sup>-</sup>) phenotype, whereas those arising from the TL-CFU II contained T4<sup>+</sup> and T8<sup>+</sup> cells in the usual peripheral blood ratio. It appeared from morphological analysis, that both the TL-CFU I and the TL-CFU II colony cells resembled lymphoblasts.

#### *Determination of IL-2 Activity in LT BMC*

Supernatants of two LT BMC were assayed for IL-2 at different times of culture. We detected no IL-2 activities in the media at weeks 2, 3, and 4 of these cultures.

#### DISCUSSION

In this article, we have described experiments on T lymphocyte kinetics in human Dexter-type long-term bone marrow cultures (LT BMC) and have distinguished between three stages of T cell differentiation (ie, a putative pre-TL-CFU, TL-CFU I, and TL-CFU II) on the basis of PHA-induced *in vitro* colony formation. Our results demonstrate that T lymphocytes are maintained in LT BMC when an unfractionated light density marrow sample is inoculated (Fig 1). However, when an E-depleted marrow fraction is used, cells with E, T3, T4, T6, or T8 markers are not detected in significant numbers in these cultures. Thus, differentiation from immature precursors to T cells with a more mature immunologic phenotype does not occur. Similar findings have been described in the mouse system.<sup>8</sup>

T lymphocyte colony-forming cells are produced in LT BMC. This was evident in nondepleted cultures (Fig 2) as well as in cultures set up with marrow from which T lymphocytes had been removed prior to inoculation in LT BMC (Fig 3, Table 2). The T cell-depleted marrow had been rendered devoid of TL-CFU (Table 1). The TL-CFU newly formed in LT BMC in the absence of T lymphocytes stemmed from immature (E<sup>-</sup>, T3<sup>-</sup>) cells without colony-forming capacities. This step did not require the addition of mitogens to the system and occurred in the absence of assayable amounts of interleukin 2 in the culture medium.

On the other hand, the generation of TL-CFU in E<sup>-</sup> LT BMC was dependent on the presence of stroma cells. Typically, cultures set up in the absence of a preestablished adherent cell layer failed to produce significant numbers of E<sup>-</sup> TL-CFU (data not shown). Thus, the production of TL-CFU was stimulated by

the marrow stroma analogue in these cultures, similar to the production of the granulocyte/macrophage colony-forming cells.<sup>18</sup> We have no data to indicate the identity of the stroma-derived factors needed for this production. A recently discovered interleukin, IL-3, is a possibility, as Hapel and coworkers<sup>21</sup> have demonstrated that, in mice, IL-3 promotes the commitment of Thy-1<sup>-</sup> precursors to T cell differentiation in the absence of IL-2. It should be emphasized that in concordance with the IL-3-induced progenitor cells, the LT BMC-derived immature TL-CFU in mice<sup>5</sup> and in man (Table 2) produced no significant amounts of cytotoxic/suppressor phenotype T lymphocytes, but mainly, if not only, helper phenotype progeny. We designated the E<sup>-</sup> TL-CFU, which is generated in the human Dexter culture and is capable of the production of helper (T4<sup>+</sup>) colonies, TL-CFU I.

It was found that TL-CFU detectable in uncultured marrow samples carry a mature T cell (ie, E<sup>+</sup> and T3<sup>+</sup>) phenotype, which is in agreement with a previous report.<sup>22</sup> We have now designated these as TL-CFU II. Similar results have been published for peripheral blood-derived TL-CFU.<sup>22-24</sup> By contrast, other investigators have described the presence of E<sup>-</sup>, T3<sup>-</sup> TL-CFU in the peripheral blood<sup>25</sup> and marrow,<sup>26</sup> using somewhat different colony culture techniques. The relationship between these and the Dexter culture-derived TL-CFU I is not yet clear, but the results of colony phenotyping by Triebel et al<sup>26</sup> reveal progeny of both the helper (T4<sup>+</sup>) and the suppressor/cytotoxic (T8<sup>+</sup>) phenotypes and, therefore, suggest that they are different cell types.

At present, we propose that the production of E<sup>-</sup> TL-CFU (TL-CFU I) in LT BMC reflects an early step in bone marrow T lymphocyte differentiation. This hypothesis is also of interest in view of the observations of Messner et al<sup>27</sup> that multilineage *in vitro* colony-forming cells from normal bone marrow are capable of T lymphocyte differentiation, notably only towards the helper (T4<sup>+</sup>, T8<sup>-</sup>) subset.

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