

T-Cell Subsets and Suppressor Cells in Human Bone Marrow

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To characterize immune suppressive and hematopoietic features of enriched subsets of human marrow cells, we separated these cells on Percoll density gradients. CD4⁺ and CD8⁺ T cells (CD3⁺) were enriched in the high-density marrow cell fractions and reduced in low-density fractions. CD4⁻CD8⁻ (CD3⁺) T cells expressing the $\alpha\beta$ T-cell antigen receptor were at least 10 times less numerous than the CD4⁺ and CD8⁺ T cells in all fractions. Purified populations of the CD4⁻CD8⁻ $\alpha\beta$ ⁺ T cells obtained by flow cytometry suppressed the mixed leukocyte reaction (MLR). Another population of suppressor cells that expressed neither T-cell (CD3) nor natural killer cell (CD16) surface markers was also identified. The latter cells had the phenotypic and functional characteristics of "natural suppressor" cells. Suppressor cell

activity was enriched in the low-density fractions along with hematopoietic progenitors (colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid). The progenitor and suppressor cell activities were depleted in high-density fractions. The latter fractions made vigorous responses in the MLR. The low-density fractions, which accounted for less than 10% of the input marrow cells, suppressed the MLR and did not respond. Further evaluation of the low-density fractions may be of value in allogeneic bone marrow transplantation due to the reduction of CD4⁺ and CD8⁺ T cells and the enrichment of hematopoietic progenitors as well as immune suppressor cells that may inhibit graft-versus-host disease.

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ALLOGENEIC bone marrow transplantation (BMT) is the therapy of choice for various hematologic neoplasias in humans.^{1,2} However, one of the main obstacles is graft-versus-host disease (GVHD). Approaches taken to reduce or eliminate GVHD in animals and humans after allogeneic BMT include: (1) elimination of T cells from the donor marrow³⁻⁷; (2) posttransplant drug therapy of GVHD⁸⁻¹²; (3) use of mixed allogeneic and syngeneic marrow cells¹³⁻¹⁵; and (4) conditioning the host with total lymphoid irradiation (TLI).¹⁶⁻¹⁸ Although T-cell depletion of donor marrow lowers the risk of GVHD in humans, a lower rate of engraftment and higher malignancy relapse rate for certain tumors has been reported.¹⁹ As a result, the overall survival rate of patients with HLA-matched allogeneic BMT for leukemia has not been significantly improved by T-cell depletion.¹⁹

An alternative approach to T-cell depletion for control of GVHD is the enrichment of donor marrow with natural suppressor (NS) cells, which can inhibit T-cell activation by allogeneic lymphocytes or mitogens in vitro and GVHD in vivo.²⁰⁻²² In mice and humans, NS cells express the "null" surface phenotype, and inhibitory activity is neither antigen specific nor major histocompatibility complex (MHC) restricted.^{18,20-23} NS cells are enriched in tissues with high hematopoietic activity.^{18,24-26} Murine NS cells can be enriched in low-density fractions after Percoll density gradient centrifugation of BM cells.^{21,23,27,28}

A second population of murine cells that can inhibit the activation of T cells by allogeneic lymphocytes in vitro

(mixed leukocyte reaction [MLR]), and acute lethal GVHD in vivo are CD4⁻CD8⁻ T cells that express the $\alpha\beta$ T-cell antigen receptor (TCR).²⁹⁻³¹ Recent studies in mice show that the majority of T cells in the normal BM express the CD4⁻CD8⁻ $\alpha\beta$ ⁺ surface phenotype and are enriched in low-density fractions.^{29,32,33} Hematopoietic precursor cells that can reconstitute lethally irradiated mice copurify in these low-density fractions.³⁴ Purified populations of CD4⁻CD8⁻ $\alpha\beta$ ⁺ marrow T cells obtained by flow cytometry suppress the MLR and GVHD²⁹ (Palathumpat et al, in press). Cloned lines of mouse CD4⁻CD8⁻ $\alpha\beta$ ⁺ T cells also suppress the MLR and GVHD.^{35,36} The cloned cells secrete a unique cytokine that inhibits interleukin-2 (IL-2) secretion in the MLR and the function of antigen-presenting cells.³⁷

To determine whether similar populations of "null" and CD4⁻CD8⁻ $\alpha\beta$ ⁺ suppressor cells can be found in humans, human marrow cells were fractionated by density gradients and the surface phenotype of suppressor cells was determined as well the frequency of hematopoietic progenitor cells in each fraction.

MATERIALS AND METHODS

Patients and preparation of BM samples. BM was obtained from normal adult donors after informed consent had been given according to guidelines established by the Stanford Committee for Human Subjects. Human BM cells were either aspirated or taken from cores of BM removed from the iliac crest. Single-cell suspensions were separated using Ficoll-Hypaque gradients (Lymphoprep; Nycomed AS, Oslo, Norway). These gradients are subsequently referred to as Ficoll gradients. Cells were depleted of monocytes by incubation twice on plastic Petri dishes for 45 minutes at 37°C as previously described.³⁸ Medium for incubation consisted of RPMI-1640 (Applied Scientific, San Francisco, CA), 10% fetal calf serum (FCS; Hyclone, Logan, UT), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ mol/L 2-mercaptoethanol (2-ME).

Depletion of myeloid and erythroid cells from BM samples. BM cells obtained from Ficoll gradients were depleted of morphologically identifiable myeloid and erythroid mononuclear cells in some experiments to enrich for lymphocytes. Ficoll purified buoyant mononuclear cells were washed and incubated with phycoerythrin-conjugated mouse monoclonal antibodies (MoAbs) directed against human CD33 (Leu-M9; clone P67.6; Becton Dickinson, Mountain View, CA) and with mouse MoAbs to human glycoporphin A (gift of

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J. Griffin, Dana Farber Cancer Center, Boston, MA) at 4°C for 30 minutes. To remove excess antibody, the incubation mixture was layered on a 1-mL cushion of calf serum and centrifuged at 250g for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS) containing sheep antimouse antibody coupled to magnetic particles (Dynal, Inc, Great Neck, NY) and incubated as before. Cells were put on a magnetic particle concentrator (Dynal) for 5 minutes. Nonbound cells were removed and washed twice. In some cases, mouse MoAbs directed against CD11b (Mo-1; gift of J. Griffin) and against glycoprotein A were used to remove myeloid and erythroid cells by "panning," as has been described in detail elsewhere.³⁸

Fractionation of BM cells on Percoll density gradients. Percoll gradients were prepared using 2.5% steps ranging from 40% to 55% of stock solutions of Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden). Stock solutions were made with 12 parts Percoll and 1 part of 10× concentrated PBS. Dilutions of the stock solution were made with a physiologic solution of PBS similar to that described by Timonen and Saksela.³⁹ Before use, gradients were checked for pH, osmolarity, and exact density by refractometry (C. Zeiss refractometer; Zeiss, Oberkochen, Germany). Osmolarity was in the range of 280 to 290 mOsm/L and pH was adjusted to 7.3 to 7.4. The Ficoll purified, nonadherent or lymphocyte-enriched marrow cells were mixed with the lowest-density Percoll, placed on top of the discontinuous Percoll gradient, and centrifuged (550g) for 30 minutes at 20°C. Cells at each interface were collected and washed before phenotypic analysis and testing in the MLR or in the hematopoietic progenitor assays.

Preparation of peripheral blood lymphocytes (PBL). PB obtained from the BM donors or from normal individuals were separated on a Ficoll gradient, and the mononuclear cells from the interface were depleted of monocytes by adherence to plastic as described previously.³⁸ The latter cells were referred to as PBL.

Immunofluorescent staining. BM cells or PBL were stained with various MoAbs against human surface antigens with one- or two-stage techniques. Mouse MoAbs included those directed against human CD3 (anti-Leu-4), CD4 (anti-Leu-3), CD8 (anti-Leu-2), CD16 (anti-Leu-11) (Becton Dickinson), and human αβ TCR (WT31; T Cell Sciences, Cambridge, MA).

In the two-stage procedure, 1×10^6 cells were incubated with antibody at saturating concentration for 30 minutes at 4°C. To remove excess antibody, the incubation mixture was layered on a 1-mL cushion of calf serum and centrifuged at 250g for 10 minutes. Irrelevant mouse MoAbs directed against mouse surface receptors were used as controls. For the second stage, the pellet was resuspended in PBS containing fluoresceinated goat antimouse antibody (Becton Dickinson) at saturating concentrations and incubated as before. The second-stage antibody was absorbed with human BM cells for 30 minutes at 4°C to reduce background staining. Stained cells were washed and analyzed with a fluorescence-activated cell sorter (FACS III; Becton Dickinson) linked to a micro-VAX computer (Digital Electronic Company, Santa Clara, CA) equipped with FACS-DESK software (Becton Dickinson). Background staining with the control first-stage antibody was usually less than 2%.

One-stage staining was performed with fluorescein- or phycoerythrin-conjugated MoAbs obtained from the same source as the unconjugated antibodies. Staining was performed at saturation as described above. Background staining was determined by using irrelevant conjugated antibodies (fluorescein-conjugated monoclonal mouse antimouse V_β antibodies [Pharmingen, Inc, San Diego, CA] and phycoerythrin-conjugated monoclonal mouse anti-rat CD8 antibodies [Caltag Laboratories, South San Francisco, CA]) and was also less than 2%.

Cell sorting. In some experiments, purified populations of

marrow cells were obtained after immunofluorescent staining and sorting on a FACStar (Becton Dickinson). Cells were stained with MoAbs against the αβ TCR coupled to fluorescein in the first stage, and a combination of anti-CD4 and anti-CD8 antibodies coupled to phycoerythrin in the second. Staining was performed as stated above. Cells were sorted into αβ⁺ CD4⁻ CD8⁻ and into αβ⁺ CD4⁻ CD8⁺ cells using the dual-laser FACS. Thresholds for αβ⁺ and αβ⁻ cells were set at the background staining with irrelevant MoAbs and an interval of 20 fluorescence channels was used to separate the sorted cells. Cells falling in that interval were discarded. Thresholds for CD4⁻ CD8⁻ cells were 10 channels below the background staining level. There was less than 5% contamination of dull in bright cells, and of bright in dull cells on reanalysis.

MLR. Responder and irradiated stimulator cells were cultured at a concentration of 1×10^5 cells each in a final volume of 0.2 mL/well in U-bottomed microculture plates (Costar, Cambridge, MA). Cells were incubated for 120 hours at 37°C with 5% CO₂. Stimulator cells were irradiated with 3,000 cGy from a ¹³⁷Cs source (Mark I model 125 irradiator; J.L. Shepherd and Associates, Glendale, CA).

DNA synthesis was assayed by the addition of 1 μCi of tritiated thymidine (³H-TdR; specific activity, 6.7 Ci/mmol/L; New England Nuclear Corp, Boston, MA) to each well during the final 18 hours of the incubation period. Radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Inc, Fullerton, CA). Experiments were performed in triplicate and values are expressed as means.

MLR suppressor assay. Irradiated (3,000 cGy) putative suppressor cells were added at graded concentrations to 96-well, U-bottomed microtiter plates containing 1×10^5 responder and 1×10^5 irradiated (3,000 cGy) stimulator cells/well in a final volume of 200 μL. Putative suppressor cells were irradiated (1,500 cGy) before coculture, because unirradiated cells added to the autologous MLR (responder and stimulator cells obtained from the marrow donor) significantly increased ³H-TdR incorporation, but irradiated cells did not. Controls included the addition of irradiated responder cells instead of putative suppressor cells, or no addition of cells to the cell mixtures. Cultures were incubated at 37°C in 5% CO₂. After 120 hours, plates were pulsed with 1 μCi/well ³H-TdR as described above. All experiments were performed in triplicate. Percent suppression was calculated as $(1 - \text{cpm with cocultured cells/cpm without cocultured cells}) \times 100$. Suppression with irradiated responder cells was uniformly less than 20%.

Natural killer (NK) assay. NK activity was evaluated with a 4-hour ⁵¹Cr release assay. The K562 cell line, a human erythroleukemia cell line, was used as the target.³⁴ ⁵¹Cr-labeled target cells (10^4) and graded numbers of effector cells derived from BM Percoll fractions were added to each well and the cultures were incubated for 4 hours. ⁵¹Cr released into the supernatant was harvested with a Supernatant Collection System (Skatron Inc, Sterling, VA). Spontaneous and maximum ⁵¹Cr release were determined by incubating labeled target cells in culture medium or 1% NP-40, respectively. Percent Cr⁵¹ release was calculated as $(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}) \times 100$. All experiments were performed in triplicate.

Assay for colony-forming unit-granulocyte-macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E). BM cells or cell fractions depleted of adherent cells or of T cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with 15% FCS, 0.9% bovine serum albumin, 50 μmol/L 2-ME, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine, 1 U/mL recombinant human erythropoietin (Epo; Ortho Pharmaceutical Co, Raritan, NJ), 1% Mo conditioned medium (provided by D. Golde, UCLA, Los Angeles, CA) as a burst-promoting activity source, and

methylcellulose at a final concentration of 1.1% for enumeration of BFU-E as described previously.^{14,16} After 14 days of incubation at 37°C with 5% CO₂, cultures were examined under an inverted microscope and BFU-E colonies were scored. Cultures for CFU-GM were plated as above with the use of 15% placental conditioned as a source of myeloid colony-stimulating factor medium,^{38,40} instead of Epo and Mo conditioned medium. CFU-GM colonies were scored on days 9 and 14.

RESULTS

T-lymphocyte surface markers in human BM. Human BM aspirates were purified by Ficoll gradients, removal of adherent cells, and density centrifugation in Percoll. Gradients contained seven layers of Percoll starting with a 40% solution and ending with 55% solution in steps of 2.5% each. Fractions were identified as follows: fraction I (FR.1) and FR.2, (bands at 40% to 42.5% and 42.5% to 45% interfaces; FR.3 and FR.4, 45% to 47.5% and 47.5% to 50% interfaces; FR.5 and FR.6, 50% to 52.5% and 52.5% to 55% interfaces; FR.7, cell pellet in 55% layer. The combined yield of Percoll FR.1 and FR.2 gave a mean of 0.9% of the original BM samples, and that of FR.3 and 4 gave a mean of 4% (Table 1). This represented 3% and 11% of the nonadherent mononuclear cells, respectively. The combined yield of Percoll FR.5 and FR.6 gave a mean of 7% of the original samples and that of FR.7 gave a mean of 8%. This represented 20% and 24% of the nonadherent cells, respectively. The total yield of all fractions was 58% of the nonadherent mononuclear cells. CD3⁺ cells (T cells) were decreased in low-density fractions (mean, 6% in FR.1) as compared with BM separated by Ficoll (mean, 17%). In contrast, CD3⁺ cells were increased in high-density fractions (33% in FR.7; Table 1). Similarly, CD4 and CD8 T-cell subsets were reduced in the low-density fraction (both 2% in FR.1) as compared with BM separated by Ficoll (both 9%) and with high-density Percoll fractions (both 15% in FR.7). The sum of the CD4⁺ and CD8⁺ cells was similar to that of the CD3⁺ cells in the fraction analyses (Table 1). CD16, an NK cell marker,^{41,42} was studied also. CD16⁺ cells were enriched in low-density fractions (18% to 21% in FR.1 and FR.3) as compared with high-density fractions (4% in FR.7). Percentages of T-cell subsets in FR.2, FR.4, and FR.6 were intermediate to those in FR.1, FR.3, FR.5, and FR.7 (data not shown).

To examine the surface markers of human marrow T lymphocytes for coexpression of CD4, CD8, and αβ TCR markers, BM cells from two additional donors were depleted of myeloid cells by "panning" or magnetic bead separation using anti-CD33 and antiglycophorin A antibodies, and purified by Percoll density gradient fractionation with 2.5% steps (40% to 55%). Thereafter, cells were stained with phycoerythrin- or fluorescein-conjugated antibodies. Table 2 shows that the percentage of CD4⁺ and CD8⁺ αβ⁺ T cells (sum of both subsets) were depleted in FR.1 (12%) and FR.3 (17%) as compared with those in Ficoll-purified cells (24%). Only FR.5 showed an enrichment of these T cells (34%). The percentages of CD4⁺ and CD8⁺ αβ⁺ cells in each fraction were similar to the percentage of CD3⁺ cells (data not shown). The percentage of CD4⁻ CD8⁻ αβ⁺ cells was maximum in FR.5 (1.2%). In all fractions, at least 90% of the αβ⁺ cells were CD4⁺ and CD8⁺. The highest ratio of CD4⁺ and CD8⁺:CD4⁻CD8⁻ αβ T cells was found in FR.7 (48:1) and the lowest was found in FR.1 (15:1). PB mononuclear cells (PBL) from the same donor showed that 65% of cells were CD4⁺ and CD8⁺ αβ⁺ T cells, and 1% were CD4⁻ CD8⁻ αβ⁺ cells.

Suppression of the MLR by human marrow CD4⁻ CD8⁻ αβ⁺ and CD4⁻ CD8⁻ αβ⁻ cells. Because sorted CD4⁻ CD8⁻ αβ⁺ and CD4⁻ CD8⁻ αβ⁻ cells from normal mouse BM were able to suppress the MLR,²⁹ purified populations of sorted human CD4⁻ CD8⁻ αβ⁺ and CD4⁻ and CD8⁻ αβ⁻ cells were tested for their suppressive activity in the MLR. To obtain sufficient numbers of the CD4⁻ CD8⁻ αβ⁺ T cells, marrow cells were obtained from Ficoll gradients and then depleted of myeloid cells with anti-MO-1 (anti-CD11b) and antiglycophorin A MoAbs using "panning" or magnetic bead separation. The nonadherent cells were stained with fluorescein-conjugated anti-αβ TCR and phycoerythrin-conjugated anti-CD4 and anti-CD8 antibodies (Fig 1A). Two-color analysis of these cells is compared with similarly stained PBL from the same donor in Fig 1B.

A discrete population of CD4⁻ CD8⁻ αβ⁺ cells was observed in the lower right quadrant of Fig 1A with BM cells of this donor, but not with PBL. Two of five donors showed this discrete population of CD4⁻ CD8⁻ αβ⁺ cells with concentric contours. The CD4⁻ CD8⁻ αβ⁺ and CD4⁻ CD8⁻ αβ⁻ populations of marrow cells in Fig 1A were

Table 1. Yield and Subsets of Marrow Cells After Fractionation

Marrow Cell Population	% Cells Expressing Surface Marker				% Yield†
	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD16 ⁺ *	
Unfractionated					100
Ficoll Hypaque	17 ± 10	9 ± 3	9 ± 3	ND	52 ± 11
Plastic nonadherent	20 ± 14	10 ± 6	10 ± 2	17	34 ± 7
Percoll fractions:					
FR.1 (40% to 42.5%)	6 ± 5	2 ± 1	2 ± 1	18	0.9 ± 0.7 (FR.1 + 2)
FR.3 (45% to 47.5%)	11 ± 10	5 ± 3	5 ± 2	21	4 ± 4 (FR.3 + 4)
FR.5 (50% to 52.5%)	20 ± 14	10 ± 5	8 ± 4	2	7 ± 4 (FR.5 + 6)
FR.7 (> 55%)	33 ± 24	15 ± 6	15 ± 6	4	8 ± 6 (FR.7)

Abbreviation: ND, not done.

*Average percentage of CD16⁺ cells in two experiments that were separate from those with other markers.

†Mean percent yield ± SEM as compared with the number of unfractionated marrow cells in four experiments.

Table 2. Percentages of CD4⁺ or CD8⁺ αβ⁺ and CD4⁻ CD8⁻ αβ⁺ Cells in Fractionated BM and PBL

Fraction	% Cells Expressing Surface Marker*		A:B Ratio
	CD4 ⁺ αβ ⁺ and CD8 ⁺ αβ ⁺ (A)	CD4 ⁻ CD8 ⁻ αβ ⁺ (B)	
Ficoll gradient and "panning"	24	0.54	44:1
Percoll gradient			
FR.1	12	0.8	15:1
FR.3	17	0.6	28:1
FR.5	34	1.2	28:1
FR.7	19	0.4	48:1
PBL	65	1.0	65:1

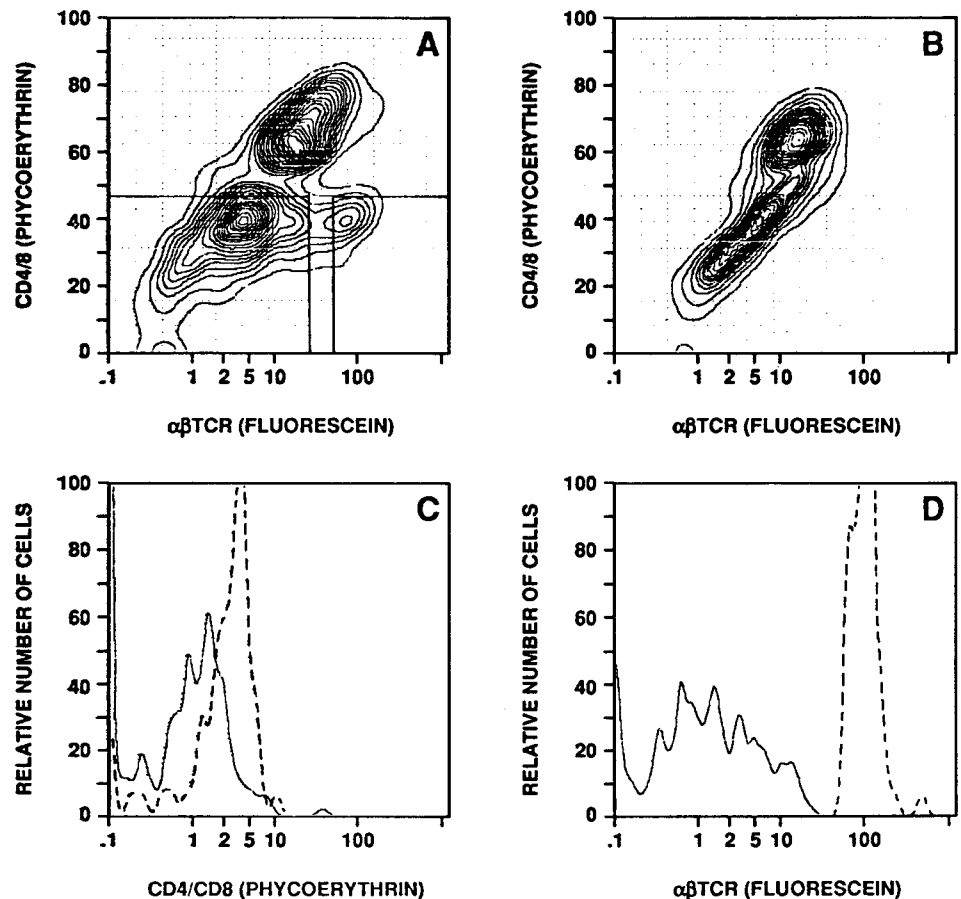
*Percentage of cells staining positively for given surface marker in one of two similar experiments in which T cells in BM were enriched by depletion of CD33⁺ and glycophorin A⁺ cells using magnetic beads. The same thresholds were used in all experiments.

purified by flow cytometry, and reanalyzed for their surface marker patterns. Figure 1C shows that the intensity of staining for the CD4 and CD8 markers was dull in both populations and overlapped. However, the pattern for αβ TCR staining showed a clear separation of bright and dull cells (Fig 1D), such that less than 5% contamination was noted. Graded numbers of CD4⁻ CD8⁻ αβ⁻ and CD4⁻ CD8⁻ αβ⁺ cells irradiated in vitro (3,000 cGy) were added to the MLR as shown in Fig 2A. Although both populations suppressed the ³H-TdR incorporation by about 40%, the

CD4⁻ CD8⁻ αβ⁺ cells were approximately five times more efficient than the CD4⁻ CD8⁻ αβ⁻ population. Unsorted marrow cells separated on Ficoll and "panned" as above failed to suppress the MLR over the dose range tested. The lack of suppression by the unsorted cell may be related to the presence of CD4⁺ or CD8⁺ cells that may enhance the MLR and to the low percentage of CD4⁻ CD8⁻ αβ⁺ cells.

Suppression of the MLR by fractionated BM cells. The suppressive activity of human BM cells that were unfractionated (red blood cells depleted), purified on a Ficoll gradient, or purified on a Percoll gradient was tested in the MLR. An example of the effect of unfractionated cells and various Percoll fractions added to the MLR at a 1:1 ratio to autologous mononuclear responder cells from the blood is shown in Fig 3. In contrast to the results in mice,^{21,29} unfractionated irradiated human BM cells enhanced ³H-TdR incorporation by about 10% when added to the MLR (Fig 3). In eight additional experiments, unfractionated marrow cells enhanced the MLR by a mean of 12%. An aliquot of the lowest-density marrow cells (FR.1) suppressed the MLR by about 50% (Fig 3). Aliquots of high-density marrow cells of FR.5 and FR.7 enhanced ³H-TdR incorporation by about 40% in this experiment. In two additional experiments, all the Percoll fractions were tested for suppression of the MLR. The mean percent suppression (±SE) of aliquots from FR.1 and FR.3 from all three experiments were 43% ± 8% and 22% ± 5%,

Fig 1. Purification of CD4⁻ CD8⁻ αβ⁺ and CD4⁻ CD8⁻ αβ⁻ BM cells by cell sorting. Human BM cells were separated on a Ficoll gradient and myeloid cells were removed by magnetic bead adherence (see Materials and Methods). Cells were subsequently stained with a combination of anti-CD4 and anti-CD8 MoAbs conjugated with phycoerythrin and with anti-αβ TCR MoAbs conjugated with fluorescein. (A) Two-color analysis of the stained marrow cells. (B) Analysis of autologous unfractionated PBL for comparison. The boxes in (A) show the CD4⁻ CD8⁻ αβ⁺ cells (left) and the CD4⁻ CD8⁻ αβ⁻ cells (right). The latter cells are not observed in (B). (C) One-color analysis for CD4 and CD8 surface markers on sorted CD4⁻ CD8⁻ αβ⁺ (----) and CD4⁻ CD8⁻ αβ⁻ (—) cells. (D) One-color analysis for the αβ TCR on the sorted CD4⁻ CD8⁻ αβ⁺ and CD4⁻ CD8⁻ αβ⁻ cells. Cells were sorted as described in Materials and Methods and reanalyzed after the sort. The figure shows an example of marrow donor with a discrete population of CD4⁻ CD8⁻ αβ⁺ cells.



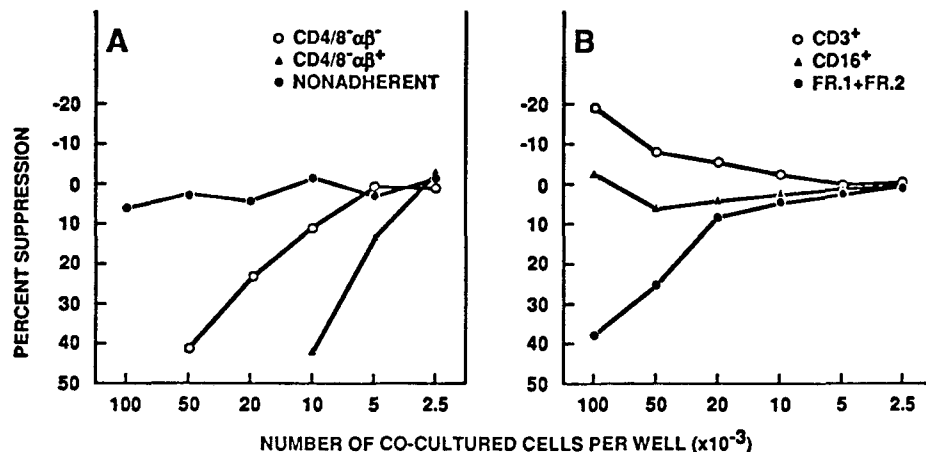


Fig 2. Suppression of the MLR with different populations of sorted BM cells. Various concentrations of sorted cells derived from human BM were added to the MLR with 1×10^5 autologous PBL responder cells and 1×10^5 irradiated allogeneic stimulator cells per well in a volume of 0.2 mL in 96-well round-bottomed plates. Stimulator and cocultured cells were irradiated with 3,000 cGy and 1,500 cGy, respectively, from a ^{137}Cs source. (A) Cocultured BM cells were either unsorted BM cells (Ficoll separated and myeloid cell depleted) or sorted $\alpha\beta$ TCR⁺, CD4⁻, CD8⁻ or $\alpha\beta$ TcR⁻, CD4⁻, CD8⁻ cells. Reanalysis after sorting showed that contamination was less than 3% in each case. Suppression was calculated as stated in Materials and Methods. Mean background cpm (responder cells plus irradiated autologous stimulator cells) was $1,394 \pm 155$. Mean experimental cpm without cocultured cells was $17,928 \pm 1,031$. The figure represents data from one set of two independent experiments with a similar pattern. (B) Cocultured BM cells were either unsorted low-density (FR.1 plus FR.2) BM cells or low-density sorted CD3⁺ or CD16⁺ marrow cells. Mean background cpm (responder plus irradiated autologous stimulator cells) was $2,291 \pm 162$. Mean experimental cpm without cocultured cells was $21,392 \pm 1,320$. The figure represents data from one of two independent experiments with a similar pattern. Negative values represent enhanced ^3H -thymidine incorporation. Standard errors of the mean were less than 15% of the mean values in the experiments in (A) and (B).

respectively. The mean percent enhancement from FR.5 and FR.7 were $26\% \pm 13\%$ and $24\% \pm 12\%$, respectively. The changes induced in the MLR by cells from Percoll FR.1 and FR.3 were statistically significantly different from those by FR.5 and FR.7 as judged by the Rank Sum Test ($P < .01$). In four more experiments, the suppression of the MLR by Ficoll-purified BM cells showed a mean of 17%.

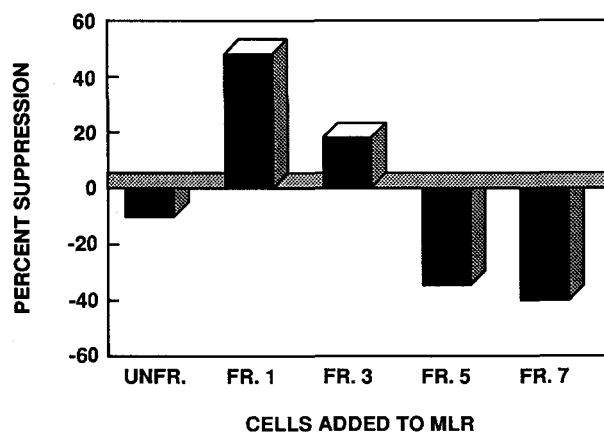


Fig 3. Suppression of the MLR with unfractionated (UNFR) and Percoll-fractionated BM cells. Cells (1×10^5) of different Percoll fractions derived from human BM were added to an MLR with 1×10^5 autologous PBL responder cells and 1×10^5 irradiated allogeneic stimulator cells. Unfractionated cells from the same donor (UNFR) were also assayed. Mean background cpm (responder cells plus irradiated autologous stimulator cells) was $4,862 \pm 570$. Mean ^3H -TdR incorporation with responder cells plus irradiated allogeneic stimulator cells was $21,752 \pm 2,874$ cpm. Suppression was calculated as stated in Materials and Methods. Negative values represent enhancement of ^3H -TdR incorporation. Standard errors were less than 15% of the mean values.

No significant differences were observed between the changes induced by the addition of cells from unfractionated BM, Ficoll-purified BM, or Percoll FR.5 and FR.7 ($P > .05$).

Inability of CD16⁺ marrow cells to suppress the MLR. The low-density marrow cell fractions from the Percoll gradients suppressed by MLR and were enriched for cells expressing CD16, a surface marker of NK cells. A previous report by Timonen et al,³⁹ showed that NK activity is enriched in low-density Percoll fractions. Accordingly, the unfractionated and Percoll-fractionated marrow cells were tested for cytolytic activity against Cr⁵¹-labeled K562 cells in a 4-hour Cr⁵¹ release assay in two experiments. As expected, the mean percent lysis by low-density, FR.1 cells (58% lysis) was considerably higher than that of either high-density, FR.7 cells (6% lysis) or of unfractionated cells (4% lysis) at a 40:1 effector:target ratio.

To determine whether CD16⁺ cells in the low-density Percoll fraction had suppressive activity in the MLR, purified CD16⁺ (bright) cells were obtained by flow cytometry from low-density marrow cells (FR.1 and FR.3) and added in graded doses to the MLR using autologous responder PBL and allogeneic stimulator PBL. In control experiments, purified CD3⁺ (bright) cells from the low-density fractions, as well as unsorted low-density cells were added to the MLR. Figure 2B shows that neither the sorted CD16⁺ nor CD3⁺ cells inhibited ^3H -Tdr over the dose range tested. However, the unsorted low-density cells were able to suppress the response, and about 40% suppression was observed with 1×10^5 cocultured cells (Fig 2B).

Response of Percoll-fractionated BM cells in the MLR. Cells from various Percoll fractions were added to irradiated allogeneic stimulator cells and tested as responder

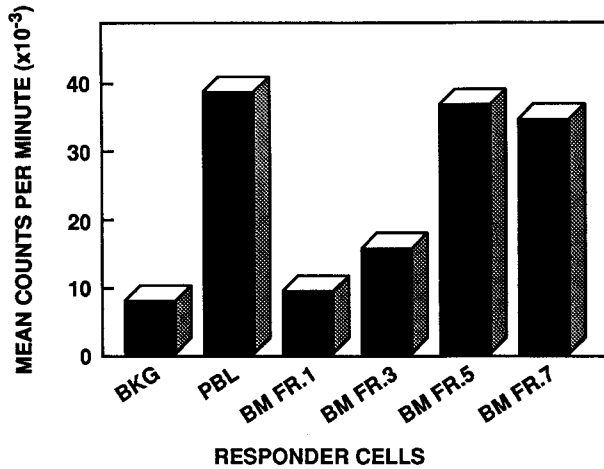


Fig 4. Response of Percoll-fractionated BM cells in the MLR. ³H-thymidine incorporation in counts per minute (mean of triplicate values) using different Percoll fractions (BM FR.1 through FR.7) derived from human BM as responder cells. Responder cells (1 × 10⁵) were tested in the MLR with 1 × 10⁵ irradiated allogeneic stimulator cells (PBL). Background (BKG) shows responder PBL plus irradiated autologous stimulator cells. PBL shows responder PBL plus irradiated allogeneic stimulator cells. One representative experiment of three is shown. Standard errors were less than 15% of the mean value.

cells in the MLR. In one experiment shown in Fig 4, low-density Percoll fractions were poor responders and ³H-TdR incorporation was similar to background. High-density fractions gave vigorous responses that were about 3.5 times greater than that of the former. The response of high-density marrow cells was similar to that of PBL (Fig 4). In three additional MLR experiments, the mean cpm ± SE of FR.1, FR.3, FR.5, and FR.7 as responder cells were 9,544 ± 1,134, 14,471 ± 2,410, 22,001 ± 8,156, and 23,647 ± 4,893. The responses of FR.7 were statistically significantly increased as compared with FR.1 (*P* < .01) as judged by the Rank Sum Test.

Frequency of CFU-GM and BFU-E in Percoll-fractionated BM. In several experiments, the distribution and recovery of hematopoietic progenitors in the Percoll density fractions of marrow was determined. For convenience, adjacent Percoll FR.1 and FR.2 (FR.1 + 2), FR.3 and FR.4 (FR.3 + 4), and FR.5 and FR.6 (FR.5 + 6) were combined. Table 3 shows the number of CFU-GM colonies per 10⁵ cells plated from various populations of marrow cells during the purification procedures. There was a 10-fold increase in CFU-GM activity in the Ficoll-purified marrow cells as compared with the unprocessed BM. Removal of adherent cells increased the CFU-GM activity to 13 times that of the unprocessed cells. An approximate 18- to 37-fold increase in CFU-GM was observed in the low-density fractions from the Percoll gradient (FR.1 + 2 and FR.3 + 4), and 89% of the CFU-GM activity was in these fractions. CFU-GM activity decreased in the highest-density fractions, and were not detectable in FR.7. Recovery of CFU-GM in the lower-density Percoll fractions was approximately 123% (FR.1 + 2 and FR.3 + 4 combined). The enhanced recovery of CFU-GM of activity after Ficoll separation and adherence may be explained by the removal

Table 3. Effect of Marrow Cell Separation Procedures on Cellular/Hematopoietic Progenitor Cell Enrichment and Recovery

Purification Procedures	All Cells		CFU-GM			
	Recovery* ×10 ⁶	%	Incidence per 10 ⁶ Cells†	Fold Enrichment‡	Recovery§ ×10 ³ %	
Unfractionated	253 ± 99	100	8 ± 2	1.0×	20.2	100
Ficoll gradient	130 ± 21	59 ± 18	80 ± 26	10.0	104	515
Removal of adherent cells	95 ± 30	39 ± 4	104 ± 30	13.0	98.8	489
Percoll gradient						
FR.1 + 2	2.9 ± 1.1	1.2 ± 0.3	295 ± 261	36.9	8.6	43
FR.3 + 4	11 ± 3	6 ± 6	146 ± 79	18.3	16.1	80
FR.5 + 6	21 ± 7	9 ± 1	15 ± 9	1.9	3.2	16
FR.7	19 ± 12	6 ± 3	0	0	0	0

*Mean recovery of cells ± SEM presented as total cell number and percent recovery as compared with unfractionated BM in three separate experiments.

†Mean number of CFU-GM ± SEM per 10⁶ cells in three experiments.

‡Fold enrichment of CFU-GM as compared with unfractionated BM.

§Mean recovery of CFU-GM presented as total recovery and as percent recovery as compared with unfractionated BM.

of inhibitory cells during the separation procedures. Approximately 50% of all cells applied to the Percoll gradient were recovered (Table 3).

Activity of BFU-E followed a pattern similar to that of CFU-GM (Table 4). There was a sixfold to eightfold enrichment of BFU-E activity after Ficoll separation and plastic adherence. The low-density Percoll fractions were enriched for BFU-E 10- to 20-fold as compared with unprocessed cells. A high proportion of the BFU-E (81%) recovered from the Percoll gradient was in FR.1 + 2 and FR.3 + 4. Again, the high-density cells were depleted of progenitor activity, and only 3% of BFU-E were recovered in FR.7. Ninety-three percent of the BFU-E present in the unprocessed marrow was recovered after the Percoll gradient.

Table 4. Effect of Marrow Cell Separation Procedures on Cellular/Hematopoietic Progenitor Cell Enrichment and Recovery

Purification Procedures	Cells		BFU-E			
	Recovery* ×10 ⁶	%	Incidence per 10 ⁶ Cells†	Fold Enrichment‡	Recovery§ ×10 ³ %	
Unfractionated	253 ± 99	100	9 ± 6	1.0×	22.7	100
Ficoll gradients	130 ± 21	59 ± 18	70 ± 62	7.8	91.0	401
Removal of adherent cells	95 ± 30	39 ± 4	55 ± 29	6.1	52.3	230
Percoll gradient						
FR.1 + 2	2.9 ± 1.1	1.2 ± 0.3	211 ± 43	23.4	6.1	27
FR.3 + 4	11 ± 3	6 ± 6	98 ± 79	10.9	11.0	48
FR.5 + 6	21 ± 7	9 ± 1	16 ± 13	1.8	3.4	15
FR.7	19 ± 12	6 ± 3	4 ± 4	0.4	0.7	3

*Mean recovery of cells ± SEM presented as total cell number and percent recovery as compared with unfractionated BM in three separate experiments.

†Mean number of BFU-E ± SEM per 10⁶ cells in three experiments.

‡Fold enrichment of BFU-E as compared with unfractionated BM.

§Mean recovery of BFU-E presented as total recovery and as percent recovery as compared with unfractionated BM.

DISCUSSION

The current work examined the T-cell subsets and immune regulatory cells in human BM. CD4⁺ and CD8⁺ T cells obtained from Percoll gradients were enriched about twofold in the high-density fractions of marrow cells and depleted by about fourfold to fivefold in the lowest-density fraction (FR.1) as compared with Ficoll-purified cells. T cells (CD3⁺) with the CD4⁻ CD8⁻ αβ⁺ surface phenotype were less than 2% of cells in the BM fractions from the Percoll gradients. The CD4⁺ and CD8⁺ T cells were at least 10 times more numerous than the CD4⁻ CD8⁻ αβ⁺ cells. These results differ from those in mice, because the majority of T cells in mouse marrow are CD4⁻ CD8⁻ αβ⁺.^{29,31,32} The ratio of CD4⁻ CD8⁻ to CD4⁺ and CD8⁺ T cells in the mouse marrow is as high as 10:1. The differences in the concentrations of CD4⁺, CD8⁺, and CD4⁻ CD8⁻ T cells with the αβ TCR in human as compared with the mouse BM may be due to the marked contamination of human marrow samples with PB as the marrow is aspirated from the pelvis.

The enrichment of CD4⁺ and CD8⁺ T cells in the high-density marrow fractions was associated with a vigorous proliferative response in vitro to allogeneic PBL. Depletion of CD4⁺ and CD8⁺ cells in the low-density fraction was associated with a minimal response. This is not surprising because others have shown that both CD4⁺ and CD8⁺ cells contribute to the MLR.⁴³ The high proportion of CD4⁺ and CD8⁺ cells among T cells in human as compared with mouse BM may explain the vigorous nature of GVHD in human allogeneic marrow transplant recipients and mild GVHD in murine recipients. The addition of small quantities of PBL to BM transplants in mice results in a more vigorous acute GVHD.⁴⁴

In view of murine studies suggesting that CD4⁻ CD8⁻ αβ⁺ BM cells can suppress the MLR, several experiments were performed in which human marrow cells depleted of myeloid cells were sorted into CD4⁻ CD8⁻ αβ⁻ and CD4⁻ CD8⁻ αβ⁺ cells by flow cytometry. The sorted cells were added to an MLR with autologous responder cells. The CD4⁻ CD8⁻ αβ⁺ cells suppressed the MLR about 40% at a 10:1 ratio of responder to suppressor cells. Similar suppression was observed with about five times more CD4⁻ CD8⁻ αβ⁻ sorted marrow cells. The latter cells may be similar or identical to "natural suppressor" cells, which are non-T cells that suppress a variety of in vitro proliferative responses of lymphocytes.^{20-23,45}

Although low-density marrow cells suppressed the MLR, sorted T cells (CD3⁺) from the low-density marrow fraction failed to suppress the MLR over the range of cell doses tested. In fact, some enhancement of the response was observed with the highest numbers of CD3⁺ T cells. The latter cells are almost all CD4⁺ and CD8⁺ T cells. Purified populations of CD16⁺ low-density BM cells also failed to inhibit the MLR over the dose range tested.

The results are consistent with previous reports that a population of non-T and non-NK cells in the low-density fractions of monkey and murine BM have immune suppres-

sive activity.^{20-23,27,45} The results also are similar to those obtained in mice, because both sorted CD4⁻ CD8⁻ αβ⁺ T cells and non-T cells in the low-density marrow fractions inhibit the MLR.²⁹ Thus, differences in mouse and human marrow appear to be related to differences in the ratio, but not function of the cell subsets. In the current experiments with human marrow, the suppressive activity of low-density cells appears to be due to the presence of non-T cells in these fractions. The suppressive activity of the CD4⁻ CD8⁻ αβ⁺ T cells was shown only after they were separated from CD4⁺ and CD8⁺ marrow cells by sorting. In both human and mouse studies, putative suppressor cells were irradiated before addition to the MLR, because unirradiated cells significantly increased ³H-TdR incorporation after addition to the autologous or syngeneic MLR. The high background levels precluded an accurate assessment of suppressor function. It is possible that irradiation reduces the suppressive activity and that the data underestimate this activity. It is unlikely that suppressive activity is a result of toxic products released by irradiated cells per se, because the suppressive activity is observed with some subsets of cells (CD4⁻ CD8⁻ αβ⁺) and not with others (CD3⁺). In the case of mice, in vitro suppression is correlated with the capacity to suppress lethal GVHD.³⁶

The human BM fractions were studied also for the presence of CFU-GM and BFU-E. The lowest-density fractions (FR.1 + 2 and FR.3 + 4) showed an enrichment in the frequency of CFU-GM of approximately 18- to 37-fold as compared with unfractionated BM cells. This was due, in part, to a 13-fold enrichment after purification of marrow cells on a Ficoll gradient and the removal of adherent cells. In the case of BFU-E, there was a 10- to 20-fold enrichment in the low-density marrow fractions. About 8% of cells were recovered in the low-density fractions as compared with the starting marrow sample, but the yield of CFU-GM and BFU-E was 123% and 75%, respectively, of the original number. The high yield of hematopoietic precursors in the low-density fractions may be due to the loss of inhibitory cells during the purification procedure.

The enrichment of CFU-GM and other more primitive hematopoietic progenitors, including long-term repopulating cells, in the lower density fractions of animal and human BM has been reported previously by other laboratories.^{46,47} In some instances, long-term repopulating cells were of higher density than the more mature progenitor cells (CFU-GM),⁴⁷ but neither population showed the high-density characteristics of small lymphocytes. In preliminary experiments, the Percoll density fractions described in the present study were assayed not only for CFU-GM and BFU-E progenitors, but also for pluripotent progenitors in an in vitro assay that detects myeloid, erythroid, and lymphoid colonies (C. Baum, Systemix Inc, Palo Alto, CA, unpublished observations) as well as an in vivo assay that detects long-term reconstitution of these three lineages in SCID/Hu mice.⁴⁸ The distribution of these pluripotent progenitors was similar to that of the CFU-GM and BFU-E, because both early and late progenitors were

enriched in the low-density fractions (FR.1, FR.2, and FR.3), and depleted in the high-density fractions (FR.5, FR.6, and FR.7) (Baum, C. and I. Schmidt-Wolf, unpublished observations).

The relationship between the immune suppressive cells and hematopoietic progenitors in the low-density fraction of human BM and the role of the suppressor cells in the regulation of hematopoiesis remains unclear. Recent studies in mice show that low-density fractions of spleen and BM mixtures are able to reconstitute lethally irradiated recipients without inducing acute GVHD.³⁴ The latter fractions maintain graft-versus-leukemia activity despite the inability to induce GVHD.³⁴ Thus, the enrichment of

immune suppressor and hematopoietic progenitor cells and depletion of CD4⁺ and CD8⁺ in the low-density marrow fractions may provide a favorable marrow inoculum for allogeneic marrow transplantation. Human marrow transplants fractionated by density gradients and elutriation to obtain low-density cells have been reported to induce less severe GVHD than unfractionated marrow.⁴⁹

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