Published in final edited form as:

J Immunol Methods. 2009 January 30; 340(2): 102–115. doi:10.1016/j.jim.2008.10.004.

A Novel Method for Long Term Bone Marrow Culture and Genetic Modification of Murine Neutrophils via Retroviral Transduction^{1,2,3}

Rachel L. Zemans^{*,†}, Natalie Briones^{*}, Scott K. Young^{*}, Kenneth C. Malcolm^{*}, Yosef Refaeli^{‡,§,||}, Gregory P. Downey^{*,†,§}, and G. Scott Worthen^{||}

^{*}Department of Medicine, National Jewish Health, Denver, CO

[‡]Department of Pediatrics, National Jewish Health, Denver, CO

[†]Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, University of Colorado Health Sciences Center, Aurora, CO

[§]Integrated Department of Immunology, University of Colorado Health Sciences Center, Aurora, CO

^{||}Cancer Center, University of Colorado Health Sciences Center, Aurora, CO

^{||}Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA

Abstract

Neutrophils are a critical component of the innate immune response to invading microbial pathogens. However, an excessive and/or prolonged neutrophil response can result in tissue injury that is thought to underlie the pathogenesis of various inflammatory diseases. The development of novel therapeutic strategies for inflammatory diseases depends on an improved understanding of regulation of neutrophil function. However, investigations into neutrophil function have been constrained in part by the difficulty of genetically modifying neutrophils using current techniques. To overcome this, we have developed a novel method for the genetic modification of murine bone marrow derived progenitor cells using retroviral transduction followed by long term bone marrow culture to generate mature neutrophils. These neutrophils are functionally mature as determined by morphology, surface marker (Gr1, CD11b, CD62L and CXCR2) expression, and functional attributes including the ability to generate superoxide, exocytose granule contents, chemotax, and phagocytose and kill bacteria. Further, the *in vitro* matured neutrophils are capable of migrating to an inflammatory site *in vivo*. We utilized this system to express the Bcl-2 transgene in mature neutrophils using the retroviral vectors pMIG and pMIT. Bcl-2 overexpression conferred a substantial delay in spontaneous apoptosis of neutrophils as assessed by annexin V and 7-amino-actinomycin D (7AAD) staining. Moreover, Bcl-2 overexpression did not alter granulopoiesis, as assessed by morphology and surface

²Abbreviations used in this paper: LTBM, long term bone marrow culture; 7AAD, 7-amino-actinomycin D; RPMI, Roswell Park Memorial Institute; 5-FU, 5-fluorouracil; KRPD, Kreb's Ringers phosphate with dextrose; HIPPP, heat inactivated platelet poor plasma; MPO, myeloperoxidase; BAL, bronchoalveolar lavage.

³Grant Support: This work was supported by National Institutes of Health grants R01HL061407-08 and R01HL068876 (G.S.W.) and R01HL090669 (G.P.D.), USPHS grant CA-117802 from the NCI and a Translational Research Award from the Leukemia and Lymphoma Society (Y.R.), and a Young Clinical Investigator Award from the Flight Attendants Medical and Research Institute (R.L.Z.).

1Address correspondence and reprint requests to: Dr. Gregory P. Downey, Drs Harold and Mary Zirin Chair in Pulmonary Biology, K701b, National Jewish Health, 1400 Jackson Street, Denver, CO USA 80206. Phone: 303-398-1436 FAX: 303 270-2243. Email: downeyg@njc.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

marker expression. This system enables the genetic manipulation of progenitor cells that can be differentiated *in vitro* to mature neutrophils that are functional *in vitro* and *in vivo*.

Keywords

Neutrophils; Rodent; Inflammation; Apoptosis; Hematopoiesis

INTRODUCTION

Neutrophilic polymorphonuclear leukocytes (neutrophils) play a critical role in the innate immune response to invading microorganisms. As neutrophils migrate to an inflammatory focus, they are activated to perform antimicrobial ('effector') functions, including superoxide production, granule release, and phagocytosis, which result in the eradication of the invading pathogens. However, an unchecked and excessive neutrophil response can result in tissue injury, manifesting clinically in inflammatory disease states such as acute lung injury (Ware and Matthay, 2000), cystic fibrosis (Elizur et al., 2008), inflammatory bowel disease (Edens and Parkos, 2003; Chin and Parkos, 2007), and autoimmune diseases such as rheumatoid arthritis (Liu and Pope, 2004). The design of novel therapeutic strategies to mitigate tissue injury in these inflammatory diseases depends on the ability to dissect at the molecular level the signaling pathways that regulate the microbicidal and cytotoxic responses of neutrophils.

The capacity to study neutrophil function has been hindered by the inability to apply current techniques of genetic modification to neutrophils. As neutrophils have a short half-life *ex vivo* and are terminally differentiated, attempts at genetic modification of mature cells using current techniques have been largely unsuccessful. One approach has been the study of signaling pathways and effector functions of neutrophils isolated from transgenic or knockout mice. However, this is expensive, time consuming, and can ultimately prove futile if the mutation is embryonic lethal, disrupts granulopoiesis, or if the animals (and isolated neutrophils) have no discernable phenotype. Alternatively, transfection of myeloid cell lines has been achieved (Redell et al., 2007) but the biological behavior of cell lines may not accurately reflect that of primary cells. Therefore, the study of neutrophil function frequently necessitates the use of pharmacologic inhibitors (Arndt et al., 2004), which often lack specificity, or protein transduction (Choi et al., 2003; Fessler et al., 2007), which is constrained by limited duration of action. For these reasons, the ability to genetically modify neutrophils would greatly enhance our ability to dissect the molecular pathways regulating neutrophil activation. Herein, we describe a method for the genetic manipulation of bone marrow-derived hematopoietic progenitor (stem) cells using retroviral transduction followed by culture in a novel long term bone marrow culture (LTBMC), producing genetically modified mature neutrophils.

Similar to the culture system described by Dexter and colleagues (Dexter et al., 1977; Moore et al., 1979; Allen and Dexter, 1983), our LTBMC system allows for the *in vitro* differentiation of murine neutrophils from progenitor cells. However, our system more closely replicates granulopoiesis in the native bone marrow than do previously described culture systems. Furthermore, while freshly isolated murine bone marrow neutrophils are currently the standard for investigation due to technical difficulties in isolating large numbers of peripheral blood neutrophils from mice (Boxio et al., 2004), the use of freshly isolated bone marrow derived neutrophils is limited by low numbers of cells isolated per mouse as well as contamination with monocytic cells (Biermann et al., 1999). The LTBMC system described herein yields greater numbers of mature neutrophils of higher purity as compared to fresh isolation from bone marrow and therefore is ideally suited for functional studies of mature neutrophils as well as for studies of granulopoiesis. Most importantly, our LTBMC allows for the genetic

modification of neutrophils via retroviral transduction of bone marrow progenitor cells followed by culture in the LTBMCM system, allowing for the persistence of transgene expression as the cells differentiate into mature neutrophils. We utilized this method to overexpress the Bcl-2 transgene in murine progenitor cells, which resulted in delayed apoptosis of mature neutrophils without affecting granulopoiesis.

MATERIALS AND METHODS

Reagents

Endotoxin-free reagents and plastic ware were used in all experiments. Antibodies to CD11b-PE-Cy5, Gr1-APC, CD62L-FITC, Sca1-FITC, Thy 1.1-FITC, and IgG2b-APC and HTS FluoroBlok 96 well plates were purchased from BD Bioscience. Annexin V-Pacific Blue, c-kit-APC antibody, pHrodo E. coli Bioparticles, calcein-AM, Vybrant DyeCycle Green, AlamarBlue, Gibco L-glutamine, Penicillin, and Streptomycin, and the 293FT cell line were purchased from Invitrogen. Antibodies to IgG2a-FITC and IgG2b-PE-Cy5 and recombinant murine IL-3, IL-6, and stem cell factor were purchased from ebioscience. Antibodies to CXCR2-PE and IgG2a-PE were purchased from R & D Systems. Bcl-2 antibody was obtained from Santa Cruz. Percoll was purchased from GE Healthcare. Chemicon Fischer's Complete Medium was purchased from Millipore. Cytochalasin D, cytochrome C, fMLP, PMA, LPS (*Escherichia coli*, 0111:B4), polybrene, and o-dianisidine were obtained from Sigma. Hydrogen peroxide was obtained from Kierkegaard & Perry Laboratories. Roswell Park Memorial Institute (RPMI) 1640 was purchased from BioWhittaker. Horse serum and hydrocortisone were obtained from StemCell Technologies. FBS was purchased from Gemini Bio-Products. 5-fluorouracil (5-FU) was obtained from Abraxis Pharmaceuticals. 7AAD and protease inhibitor cocktail III were obtained from Calbiochem. OptiCell chambers were purchased from USA Scientific. Hema 3 was purchased from Fisher Scientific. Rat anti-mouse Fc block was generated as previously described (Unkeless, 1979). pMIG and pMIG-Bcl-2 were provided by Dr. Y. Refaeli (Refaeli et al., 2002), and pMIT (Mitchell et al., 2001) and pMIT-Bcl-2 (Jorgensen et al., 2007) were a generous gift of Dr. P. Marrack.

Animals

C57BL/6 mice, aged 8–12 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were maintained in an animal care facility on a 12-hr light/dark cycle with full access to food and water. Animal protocols were approved by the Animal Care and Use Committee at National Jewish Health.

Long-Term Bone Marrow-Derived Cultures

Mice were euthanized, and bone marrow was harvested by flushing the femurs and tibias with LTBMCM media (79% by volume Fischer's medium, 20% horse serum, 1 μ M hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin). OptiCell chambers were seeded with 5×10^7 nucleated cells in 10 ml LTBMCM media and were incubated at 33°C with 5% CO₂. Once a week, 5 ml of media were removed from the chamber and replaced with 5 ml of fresh media. After 3 weeks in culture, 5 ml of media were removed, and the chambers were seeded with an additional 5×10^7 nucleated cells, or 0.5×10^6 nucleated cells for bone marrow transduced with retrovirus in 5 ml media. Two weeks after the second seeding, 4 ml of air was instilled into the chamber, the chamber was inverted 5 times, and 5 ml of cells were removed for analysis.

Gradient purification of LTBM or bone marrow derived neutrophils

Cells harvested from the LTBM or flushed from femurs with PBS were separated by centrifugation over a 3 layer discontinuous Percoll gradient as previously described (Nick et al., 2000).

Cytospin

The gradient purified cells from the LTBM or from fresh murine bone marrow were sedimented by cytocentrifugation and stained with Hema 3. Two hundred cells were counted by an investigator blinded to the conditions, and the percentage of morphologically mature neutrophils was calculated.

Light microscopic evaluation

Mature LTBM in the OptiCell chamber were photographed using a Zeiss Axiomat 200 microscope equipped with DIC optics. Cytospins were photographed using an Olympus DC70 microscope.

Retroviral Transduction

Mice were given 5-FU (5 mg/mouse) intravenously. Five days later, the mice were euthanized, and bone marrow was harvested from their femurs and tibias by flushing with DMEM supplemented with 10% FBS. Bone marrow cells were resuspended (1.5×10^6 cells/ml) in Bone Marrow Media (DMEM supplemented with 15% FBS, 20 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml stem cell factor), plated in a 24-well tissue culture plate (1.5×10^6 cells/well), and incubated at 37°C with 5% CO₂. The cells were infected daily for 3 days with retrovirus produced in 293FT cells, as previously described (Refaeli Y, 2008). Briefly, cells were centrifuged at 2000 rpm for 1 hr at RT in the presence of supernatant derived from transfected 293FT cells supplemented with 4 µg/ml polybrene. This procedure was repeated 24 and 48 hr later. The viral constructs used here include pMIG (Refaeli et al., 2002), pMIG-Bcl-2, pMIT (Mitchell et al., 2001) and pMIT-Bcl-2 (Jorgensen et al., 2007).

Spontaneous Apoptosis Assay

The gradient purified cells were resuspended in fresh LTBM media (5×10^6 cells/ml) in polypropylene tubes, and incubated at 37°C, 5% CO₂ for the indicated time periods.

FACS Analysis

The gradient purified cells from the LTBM were incubated on ice for 30 min with rat anti-mouse Fc block followed by incubation with antibody (1 µg/ 1×10^6 cells/ml) in PBS with 20% FBS on ice for 30 min. For the apoptosis experiments, cells were then resuspended (1×10^6 /ml) in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4) with annexin V (1:20 dilution) and 7AAD (1:125 dilution) and incubated on ice for 15 min. Samples were analyzed by flow cytometry using a FACScan, FACScalibur, LSR II (Becton Dickinson), or Cyan (Cytomation), and results were analyzed with CellQuestPro (Becton Dickinson) or FlowJo (Treestar) software. Gates were set such that 97.5 % of cells stained with an isotype control antibody, or unstained cells in the case of annexin V and 7AAD, were negative. For the apoptosis assay, cells that were GFP or Thy 1.1+ and Gr1+ were gated and analyzed for annexin V and 7AAD staining.

Superoxide Assay

Gradient purified cells from the LTBM or bone marrow derived neutrophils were resuspended in Krebs's Ringers phosphate with dextrose (KRPD) with 1% heat inactivated platelet depleted

(poor) plasma (HIPPP) at a concentration of 2×10^6 /ml, and superoxide release was quantified using cytochrome *c* reduction as previously described (Guthrie et al., 1984).

Myeloperoxidase Assay

Gradient purified cells from the LTBM or bone marrow derived neutrophils were resuspended in KRPD at 10×10^6 cells/ml and incubated with cytochalasin D (10 μ g/ml) or DMSO (0.1%) for 3 min. Cells were then stimulated with fMLP (10^{-6} M) or DMSO (0.1%) for 1 h at 37°C or lysed in ice-cold lysis buffer (50 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, and with protease inhibitor cocktail 3 per the manufacturer's instructions). Cells or lysates were centrifuged at $13,000 \times g$ for 30 s. Myeloperoxidase (MPO) concentration in the supernatant was quantified as described (Nick et al., 2000). Results are expressed as MPO released as a fraction of total cellular MPO content.

Phagocytosis Assay

Cells (1×10^6 /ml) were incubated in RPMI 1640 with 1% HIPPP at 37°C with 5% CO₂ for 1 h, followed by a 10 min incubation with cytochalasin D (10 μ g/ml) or DMSO (0.1%) as a diluent control. Cells were centrifuged at $300 \times g$ for 6 min and then incubated at 37°C with pHrodo *E.coli* bioparticles for 30 min per the manufacturer's instructions, followed by analysis by flow cytometry on the FACScan. To distinguish between particles bound to the cell surface and those internalized by the cell, results are expressed as percentage of cells that are fluorescent, percentage normalized to the fluorescence of cells pretreated with cytochalasin D.

Bacterial killing assay

Gradient purified cells from the LTBM or bone marrow derived neutrophils were suspended in RPMI 1640, 10 mM HEPES, pH 7.4, and 1% HIPPP, seeded in duplicate wells of a clear-bottom, black 96-well plate (Corning 3606) (1×10^5 per well in 0.1 ml), and allowed to settle for 30 min at 37°C. A suspension of *S. aureus* (1×10^5 cfu in 10 μ l) or saline control was added, and the plate was centrifuged for 3 min at $110 \times g$ to allow for synchronized contact of the bacteria and neutrophils. A standard curve of bacteria was generated (approximately 10^3 to 10^7 cfu in 10 μ l) to calibrate the assay. After 1 hr, cells were lysed with 1% Triton X-100, and brought to a final volume of 200 μ L with saline, 1 $\times$ LB broth and 20 μ l AlamarBlue. The plate was incubated in a fluorometric plate reader (BioTek Instruments FLX800) at 37°C. AlamarBlue reduction was measured at an excitation of 540 nm and emission of 600 nm, with the sensitivity set at 60 and constant shaking using KC Jr (BioTek Instruments). Readings were obtained every 30 min for 5 to 7 hr to generate sigmoidal growth curves. The time to half-maximal growth was calculated and compared to that of the standard curve growth.

In Vitro Chemotaxis

Cells (8×10^6 /ml) were labeled with Calcein-AM (1 ng/ml) in HBSS at 37°C for 40 min, washed once with HBSS, and resuspended (5×10^6 /ml) in KRPD with 1% BSA. Chemotaxis to MIP-2 (50 ng/ml) or nondirected migration to KRPD with 1% BSA was assessed using modified Boyden chambers as previously described (Arndt et al., 2006). Migration was reported as a percentage of total fluorescence of cells.

In Vivo Migration

Mice were exposed to aerosolized LPS (300 μ g/ml in 0.9% saline) for 20 min as described previously (Nick et al., 2002). Four hours later, LTBM cells were labeled with Vybrant DyeCycle Green stain (5 μ M) for 15 min at 37°C. Cells (16×10^6 in 200 μ l PBS) or 200 μ l PBS were injected via tail vein into the mice. Twenty-four hours after LPS exposure, bronchoalveolar lavage (BAL) was performed as previously described (Nick et al., 2000), and BAL cells were analyzed by flow cytometry.

Immunoblotting

Cells (1×10^6) removed from the LTBMC were lysed in 200 μ l ice-cold lysis buffer (50 mM Tris (pH 7.5), 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4 , 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) and boiled in Laemmli buffer. The proteins were separated using SDS-PAGE with a 14% gel, electrophoretically transferred to nitrocellulose, and blotted with antibodies to Bcl-2.

Statistical Analysis

Data are expressed as means $\pm$ S.E.M. Multiple comparisons were performed by one-way ANOVA with Tukey (post hoc) test for determination of differences between groups. Statistical analysis was also performed by Student's paired or unpaired *t* test or one sample *t* test where indicated. A *p* value less than 0.05 was considered significant. GraphPad PRISM software was used for all statistical calculations.

RESULTS

LTBMC structurally resemble bone marrow

Murine bone marrow derived cells cultured in OptiCell chambers under the conditions described yielded an adherent stroma with morphological features that resembled bone marrow trabecula with luminae containing loosely adherent cells (Fig. 1A). The two gas permeable membranes of the chamber provide dual growth surfaces (Fig. 1B), thus allowing for a three-dimensional culture that closely approximates the structure of native bone marrow stroma.

LTBMC generate morphologically mature neutrophils

Initial harvest of the LTBMC yields approximately 10×10^6 cells, of which $74 \pm 5\%$ were mature neutrophils, as determined by a morphological appearance of ring-shaped, segmented nuclei with modified Wright-Giemsa staining. This morphologic appearance is identical to that of murine neutrophils isolated from peripheral blood (Boxio et al., 2004). Approximately 85% of these cells were Gr1+ as determined by flow cytometry. A subsequent purification step using discontinuous (3 layer) Percoll gradients recovered approximately 40% of these cells; this purified population contained $95.2 \pm 0.8\%$ morphologically mature neutrophils (Fig. 2, A and C). In comparison, neutrophils freshly isolated from bone marrow using an identical discontinuous Percoll gradient were $71.1 \pm 4.0\%$ morphologically mature (Fig. 2, B and C). Furthermore, when analyzed by flow cytometry, the cultured cells displayed similar forward and side scattering properties to freshly isolated bone marrow derived neutrophils (Fig. 3A). It is noteworthy that the gradient purified cells from the bone marrow contained a subpopulation that had slightly different light scattering properties (lower side scatter but similar forward scatter). The smallest population likely represents erythroid precursor cells and erythrocytes and was excluded from cell surface marker analysis. Importantly, the LTBMC cells displayed cell surface markers characteristic of mature neutrophils: $98.3 \pm 0.6\%$ Gr1+, $99.3 \pm 1.1\%$ CD11b+, $90.0 \pm 6.0\%$ CD62L+, and $67.2 \pm 13.2\%$ CXCR2+ (Fig. 3B). These cells did not express cell surface markers of hematopoietic progenitor cells, with $1.0 \pm 0.8\%$ c-kit+ and $1.4 \pm 0.9\%$ Sca1+. By comparison, while freshly isolated murine bone marrow derived neutrophils displayed the cell surface markers of mature neutrophils, the levels of expression were equal to or less than that of the cultured cells (Fig. 3, C and D). This suggests that the LTBMC neutrophils are at least as pure and mature as bone marrow derived neutrophils (see Discussion). The LTBMC conditions were able to sustain myelopoiesis for extended periods of time. Cells harvested weekly for up to 5 weeks displayed similar morphology and expression of cell surface markers (data not shown).

Generation of functional neutrophils

To verify that the gradient-purified LTBM neutrophils were functionally mature, we assessed their ability to perform antimicrobial functions. The culture-derived cells produced superoxide (Fig. 4A) and released myeloperoxidase (Fig. 4B) in response to agonist stimulation. Similarly, the cultured neutrophils phagocytosed (Fig. 4C) and killed (Fig. 4D) bacteria effectively. Importantly, their microbicidal functions were comparable to or greater than that of freshly isolated bone marrow neutrophils. As shown in Fig. 4E, the cultured neutrophils displayed efficient chemotaxis in response to a gradient of MIP-2. Finally, the cultured neutrophils were capable of migrating to an inflammatory site *in vivo*, as demonstrated by recovery of cells infused intravenously in the BAL fluid of mice exposed to aerosolized LPS (Fig. 4F). Labeled cultured neutrophils were also detected in the bone marrow of these mice (data not shown). Thus, the cultured neutrophils were morphologically and functionally at least as mature as freshly isolated murine bone marrow derived neutrophils.

Generation of Genetically-Modified Neutrophils

Next, we endeavored to generate genetically modified neutrophils by transducing bone marrow progenitor cells with the bicistronic retroviral vectors pMIG or pMIG-Bcl-2 prior to seeding in the LTBM. As shown in Fig. 5A, the retrovirus successfully integrated into the genome of 28-74% of bone marrow cells, as determined by GFP expression. The percentage of bone marrow cells successfully transduced with the retrovirus varied depending on the specific virus used: $35.4 \pm 9.3\%$ for pMIG and $53.3 \pm 15.2\%$ for pMIG-Bcl-2. Once successful transduction was confirmed, the OptiCell chambers were seeded with the retrovirally-transduced bone marrow progenitor cells and maintained in culture for two weeks. Neutrophils harvested from the LTBM were determined to be stably transduced with the retrovirus, as evidenced by persistent GFP expression (Fig. 5B). Expression of recombinant Bcl-2 by LTBM neutrophils was also confirmed by immunoblot analysis of cell lysates (Fig. 5C).

Bcl-2 Overexpression Delays Spontaneous Neutrophil Apoptosis

To determine if this method allowed expression of a functional transgene that would mediate functional alterations in the cultured neutrophils, we chose to express Bcl-2. While Bcl-2 is expressed at very low levels in mature circulating neutrophils (Hockenbery et al., 1991; Delia et al., 1992), Mcl-1 and A1, closely related anti-apoptotic members of the Bcl-2 family, play a critical role in neutrophil apoptosis (Akgul et al., 2001). Furthermore, neutrophils from transgenic mice that express Bcl-2 were shown to be protected from apoptosis (Lagasse and Weissman, 1994). To confirm that the Bcl-2 protein expressed by the culture-derived neutrophils in our system was functional, the transduced neutrophils from the LTBM were incubated in media for various lengths of time to allow spontaneous apoptosis. Apoptosis and necrosis were then assessed using annexin V and 7AAD staining and gating on the population that express both Gr1 and GFP. As shown in Fig. 6A and B, neutrophils expressing the Bcl-2 transgene were largely protected from spontaneous apoptosis at 6 h, 24 h, and 48 h, compared with cells transduced with the control (empty) vector pMIG. Since GFP can leach out of apoptotic or necrotic cells, potentially skewing the data, we confirmed our observations using the retroviral vector pMIT, which is similar to pMIG except that the marker (reporter) gene is Thy 1.1 instead of GFP, and the Bcl-2 transgene is of murine rather than human origin. As above (Fig. 5A), the transduction efficiency of the bone marrow cells varied with the specific retrovirus used: $51.6 \pm 4.6\%$ for pMIT and $47.2 \pm 22.3\%$ for pMIT-Bcl-2. As shown in Fig. 6C, Bcl-2 overexpression as achieved through transduction of pMIT-Bcl-2 resulted in a considerable delay in spontaneous neutrophil apoptosis. In addition to annexin V and 7AAD staining, Bcl-2 overexpression was shown to confer an anti-apoptotic effect on neutrophils as determined by caspase-3 cleavage (data not shown).

Bcl-2 overexpression does not alter granulopoiesis

While Bcl-2 plays an important role in lymphopoiesis, less is known about the effects of Bcl-2 overexpression on granulopoiesis. We observed that Bcl-2 overexpression via retroviral transduction did not alter granulopoiesis. The retrovirally transduced LTBM cells were confirmed to be mature neutrophils, as determined by cell surface marker expression (Fig. 7) and morphology (Fig. 7B), suggesting that granulopoiesis is not altered either by Bcl-2 or by the retroviral transduction procedure.

DISCUSSION

We have developed a novel system for the long term culture and genetic modification of mature murine neutrophils. Our LTBM system is a modification of the myeloid culture system originally described by Dexter (Dexter et al., 1977; Moore et al., 1979; Allen and Dexter, 1983), but has several distinct advantages. First, because our culture system more closely resembles bone marrow structure (Allen and Dexter, 1983), with three dimensional organized trabeculae-like structures containing suspended cells that are mature neutrophils (Fig. 1), it is highly suited to study granulopoiesis. For example, by withdrawing cells from the culture at various time points during development, the functional capabilities and cell surface marker expression profile of myeloid progenitor cells throughout development could be determined. In addition, because the transparent membranes of the OptiCell chamber provide superior optical characteristics, this system can be used to study granulopoiesis using immunofluorescence microscopy. Finally, the effects of factors such as cytokines on granulopoiesis can be determined by their instillation through the chamber ports followed by sequential harvest of cells. Alternatively, perfusion of the chamber with continuous cell harvest would be particularly suited to examine rapid events, such as the effects of blocking antibodies to cell adhesion molecules on neutrophil release from the model bone marrow. In summary, the LTBM systems allows for intricate studies of granulopoiesis and its regulation.

Another advantage of our LTBM system is the ability to generate large numbers of mature neutrophils for functional studies. Our LTBM generates up to 12×10^6 cells on a weekly basis that are predominantly mature neutrophils (approximately 85% Gr1+). After several weekly harvests, the LTBM yields many times more neutrophils per mouse sacrificed than fresh isolation of murine bone marrow neutrophils. With a subsequent purification step, our LTBM system yields a highly pure population of mature neutrophils as determined by morphologic appearance, cell surface marker expression, and functional capabilities. The LTBM neutrophils described herein actually represent a more pure population of mature neutrophils than those freshly isolated from bone marrow (Fig. 2, 3), which may be contaminated with macrophages or myeloid and erythroid progenitor cells. Although it has been reported that some murine leukocytes with ring-shaped nuclei can be of monocytic lineage (Biermann et al., 1999), the gradient purified LTBM cells appear to be *bone fide* neutrophils, inasmuch as 99% express Gr1. Moreover, the LTBM system is ideally suited to functional studies because the cultured neutrophils are highly functionally mature (Fig. 4). For example, the LTBM cells possess the ability to migrate towards a chemoattractant gradient (Fig. 4E), which is known to be the final functional response to develop during granulopoiesis (Glasser and Fiederlein, 1987). Indeed, some of the functional capabilities (MPO and superoxide release) appear to be greater in LTBM cells as compared with bone marrow derived neutrophils (Fig. 4, A and B), even taking into account differences in purity (Fig. 2,3). This suggests that the LTBM neutrophils may be more mature (and not simply a more pure population) than bone marrow derived neutrophils. Since murine bone marrow derived neutrophils are of comparable maturity to murine peripheral blood neutrophils (Boxio et al., 2004), we conclude that the LTBM neutrophils can be utilized for functional studies as a surrogate for mature peripheral blood murine neutrophils. The one caveat to this is that $67.2 \pm$

13.2 % of LTBMN neutrophils express CXCR2 in contrast to nearly 100% of circulating murine neutrophils (R&D Systems murine CXCR2 antibody data sheet).

A major advantage of this system is the ability to genetically modify the bone marrow progenitor cells that yield mature neutrophils in culture. Advancements in the field of neutrophil biology have been limited in part by the inability to genetically modify primary neutrophils using current techniques. Although retroviral transduction of hematopoietic precursor cells has been achieved (Tsai and Collins, 1993), this technique does not yield a pure population of genetically modified mature neutrophils. Our inability to transfect primary neutrophils is attributable to the fact that they have a short *ex vivo* half-life and are terminally differentiated. Through retroviral transduction of hematopoietic progenitor cells, followed by *in vitro* differentiation into mature neutrophils, we have generated mature neutrophils that express both a marker gene (GFP or Thy 1.1) and a functional transgene (Bcl-2) (Fig. 5). Furthermore, genetic modification of neutrophils results in the expected functional alterations; in this case, Bcl-2 overexpression delays spontaneous neutrophil apoptosis (Fig. 6).

As mentioned above, dying cells can lose GFP fluorescence, which could potentially overestimate the protective effect of Bcl-2, since the annexin V and 7AAD staining of only GFP+, Gr1+ cells is examined. We have taken several measures to avoid this pitfall. First, we use the empty vector, pMIG, as a control for the apoptosis assay and observed that the survival benefit conferred by pMIG – Bcl-2 is not merely an artifact of selecting for live (GFP+) cells, as demonstrated by the difference in survival curves between GFP+ cells transduced with pMIG and GFP+ cells transduced with pMIG-Bcl-2 (Fig. 6, A and B). Confirming the protective effect of Bcl-2 on spontaneous apoptosis, caspase-3 cleavage was observed in control but not Bcl-2 transgenic neutrophils at 6 and 24 h (data not shown). Finally, we confirmed our results using pMIT ($\pm$ Bcl-2), which has Thy 1.1 as a marker, thus excluding the potential artifactual effect of loss of GFP from dying cells (Fig. 6C). The slight differences in the apoptosis results in the pMIG experiments as compared to the pMIT experiments may be due to the fact that pMIG-Bcl-2 contains the human Bcl-2 transgene, whereas pMIT-Bcl-2 contains the murine Bcl-2 transgene. The slight differences in survival between non-transduced or pMIT-transduced neutrophils versus neutrophils transduced with pMIG may be due to minimal loss of GFP from dying cells.

Given that neutrophils are known to have a short half-life due to spontaneous apoptosis (Akgul et al., 2001), the relatively long survival of LTBMN neutrophils without Bcl-2 expression (either non-transduced or pMIG/pMIT-transduced) was unanticipated (Fig. 6). However, the evidence for the short half-life of neutrophils derives from experiments on circulating neutrophils, which undergo apoptosis much more quickly than murine bone marrow neutrophils (Boxio et al., 2004). Our results are similar to previously published data, which show that wild-type murine bone marrow neutrophils display low levels of annexin V binding at 24 h in spontaneous apoptosis assays, due to expression of the anti-apoptotic Bcl-2 family member Mcl-1 (Dzhagalov et al., 2007). Furthermore, our data reveal that by 48 h after removal from the LTBMN, the majority of wild-type neutrophils have undergone spontaneous apoptosis (Fig. 6). The fact that bone marrow derived neutrophils and LTBMN neutrophils undergo apoptosis more slowly than peripheral neutrophils and that there are virtually no apoptotic neutrophils present in the LTBMN (Fig. 6, see 6 h data) implies that anti-apoptotic factors (e.g., G-CSF) are present in the native bone marrow and LTBMN environment but absent in the circulation. Interestingly, as seen in Fig. 6A, the delay of spontaneous apoptosis induced by Bcl-2 overexpression may ultimately result in an alternative necrotic death, as evidenced by the higher percentage of annexin V+, 7AAD+ cells than annexin V+, 7AAD- cells at 48 h.

Our data suggest that Bcl-2 overexpression does not alter granulopoiesis. Based on both morphology and Gr1 expression, the LTBMN environment generates mature neutrophils

whether or not Bcl-2 is overexpressed (Fig. 7). Since apoptosis is a critical phenomenon during developmental processes, Bcl-2 overexpression might theoretically alter granulopoiesis. In addition, independent of its effects on apoptosis, Bcl-2 is known to control cell cycle entry (O'Reilly et al., 1996) and to play a critical role in lymphopoiesis (Matsuzaki et al., 1997; Ogilvy et al., 1999), although less is known about the effects of constitutive Bcl-2 expression on granulopoiesis. Mice that are deficient in Bcl-2 (Matsuzaki et al., 1997; Villunger et al., 2003) or mice expressing a Bcl-2 transgene (Lagasse and Weissman, 1994; Innes et al., 1999; Ogilvy et al., 1999) have normal peripheral and bone marrow neutrophil counts and morphology (Ogilvy et al., 1999). Further, overexpression of Bcl-2 in the HL-60 myeloid leukemia cell line does not alter differentiation to granulocytes in response to retinoic acid (Park et al., 1994). Consistent with these reports, in our experiments, Bcl-2 overexpression did not affect development of cells into mature neutrophils within the LTBM (Fig. 7). This result highlights the utility of this system for the culture of genetically modified progenitor cells into mature neutrophils as a method for studying granulopoiesis.

We anticipate that this system can be utilized to examine the effects of modification of diverse neutrophil genes on antimicrobial effector functions and on hematopoiesis. In addition, because the LTBM neutrophils are capable of migrating to an inflammatory focus *in vivo* (Fig. 4F), this system can be applied to study the effect of modification of a given gene on neutrophil responses such as adhesion and migration in intact animals via adoptive transfer. Our system is suitable for expression of functional or inactive (dominant negative) transgenes as well as for gene silencing using RNA interference with short hairpin RNA using retroviral vectors such as murine stem cell virus (Dickins et al., 2005). In this system, the efficiency of retroviral transduction ranges from 28-74% (Fig. 5A). We believe that one advantage of our system is the existence of non-transduced neutrophils (GFP or Thy 1.1 negative cells) as an integrated control for flow-based or immunofluorescence studies. Alternatively, if a homogenous population of transgenic neutrophils is desired, the transduced bone marrow progenitor cells can be purified via fluorescence cell sorting, followed by seeding of the LTBM chambers with the purified population. Genetically modified neutrophils can be produced using the LTBM with relative ease and rapidity compared to the generation of gene-disrupted animals. Furthermore, a genetic modification resulting in an embryonic lethal phenotype could potentially be studied using our system, as long as there is no detrimental effect on granulopoiesis or bone marrow survival in culture. If the gene modification resulted in a survival or developmental disadvantage, use of an inducible retroviral vector (Ventura et al., 2004) would be a consideration. In the future, in addition to its utility for investigation, we anticipate that this technology can be applied to therapeutic approaches in humans, such as the infusion of neutrophils genetically modified to have heightened antimicrobial functions into neutropenic or chronic granulomatous disease patients during episodes of acute infection.

In conclusion, herein we report a novel system for the *in vitro* culture of murine bone marrow progenitor cells that yields, on a weekly basis, large numbers of morphologically and functionally mature neutrophils. This system can be utilized to generate neutrophils for functional studies or to study the regulation of granulopoiesis and/or release of mature neutrophils from the bone marrow. Further, we present a method for the genetic modification of neutrophils that historically has been technically difficult and that has the potential to facilitate the study of neutrophil function and the pathogenesis of inflammatory diseases in which neutrophils are believed to participate. Finally, our observations indicate that Bcl-2 overexpression does not alter granulopoiesis, a conclusion that is consistent with previous reports and underscores the utility of this system for the study of granulopoiesis.

Acknowledgements

We acknowledge Jonathan Lieber for his assistance with development of the LTBM system, Peter Henson for his thoughtful discussions, and Katie Poch, Annemie Van Linden, and Cory Yamashita for their technical assistance.

References

- Akgul C, Moulding DA, Edwards SW. Molecular control of neutrophil apoptosis. *FEBS Lett* 2001;487:318–22. [PubMed: 11163351]
- Allen TD, Dexter TM. Long term bone marrow cultures: an ultrastructural review. *Scan Electron Microsc* 1983;1851–66. [PubMed: 6669951]
- Arndt PG, Suzuki N, Avdi NJ, Malcolm KC, Worthen GS. Lipopolysaccharide-induced c-Jun NH2-terminal kinase activation in human neutrophils: role of phosphatidylinositol 3-Kinase and Syk-mediated pathways. *J Biol Chem* 2004;279:10883–91. [PubMed: 14699155]
- Arndt PG, Young SK, Poch KR, Nick JA, Falk S, Schrier RW, Worthen GS. Systemic inhibition of the angiotensin-converting enzyme limits lipopolysaccharide-induced lung neutrophil recruitment through both bradykinin and angiotensin II-regulated pathways. *J Immunol* 2006;177:7233–41. [PubMed: 17082641]
- Biermann H, Pietz B, Dreier R, Schmid KW, Sorg C, Sunderkotter C. Murine leukocytes with ring-shaped nuclei include granulocytes, monocytes, and their precursors. *J Leukoc Biol* 1999;65:217–31. [PubMed: 10088605]
- Boxio R, Bossenmeyer-Pourie C, Steinckwich N, Dournon C, Nusse O. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J Leukoc Biol* 2004;75:604–11. [PubMed: 14694182]
- Chin AC, Parkos CA. Pathobiology of neutrophil transepithelial migration: implications in mediating epithelial injury. *Annu Rev Pathol* 2007;2:111–43. [PubMed: 18039095]
- Choi M, Rolle S, Wellner M, Cardoso MC, Scheidereit C, Luft FC, Kettritz R. Inhibition of NF-kappaB by a TAT-NEMO-binding domain peptide accelerates constitutive apoptosis and abrogates LPS-delayed neutrophil apoptosis. *Blood* 2003;102:2259–67. [PubMed: 12763940]
- Delia D, Aiello A, Soligo D, Fontanella E, Melani C, Pezzella F, Pierotti MA, Della Porta G. bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. *Blood* 1992;79:1291–8. [PubMed: 1536952]
- Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol* 1977;91:335–44. [PubMed: 301143]
- Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, Lowe SW. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nat Genet* 2005;37:1289–95. [PubMed: 16200064]
- Dzhagalov I, St John A, He YW. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood* 2007;109:1620–6. [PubMed: 17062731]
- Edens HA, Parkos CA. Neutrophil transendothelial migration and alteration in vascular permeability: focus on neutrophil-derived azurocidin. *Curr Opin Hematol* 2003;10:25–30. [PubMed: 12483108]
- Elizur A, Cannon CL, Ferkol TW. Airway inflammation in cystic fibrosis. *Chest* 2008;133:489–95. [PubMed: 18252915]
- Fessler MB, Arndt PG, Just I, Nick JA, Malcolm KC, Worthen GS. Dual role for RhoA in suppression and induction of cytokines in the human neutrophil. *Blood* 2007;109:1248–56. [PubMed: 17018860]
- Glasser L, Fiederlein RL. Functional differentiation of normal human neutrophils. *Blood* 1987;69:937–44. [PubMed: 3814822]
- Guthrie LA, McPhail LC, Henson PM, Johnston RB Jr. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme. *J Exp Med* 1984;160:1656–71. [PubMed: 6096475]
- Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci U S A* 1991;88:6961–5. [PubMed: 1871110]

- Innes KM, Szilvassy SJ, Davidson HE, Gibson L, Adams JM, Cory S. Retroviral transduction of enriched hematopoietic stem cells allows lifelong Bcl-2 expression in multiple lineages but does not perturb hematopoiesis. *Exp Hematol* 1999;27:75–87. [PubMed: 9923446]
- Jorgensen TN, McKee A, Wang M, Kushnir E, White J, Refaeli Y, Kappler JW, Marrack P. Bim and Bcl-2 mutually affect the expression of the other in T cells. *J Immunol* 2007;179:3417–24. [PubMed: 17785775]
- Lagasse E, Weissman IL. bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. *J Exp Med* 1994;179:1047–52. [PubMed: 8113673]
- Liu H, Pope RM. Phagocytes: mechanisms of inflammation and tissue destruction. *Rheum Dis Clin North Am* 2004;30:19–39. v. [PubMed: 15061567]
- Matsuzaki Y, Nakayama K, Nakayama K, Tomita T, Isoda M, Loh DY, Nakauchi H. Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. *Blood* 1997;89:853–62. [PubMed: 9028316]
- Mitchell TC, Hildeman D, Kedi RM, Teague TK, Schaefer BC, White J, Zhu Y, Kappler J, Marrack P. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2001;2:397–402. [PubMed: 11323692]
- Moore MA, Sheridan AP, Allen TD, Dexter TM. Prolonged hematopoiesis in a primate bone marrow culture system: characteristics of stem cell production and the hematopoietic microenvironment. *Blood* 1979;54:775–93. [PubMed: 476301]
- Nick JA, Young SK, Arndt PG, Lieber JG, Suratt BT, Poch KR, Avdi NJ, Malcolm KC, Taube C, Henson PM, Worthen GS. Selective suppression of neutrophil accumulation in ongoing pulmonary inflammation by systemic inhibition of p38 mitogen-activated protein kinase. *J Immunol* 2002;169:5260–9. [PubMed: 12391245]
- Nick JA, Young SK, Brown KK, Avdi NJ, Arndt PG, Suratt BT, Janes MS, Henson PM, Worthen GS. Role of p38 mitogen-activated protein kinase in a murine model of pulmonary inflammation. *J Immunol* 2000;164:2151–9. [PubMed: 10657669]
- O'Reilly LA, Huang DC, Strasser A. The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *Embo J* 1996;15:6979–90. [PubMed: 9003774]
- Ogilvy S, Metcalf D, Print CG, Bath ML, Harris AW, Adams JM. Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc Natl Acad Sci U S A* 1999;96:14943–8. [PubMed: 10611317]
- Park JR, Robertson K, Hickstein DD, Tsai S, Hockenbery DM, Collins SJ. Dysregulated bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid-induced HL-60 granulocytes. *Blood* 1994;84:440–5. [PubMed: 8025271]
- Redell MS, Tsimelzon A, Hilsenbeck SG, Tweardy DJ. Conditional overexpression of Stat3alpha in differentiating myeloid cells results in neutrophil expansion and induces a distinct, antiapoptotic and pro-oncogenic gene expression pattern. *J Leukoc Biol* 2007;82:975–85. [PubMed: 17634277]
- Refaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon gamma is required for activation-induced death of T lymphocytes. *J Exp Med* 2002;196:999–1005. [PubMed: 12370261]
- Refaeli Y, Y R, Turner BC, Duda J, Field KA, Bishop JM. The B cell antigen receptor and overexpression of MYC can cooperate in the genesis of B cell lymphomas. *PLoS Biol* 2008;6:e152. [PubMed: 18578569]
- Tsai S, Collins SJ. A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocyte stage. *Proc Natl Acad Sci U S A* 1993;90:7153–7. [PubMed: 8394011]
- Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J Exp Med* 1979;150:580–96. [PubMed: 90108]
- Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R, Jacks T. Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A* 2004;101:10380–5. [PubMed: 15240889]
- Villunger A, Scott C, Bouillet P, Strasser A. Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. *Blood* 2003;101:2393–400. [PubMed: 12433687]
- Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–49. [PubMed: 10793167]

A.

B.

Figure 1.

LTBMC grown in OptiCell chambers closely resemble *in situ* bone marrow granulopoiesis. A) Murine bone marrow cells cultured in OptiCell chambers formed an adherent stroma reminiscent of bone marrow trabeculae with luminae containing non-adherent cells that can be removed by lavage. Live cultures were photographed using a Zeiss Axiomat 200 microscope with (10x, DIC) objective. Images shown are representative of at least three cultures. B) The OptiCell chamber consists of two parallel gas-permeable, cell culture-treated polystyrene membranes, 2 mm apart, attached to a frame. Two resealing access ports provide sterile channels for instillation of cells and media.

Figure 2.

Cells generated in the LTBM are morphologically mature neutrophils. Cells harvested from A) LTBM or B) mouse bone marrow were purified by Percoll gradient centrifugation followed by cytopsin and modified Wright-Giemsa staining. Images were photographed using an Olympus DC70 microscope at 40x original magnification. Results shown are representative of at least three separate cultures or mice. C) Two hundred cells were counted per slide on 3 slides generated from independent cultures or mice, and the percentage of morphologically mature neutrophils was calculated.

A.

Cultured PMN

BM PMN

Bone Marrow Derived PMN

Figure 3.

Culture-derived neutrophils display forward and side scatter properties and express cell surface markers similar to bone marrow-derived neutrophils. Culture-derived neutrophils and bone marrow-derived neutrophils were purified by gradient centrifugation followed by staining with antibodies for various cell surface markers or with isotype control antibodies and analysis by flow cytometry. A) Culture- and bone marrow-derived neutrophils display similar forward and side scatter properties. Cells within the circle denoted by the hatched line are likely red blood cells contaminating the bone marrow gradient prep and were excluded from cell surface marker analysis. Culture-derived cells (B, D) and bone marrow-derived neutrophils (C, D) express similar cell surface markers. *, $p < 0.05$ for cultured cell *versus* bone marrow-derived neutrophils for a given cell surface marker. Each sample was stained in triplicate. Results shown are representative of three or more experiments.

Figure 4.

Cells generated in the LTBM are functionally mature neutrophils, performing antimicrobial (effector) functions in a similar manner to bone marrow neutrophils. A) Cells (2×10^6 /ml) were stimulated with PMA (0.1 mM) for 10 min, and superoxide production was measured using a cytochrome *c* reduction assay, as previously described (Guthrie et al., 1984). *, $p < 0.05$ for PMA stimulated versus nonstimulated. †, $p < 0.05$ for cultured neutrophils + PMA versus freshly isolated bone marrow derived neutrophils + PMA. B) Cells (10×10^6 /ml) were preincubated with cytochalasin D (10 mg/ml) at 37°C followed by stimulation with fMLP (10^{-6} M) or DMSO (0.1%) for 1 h, and MPO release was quantified as previously reported (Nick et al., 2000). Results are expressed as MPO released as a fraction of total cellular MPO

content. *, $p < 0.05$ for +fMLP versus nonstimulated cultured neutrophils. †, $p < 0.05$ for cultured neutrophils +fMLP versus bone marrow derived neutrophils +fMLP. C) Cells (1×10^6 /ml) were incubated for 10 min with cytochalasin D (10 mg/ml) or DMSO (0.1%) at 37°C with 5% CO₂. Cells were then incubated at 37°C with pHrodo E. coli Bioparticles followed by analysis by flow cytometry on the FACScan. To distinguish between particles bound to the cell surface and those internalized by the cell, results are expressed as percentage of cells that are fluorescent, controlled for percentage of cells pretreated with cytochalasin D that are fluorescent. The difference between phagocytosis by cultured neutrophils and bone marrow neutrophils was not significant. D) Gradient purified cells harvested from the LTBM or directly from murine bone marrow were seeded in a 96-well plate (1×10^5 per well in 0.1 ml) and allowed to settle for 30 min at 37°C. *S. aureus* (1×10^5 CFU in 10 µl) or saline control was added and incubated at 37°C for 1 h. Cell lysates were incubated with AlamarBlue in a fluorometric plate reader at 37°C with constant shaking for 5-7 h, with AlamarBlue reduction measured every 30 min. Results are expressed as time to half-maximal growth of the standard growth curve as a percentage of time to half-maximal growth of bacteria incubated in the presence of neutrophils. *, $p < 0.05$ for cultured or bone marrow neutrophils versus control. E) Cells (5×10^6 /ml) were labeled with Calcein-AM (1 ng/ml), incubated at 37°C for 15 min, and then placed into the upper wells of modified Boyden chambers. Assessment of chemotaxis toward MIP-2 (50 ng/ml) or nondirectional movement to buffer was determined by measuring the amount of fluorescence in the lower wells. Migration is reported as a percentage of total fluorescence of cells. *, $p < 0.05$ for migration of cultured or bone marrow neutrophils toward MIP-2 versus nondirected migration. F) Mice were exposed to aerosolized LPS (300 µg/ml in 0.9% saline) for 20 min. Four hours later, LTBM cells were labeled with Vybrant DyeCycle Green stain (5 µM) for 15 min at 37°C. Cells (16×10^6 in 200 µl PBS) or 200 µl PBS were injected via tail vein into the mice. Twenty-four hours after LPS exposure, BAL was performed and BAL cells were analyzed by flow cytometry. *, $p < 0.05$.

Figure 5.

Genetically modified neutrophils were generated through retroviral transduction. Bone marrow cells harvested from 5-FU-treated mice were transduced with pMIG or pMIG-Bcl-2 or left untransduced. A) Cells were analyzed by flow cytometry for GFP expression. B) Cells (0.5×10^6) were loaded into the LTBM. Cultured cells were harvested, gradient purified, stained for Gr1, and analyzed by flow cytometry for GFP and Gr1 (not shown) expression. C) Cultured cell lysates were immunoblotted for Bcl-2 (*upper panel*). To ensure that equal amounts of cell lysates from each sample were loaded, membranes were immunoblotted for β -actin (*lower panel*).

Figure 6.

Bcl-2 overexpression through retroviral transduction delays spontaneous neutrophil apoptosis. Gradient purified cells were harvested from LTBMCM that had been seeded with bone marrow cells transduced with (A, B) pMIG or pMIG-Bcl-2 or (C) pMIT or pMIT-Bcl-2 or left non-transduced. The cells were incubated in LTBMCM Media (5×10^6 cells/ml) at 37°C , 5% CO_2 for indicated periods of time, followed by staining for (A, B, C) Gr1, annexin V, 7AAD, and (C) Thy 1.1. Annexin V and 7AAD data is reported from cells that are Gr1+ and (A, B) GFP + or (C) Thy1.1+. *, $p < 0.05$ for pMIG versus pMIG - Bcl-2 Annexin-, 7AAD- at 24 h and 48 h; §, $p < 0.001$ for pMIG versus pMIG - Bcl-2 Annexin+, 7AAD- at 48 h. †, $p < 0.01$ for control (non-transduced) cells versus pMIT - Bcl-2 Annexin-, 7AAD- at 24 h; °, $p < 0.05$ for

pMIT *versus* pMIT – Bcl-2 Annexin-, 7AAD- at 24 h. Dot plots shown are representative of at least three separate experiments.

Figure 7.

Bcl-2 overexpression does not alter granulopoiesis. Gradient purified cells harvested from the LTBM that had been seeded with bone marrow cells transduced with pMIT or pMIT-Bcl-2 were (A) stained for Gr1 and Thy 1.1 and (B) subjected to cytopsin. Differences in percentage of cells that are Gr1+ are nonsignificant. Dot plots and cytopsin are representative of at least three separate experiments.