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Research paper

A novel method for long term bone marrow culture and genetic modification of murine neutrophils via retroviral transduction ☆

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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 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*.

Abbreviations: LTBMC, 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.

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1. 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 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 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 LTBMC system, allowing for the persistence of transgene expression as the cells differential 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.

2. Materials and methods

2.1. 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, Alamar-Blue, 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.

2.2. 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-h 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.

2.3. Long-term bone marrow-derived cultures

Mice were euthanized, and bone marrow was harvested by flushing the femurs and tibias with LTBMC media (79% by volume Fischer's medium, 20% horse serum, 1 μM hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). OptiCell chambers were seeded with 5×10^7