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Characterization of neutrophils and macrophages from *ex vivo*-cultured murine bone marrow for morphologic maturation and functional responses by imaging flow cytometry

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ABSTRACT

Neutrophils and macrophages differentiate from common myeloid progenitors in the bone marrow, where they undergo nuclear morphologic changes during maturation. During this process, both cell types acquire critical innate immune functions that include phagocytosis of pathogens, and for neutrophils the release of nuclear material called nuclear extracellular traps (NETs). Primary cells used to study these functions are typically purified from mature mouse tissues, but bone marrow-derived *ex vivo* cultures provide more abundant numbers of progenitors and functionally mature cells. Routine analyses of these cells use conventional microscopy and flow cytometry, which present limitations; microscopy is laborious and subjective, whereas flow cytometry lacks spatial resolution. Here we describe methods to generate enriched populations of neutrophils or macrophages from cryopreserved mouse bone marrow cultured *ex vivo*, and to use imaging flow cytometry that combines the resolution of microscopy with flow cytometry to analyze cells for morphologic features, phagocytosis, and NETosis.

1. Introduction

Neutrophils and macrophages are myeloid cells essential to innate immunity that differentiate from hematopoietic stem cells in the bone marrow. During their differentiation, these professional phagocytes acquire specialized functions, including the capacity to recognize, bind and internalize pathogens leading to the formation of intracellular phagosomes. The activated cells then destroy ingested pathogens by producing an arsenal of antimicrobial proteases, reactive oxygen species (ROS), and nitric oxide (NO) [1]. Both cell types also undergo nuclear morphologic changes during early maturation: nuclei of early neutrophil progenitors (e.g. myelocytes) and monocytes condense while forming indented/kidney-like structures [2,3]. Myelocyte nuclei then continue to condense during late stages of maturation to form segmented or lobulated structures connected by thin strands of chromatin in mature, polymorphonuclear neutrophils (PMNs) [4]. By comparison, monocytes further mature into macrophages that form small, spherical nuclei as their nuclear-to-cytoplasmic (N/C) ratios dramatically decrease. Nuclear condensation and lobulation are architectural features thought to increase nuclear flexibility, which facilitates the capacity of neutrophils and perhaps monocytes to escape capillary beds (extravasation) and migrate between or even through endothelial cells or fibroblasts toward infected tissues [5–7]. Neutrophils are the first responders to acute infections and injuries, primarily operating in a "seek and destroy" fashion supported by the release of ROS and proteolytic enzymes. Neutrophils also produce critical pro-inflammatory cytokines that increase vascular permeability and promote capillary leakage, thereby facilitating the recruitment of more circulating neutrophils along with circulating monocytes. A unique feature of neutrophils is that activated cells can release a web of chromatin termed neutrophil extracellular traps (NETs) that help capture pathogens and expose them to localized, high concentrations of antimicrobial agents contained within the NETs. Recruited monocytes and differentiated macrophages release additional pro-inflammatory cytokines as well as digest microbes, dead neutrophils, and cellular debris within damaged tissue. Macrophages also produce anti-inflammatory cytokines that stimulate wound healing and tissue repair, along with presenting antigens that activate lymphocytes (e.g. T-cells). Combined, these myeloid cells create a partnership that provides an impressive defense mechanism to protect mammals from a

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plethora of potentially harmful pathogens including bacteria and fungi, while also promoting tissue remodeling and repair. Evidence for their importance to human biology is easily found in the symptoms of diseases that diminish their production and/or functionality, including inflammatory diseases, leukemias, or neutropenias. However, when functional activities of these cells are not properly regulated, they can be destructive. For instance, secretions produced by dysfunctional monocytes have been associated with rheumatoid arthritis and liver fibrosis [8,9]. Production of ROS, NO, and/or pro-inflammatory cytokines by aberrantly active neutrophils or macrophages contribute to disorders that include autoimmune diseases, myocardial ischemia/reperfusion injury, and atherosclerosis [10,11]. Interestingly, NETs produced by neutrophils have now been linked to pathological venous thrombosis, vasculitis, and autoimmune diseases [12,13]. By studying the maturation and functional activities of mouse myeloid lineages that accurately reflect those of humans, we may better understand normal vs. aberrant human innate immunity and inflammatory responses, or how abnormal responses affect wound repair.

Mouse myeloid cells are obtained routinely from select genetic strains, including wild-type or those genetically altered to model human diseases that affect innate immunity and/or inflammatory responses. Terminally differentiated neutrophils can be selected from mixed populations present in peripheral blood or bone marrow [14,15], whereas mature macrophages can be obtained from a peritoneal lavage following an infection response, directly from alveolar tissue, or as monocytes from bone marrow [16]. However, these sources present experimental limitations: (1) the harvested mature cells can be inadvertently stimulated either during the isolation process or have already been stimulated in the case of peritoneal macrophages [17], (2) fully matured cells cannot be used as models of myeloid differentiation, and (3) only limited numbers of cells can be isolated per mouse. Because of these issues, researchers have relied on immortalized cell lines as models of myelopoiesis for two general reasons: cultures can be easily expanded into large populations required for many experiments, and certain immature lines are inducible and allow for studies at progressive stages of differentiation. Nonetheless, as with primary cells, immortalized models also present certain limitations. Several neutrophil models have been well characterized, including the EML/EPRO and MPRO models, plus the SCF ER-Hoxb8 cell line, each modified to express either a dominant negative RARα or an estrogen receptor-regulated Hoxb8 transcription factor, respectively [18–21]. Although mature cells derived from each line express protein profiles observed in mature primary neutrophils and display several characteristic functional responses ([7,21] and unpublished observations), each model was genetically modified and the long-term culture required to generate each line may alter immune functions. Two immortalized murine monocyte/macrophage models are commonly used, RAW264.7 and J774A.1 cells, but these too are genetically altered (by the Abelson Murine Leukemia Virus or sarcoma-inducing mutations, respectively), plus are factor-independent and at advanced stages of differentiation. An alternative monocyte-like line is available that is blocked at an earlier developmental stage, GM-CSF ER-Hoxb8 cells, however these cells have not been thoroughly characterized and expression of the ER-Hoxb8 fusion protein has the potential to restrict normal functional responses of differentiated cells [18]. One method to circumvent the limitations of using myeloid cells isolated from mature tissues or derived from immortalized cell lines is to generate genetically unaltered myeloid progenitors directly from hematopoietic stem cells (HSCs) in mouse bone marrow, and then induce the progenitors into mature neutrophils or macrophages. Extracted whole bone marrow can be effectively depleted of lineage committed cells, and cultured *ex vivo* to yield an expanded population of HSCs, thereby minimizing experimental cost as well as reducing the number of mice required per assay [9,22,23]. The expanded HSC population can then be induced into common myeloid progenitors (CMPs) and myeloblasts with continued exponential cell population expansion, providing cells for analyses at an early stage of myelopoiesis. Throughout this process the cells can be genetically manipulated with viral vectors, producing progenitors that contain modified gene expression profiles potentially reflecting those associated with human diseases or developmental disorders [24]. Subsequently, expanded progenitor populations can be induced with different combinations of cytokines to drive terminal differentiation, yielding highly enriched populations of unstimulated neutrophils or macrophages [16,22]. The resulting lineages generally exhibit more robust and consistent functional responses, particularly phagocytosis, when compared to corresponding mature cells derived either from primary tissues or immortalized cell lines [17].

Accurate and quantitative assessment of the characteristic cellular features and functional responses of either wild-type or genetically modified (e.g. gene knockout or overexpression) progenitors and their mature counterparts are critical to understanding how genetic disorders lead to disease symptoms. Preliminary assessments typically use qualitative evaluation of cell morphologies by manual inspection of differentially stained cells with conventional microscopy. This technique allows for the identification of changes in nuclear structure (e.g. lobulation in neutrophils and decreased N/C ratios in macrophages) and cytoplasmic features such as increased cell size (macrophages), or accumulation of intracellular vesicles and granules (neutrophils and macrophages). Changes in the expression profile of lineage-specific cell surface markers can then be quantitatively measured by traditional flow cytometry, for either neutrophil (Gr-1 and Mac-1) or macrophage (F4/80 and Mac-1) differentiation. Analyses continue with evaluation of key functional responses, including phagocytosis or NETosis, both typically using fluorescence microscopy. For example, cells that have engulfed opsonized, fluorescence-labeled particles (e.g. bacteria or zymosan) are visually inspected for internalized fluorescence signals. Fluorescence microscopy also is used to detect changes of cellular components that are hallmarks of NETosis, such as nuclear translocation of cytoplasmic azurophilic granule proteins [e.g. myeloperoxidase (MPO) or neutrophil elastase (NE)], diffuse DNA staining indicating nuclear decondensation, or increased nuclear immunostaining with citrullinated histone-H3. Labeling of cell surface markers such as Mac-1 is then used to detect membrane blebbing events prior to the release of nuclear material, aka NETs. These processes are key indicators of "suicidal" NETosis, but whole cell imaging also can detect the more recently termed "vital" NETosis. In the latter process, nuclei also become less lobular as DNA diffuses into the cytoplasm, but cell lysis does not occur; rather, the nuclear material is simply released into an extracellular vesicle leaving behind an intact cytoplast [13,25].

The above-described tools for assessing myeloid cell morphologic maturation or functional responses have been optimized and extensively performed, but each has limitations. Traditional flow cytometry allows for multiplex analyses on a single cell plus statistical measurements from high throughput assessment of thousands of cells, but the processing used to identify positive cells lacks a spatial analysis component that can cause misclassification or misinterpretation of the results from different datasets. This limitation can be particularly problematic for certain phagocytosis studies; although analyses of phagocytosed particles can be semi-quantitatively assessed by flow cytometry, the capacity of the technology to discern internalized vs. surface-bound fluorescent particles is limited, even when quenching reagents are utilized [26–28]. The recent advent of pHrodo particles has improved accuracy of phagocytosis measurements, since the particles only