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Evaluation of a method for murine monocyte isolation by bone marrow depletion

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ABSTRACT

The study of monocyte activation and differentiation has great applications in sepsis, chronic inflammatory diseases, and cancer studies. However, despite the existence of well-established protocols for monocyte purification from human blood, the isolation of murine monocytes that can be subsequently activated has not yet been fully optimized. Here we evaluate a recently developed commercial procedure for obtaining monocytes from the bone marrow based on immunomagnetic depletion of non-monocytic cells. Moreover, we compare the advantages and disadvantages of this approach relative to other existing procedures. We found that monocytes isolates generated using this technique had equal purity to those attained via depletion from peripheral blood; however, higher yields were achieved. Furthermore, isolates from this technique have lower levels of macrophage contamination than those reported in samples generated by culturing bone marrow extracts with macrophage colony-stimulating factor (M-CSF). In addition, we demonstrate that the purified monocytes are sensitive to lipopolysaccharide (LPS)-mediated activation and, therefore, are useful for studies aimed at elucidating the molecular mechanisms involved in monocyte activation and differentiation.

The study of monocyte activation and differentiation has potential applications in multiple diseases. Monocytes are, together with neutrophils, the major components of the innate immune system, recognizing and phagocytosing pathogens as well as releasing cytotoxic compounds such as reactive oxygen species. Unlike neutrophils, activated monocytes are also able to modulate immune responses via the secretion of a wide range of prostaglandins and cytokines. However, prolonged activation has detrimental effects on the organism, and accordingly monocytes have been recently reported to play a role in several inflammatory diseases such as rheumatoid arthritis and liver fibrosis [1,2].

Monocytes are not only effectors of the immune system but also circulating reservoirs of macrophages and dendritic cells. Macrophages are the main constituents of the phagocyte system, whereas dendritic cells are important modulators of immune responses. However, the pathways that control monocyte differentiation into different macrophage subtypes remain to be characterized. It is crucial, therefore, to further explore the mechanisms that underlie monocyte activation and differentiation for further understanding of the physiopathology and pharmacology of infection, inflammation, and cancer [1,2].

To study monocytic activation and differentiation, monocytes need to be separated from other immune cells. Monocytes are hematopoietic cells that represent 10% of total leukocytes in humans and 4% in mice. They originate from the bone marrow and egress into the bloodstream via the CCR2 receptor. Blood monocytes can be identified by their expression of CD115, with two subtypes having been described: CD14++CD16− and CD14+CD16+ in humans and Ly6C^{high} and Ly6C^{low} in mice [2,3]. It has been reported that in the presence of a pathogen or an inflammatory environment, Ly6C^{high} monocytes activate and express biomarkers such as CD80 and CD86. Those Ly6C^{high} circulating monocytes that are not activated or differentiated eventually return to the bone marrow, where they turn into Ly6C^{low} monocytes [4]. In fact, a recent study showed that short-lived Ly6C^{high} monocytes are the steady-state precursor of blood-resident Ly6C^{low} monocytes; thus, the abundance of the former cells

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regulates the life span of the latter cells [5]. Therefore, monocytes can be potentially isolated from both the blood and bone marrow.

Traditionally, macrophages have been obtained by culturing bone marrow isolates for 7 days in the presence of the growth factor macrophage colony-stimulating factor (M-CSF)[1] [6]. Under these conditions, monocyte/macrophage precursors differentiate into macrophages, which then phagocytose non-monocytic cells and non-differentiated macrophages. However, to study monocyte activation and differentiation, it is necessary to start from a pure monocytic population rather than a complex mixture of bone marrow cells. Purification techniques employing immunomagnetic separation that are based on binding monocytic cell surface markers have been developed and enable a strong enrichment of monocytes. However, the resultant cells are non-functional because the targeting of these receptors during the separation process leads to cellular activation [7]. Recently, a novel method consisting of the depletion of all non-monocytic blood cells has been developed. Because monocyte cell surface markers are not targeted during this purification, the yielded monocytes are fully functional; however, the technique requires the use of high numbers of mice due to low yields [8].

Here we evaluate a new method developed by Miltenyi Biotec for monocyte isolation from bone marrow. Currently, there are no reports analyzing the advantages and disadvantages of this new approach. Furthermore, because the cells are isolated from the bone marrow, which contains high levels of immature monocytes, it is important to check that the purified cells are both mature and functional. This technique resulted in a highly pure population and, given that it is based on depletion of non-monocytic cells, yields monocytes that are capable of responding to lipopolysaccharide (LPS) stimulation. Importantly, however, the isolation from bone marrow results in a higher yield of monocytes than from blood and, thus, decreases the number of mice required per extraction. Therefore, this technique has both monetary and ethical benefits in terms of mouse numbers required as well as providing monocytes suitable for subsequent activation and differentiation studies.

Materials and methods

Animals

All animal protocols were approved by the scientific committee of the University of Seville. C57BL/6 mice were obtained from the University of Seville (Spain). Animals were kept in specific pathogen-free conditions and had free access to food and water. The animals used in the facilities associated with the experimentation service of the University of Seville were kept in accordance with both the national (RD 1201/2005) and European (609/CEE) legislation regarding the protection of animals used for experimentation and other scientific aims. Experiments were performed in accordance with the Guidelines of the European Union Directive 2010/63/EU, following Spanish regulations for the use of laboratory animals, and were approved by the scientific committee of the University of Seville.

Bone marrow harvest

For bone marrow (BM) harvest, C57BL/6 mice were sacrificed by cervical dislocation and BM was harvested by standard techniques [6]. The BM was obtained by flushing warm phosphate-buffered saline (PBS; Gibco, Invitrogen, Paisley, UK) through the medullar cavity of femurs and tibias, exposed after cutting the top edges of the bones, using a 27-gauge needle (0.4 × 25 mm) (B. Braun, Melsungen, Germany) and 20-ml syringes (Nipro Europe, Zaventem, Belgium).

The cell suspension was subsequently filtered to remove debris, washed twice in PBS, and centrifuged for 10 min at 300*g*. To analyze the distribution of cells in the BM, an aliquot of this suspension was directly stained with anti-CD45, anti-CD3, anti-CD11b, anti-CD19, anti-CCR3, anti-Ly6G, and anti-CD115 (Miltenyi Biotec, Bergisch Gladbach, Germany) (see Table 1 for antibodies used). Fluorescence was measured in a FACSCanto II (BD Biosciences, Erembodegem, Belgium) equipped with the FACSDiva program (BD Biosciences).

Monocyte purification

Monocytes were purified by negative selection using the BM Monocyte Isolation Kit (Miltenyi Biotec) following the manufacturer's procedures. Non-target cells are indirectly labeled with a cocktail of biotin-conjugated monoclonal antibodies, which are then bound to magnetic beads. After obtaining the BM cell suspension, the cells were counted and an aliquot containing 50×10^6 cells was further centrifuged (300*g*, 10 min) and resuspended in 175 µl of MACS running buffer (Miltenyi Biotec), followed by the addition of 25 µl of FcR Blocking Reagent and, immediately, 50 µl of the Monocyte Biotin–Antibody Cocktail, both contained in the BM Monocyte Isolation Kit. The cell suspension was then incubated for 5 min at 4 °C, followed by the addition of 10 ml of running buffer. After centrifuging for 10 min at 300*g* and 4 °C, the pellet was resuspended in 400 µl of running buffer and 100 µl of Anti-Biotin Microbeads was added. This suspension was then incubated for 10 min at 4 °C, after which the negative fraction was collected in a MACS separator using LS columns (see Table 2 for a detailed stepwise protocol). When purifying from more than 50×10^6 cells, the volume of kit reagents used was scaled accordingly.

To determine the purity of the isolated monocyte fraction, cells were stained with anti-CD45, anti-CD3, anti-CD11b, anti-CD19, anti-CCR3, anti-Ly6G, anti-Ly6C, and anti-CD115 (Miltenyi Biotec) (see Table 1 for antibodies used). Fluorescence was measured in a FACSCanto II (BD Biosciences) equipped with the FACSDiva program (BD Biosciences).

Table 1
Antibodies used for flow cytometry.

CD45	Hematopoietic cells	VioBlue	Miltenyi Biotec
Ly6G	Neutrophils	FITC	Miltenyi Biotec
CD4	T4 lymphocytes	FITC	Miltenyi Biotec
CD115	Monocytes/Macrophages	PE	Miltenyi Biotec
CD8	T8 lymphocytes	PE	Miltenyi Biotec
CD80	Activated monocytes and B cells	PE	Miltenyi Biotec
CCR3	Eosinophils	PerCP–Vio550	Miltenyi Biotec
F4/80	Monocytes/Macrophages	PerCP–Vio550	Miltenyi Biotec
CD11b	Monocytes/Neutrophils/Some lymphocytes	APC	Miltenyi Biotec
CD3	T lymphocytes	APC	Miltenyi Biotec
CD19	B lymphocytes	APC–Vio770	Miltenyi Biotec

[1] *Abbreviations used:* M-CSF, macrophage colony-stimulating factor; LPS, lipopolysaccharide; BM, bone marrow; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting.