Journal of Immunological Methods 385 (2012) 1–14

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Pure populations of murine macrophages from cultured embryonic stem cells. Application to studies of chemotaxis and apoptotic cell clearance

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ARTICLE INFO

Article history:
Received 5 April 2012
Received in revised form 10 June 2012
Accepted 11 June 2012
Available online 18 June 2012

Keywords:
Embryonic stem cells
Macrophages
Embryonic stem cell-derived macrophages
Chemotaxis
Apoptosis
Apoptotic cells

ABSTRACT

Embryonic stem cells provide a potentially convenient source of macrophages in the laboratory. Given the propensity of macrophages for plasticity in phenotype and function, standardised culture and differentiation protocols are required to ensure consistency in population output and activity in functional assays. Here we detail the development of an optimised culture protocol for the production of murine embryonic stem cell-derived macrophages (ESDM). This protocol provides improved yields of ESDM and we demonstrate that the cells are suitable for application to the study of macrophage responses to apoptotic cells. ESDM so produced were of higher purity than commonly used primary macrophage preparations and were functional in chemotaxis assays and in phagocytosis of apoptotic cells. Maturation of ESDM was found to be associated with reduced capacity for directed migration and increased capacity for phagocytic clearance of apoptotic cells. These results show ESDM to be functionally active in sequential phases of interaction with apoptotic cells and establish these macrophage populations as useful models for further study of molecular mechanisms underlying the recognition and removal of apoptotic cells.

1. Introduction

Macrophages play diverse roles in tissue homeostasis, development, immunity, inflammation, tissue repair and regeneration. Such functional diversity is reflected in the broadly diverse activation states that macrophages display in response to their environment. Current understanding of macrophage biology has been generated to a significant extent from reductionist investigations of macrophages and their monocyte precursors in isolation in vitro. Two main approaches have been used: (a) isolation of primary macrophages or their precursors followed by culture to mature and/or activate them, and (b) culture of continuous macrophage-lineage cell lines, such as the widely used J774 and RAW 264 lines, derived from malignant tumours (Ralph and Nakoinz, 1975; Raschke et al., 1978). Each of these approaches has its obvious advantages and disadvantages. Acquisition of primary cells requires access to living organisms, often disruptive isolation procedures, and produces issues over purity of cell populations and limitations in cell numbers. Furthermore, primary cells are inherently difficult to manipulate genetically which limits their use for studying the molecular mechanisms associated with their function. By contrast, macrophage cell lines provide supplies of limitless numbers of pure cell populations that can be readily manipulated but as they are transformed cells they are far removed from the normal state. They do, however retain certain features of normal macrophages and their precursors and consequently are widely used in macrophage research.

Rapid progress in embryonic stem cell (ESC) culture and the development of efficient differentiation strategies over

Abbreviations: BMDM, bone marrow-derived macrophages;CM, conditioned medium;DC, dendritic cell;EB, embryoid bodies;EBV, Epstein–Barr virus;ESC, embryonic stem cell;ESDM, embryonic stem cell-derived macrophages;LIF, leukaemia inhibitory factor

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doi:10.1016/j.jim.2012.06.008

recent years has provided access to another in vitro source of macrophages. Derivation of macrophages and their precursors from ESCs, at least in part, combines the advantages of tumour cell lines – including avoidance of in vivo access and isolation problems – with the preferred option to study essentially normal cells, rather than their neoplastic, transformed counterparts. ESCs can be expanded in large numbers in vitro and are highly amenable to sophisticated genetic manipulation technology so they have the potential to provide highly pure populations of wild-type or genetically manipulated macrophages and their precursors representative of tissue-specific settings. For example, murine ESC-derived macrophages (ESDM) have been applied to the investigation of macrophage activities in atherosclerotic lesions (Moore et al., 1998) and microglial cells (Napoli et al., 2009) as well as general macrophage physiology (Chawla et al., 2001; Odegaard et al., 2007). Similarly, human ES cells and haemopoietic stem cells have been used as productive sources of human macrophages for mechanistic studies (Karlsson et al., 2008; Subramanian et al., 2009; Way et al., 2009). Furthermore, cells apparently comparable with bone marrow-derived macrophages may be derived in vitro from induced pluripotent stem cells (Way et al., 2009).

Given the plasticity of macrophages and the scope for minor changes in culture conditions to change profoundly their activation status, each methodological approach requires optimisation in order to achieve reproducibility in phenotype and function of the output cells in appropriate applications. Here we describe an optimised and convenient method for the production of supplies of murine macrophages and their precursors from ESCs with particular consideration of their application to in vitro studies of macrophage chemotaxis and phagocytic clearance of apoptotic cells — a fundamentally important macrophage activity. We compare the purity of these ESDM with bone marrow-derived and peritoneal macrophage populations, the primary cells most often used in murine macrophage studies. Our findings indicate that ESDM can be produced in required numbers at high purity and can be applied effectively to in vitro studies of migration and apoptotic-cell clearance. Furthermore the results show that the migratory and phagocytic responses of these cells are reciprocally related, being dependent upon their maturation state: 'younger', less mature cells responded more vigorously than more mature ESDM to chemotactic stimuli whereas more mature ESDM were more active in phagocytosis than their precursors. These results establish ESDM as a potentially valuable in vitro model system for studies of detection and removal of apoptotic cells by mononuclear phagocytes.

2. Materials and methods

2.1. Cell lines

The murine E14 ESC line (Handyside et al., 1989) was used for most of the investigations. For some experiments the ESC line, GFP#7a, which expresses enhanced green fluorescent protein (eGFP) constitutively (Gilchrist et al., 2003), was used. Both lines behaved similarly in the macrophage differentiation protocols described. ESC lines were maintained as undifferentiated cells in 0.1% gelatin-coated tissue culture flasks in GMEM (Life Technologies Ltd, Paisley, UK) supplemented with 10% (v/v) batch tested foetal bovine serum (FBS, Globepharm Ltd, Guildford, Surrey, UK), 1% (v/v) non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, Poole, Dorset, UK) and 100 U/mL leukaemia inhibitory factor (LIF) that had been produced from *Lif*-transfected COS-7 cells as previously described (Jackson et al., 2010). We designate the media used to maintain ESCs in their undifferentiated, self-renewing state as $GMEM_{SR}$. The EBV-negative human Burkitt lymphoma (BL) cell line, BL2 and its Bcl-2-expressing counterpart transfectant line, BL2-bcl-2, were derived and cultured as described (Wang et al., 1996).

2.2. Macrophage differentiation

Macrophage differentiation of ESCs was performed in a medium we termed $ESDM_{Diff}$. This medium is similar to $GMEM_{SR}$ except that (1) LIF was excluded from the medium and (2) the FCS used for differentiation had been pre-screened for optimal haematopoietic differentiation (Jackson et al., 2010). Furthermore, $ESDM_{Diff}$ contained recombinant IL-3 (Stem Cell Technologies, Genoble, France) and L929-derived macrophage colony-stimulating factor (M-CSF; CSF-1). L929 fibroblasts were cultured in D-MEM: F12 with Glutamax (Life Technologies) supplemented with 10% FCS (Biosera Ltd, Ringmer, East Sussex, UK), 100 IU/mL penicillin (PAA Laboratories Ltd, Yeovil, Somerset,UK) and 100 μg/mL streptomycin (PAA Laboratories). Conditioned medium (CM) containing CSF-1 was harvested from adherent L929 cells 3 days following confluency, filtered through a 0.22 μm membrane to remove cell debris and stored at −20 °C as described (Burgess et al., 1985). L929 CM was included in $ESDM_{Diff}$ at a final concentration of 15% (v/v).

The culture media used for ESC culture and macrophage differentiation are summarized in Table 1.

2.3. Preparation of primary macrophages

Bone marrow-derived macrophages (BMDM) were prepared from Balb/c mouse femurs as described (Truman et al., 2004). Briefly, bone marrow was flushed into a 50 mL conical tube (BD Falcon) with $DMEM_{supp}$ (DMEM: F12 with Glutamax (Life Technologies), 100 IU/mL penicillin with 100 μg/mL streptomycin, 20% (v/v) FBS (Biosera) and 10% (v/v) L929 cell conditioned medium) using a 10 mL syringe (BD Plastipak) and a 26G needle (BD Microlance). Cells were then passed through a 23G needle and cultured in 20 mL $DMEM_{supp}$ in 95 mm diameter bacteriological grade Petri dishes at 37 °C in humidified 5% CO_2 in air. Culture medium was replaced after 2 h and again after 4 days. Differentiated macrophages were used for experiments after a further 3 days in culture. Peritoneal macrophages were prepared by peritoneal lavage of a normal Balb/c mouse with PBS. Recovered cells were centrifuged, resuspended in 20 ml $DMEM_{supp}$ and cultured at 37 °C in humidified 5% CO_2 in air in 95 mm diameter bacteriological grade Petri dishes for 2 h after which non-adherent cells were removed by washing.

2.4. Detachment of macrophages

Cell culture medium was removed and macrophages were washed twice with Hanks' BSS (without Ca^{2+}/Mg^{2+}/phenol