Immunobiology 219 (2014) 737–745

Contents lists available at ScienceDirect

Immunobiology

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

Spleen-derived macrophages are readily polarized into classically activated (M1) or alternatively activated (M2) states

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ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form 25 April 2014
Accepted 29 May 2014
Available online 6 June 2014

Keywords:
Macrophages
LCMV
Spleen
Phagocytosis
CD4

ABSTRACT

Bone marrow derived macrophages (BM-MΦ) that differentiate from precursor cells can be polarized into classically activated pro-inflammatory (M1) or alternatively activated (M2) states depending upon the cytokine microenvironment. We questioned whether tissue MΦ, such as spleen-derived MΦ (Sp-MΦ), have the ability to differentiate into M1 or M2 cells. We show in response to activation with IFN-gamma (IFN-γ) and lipopolysaccharide (LPS), that the Sp-MΦ readily acquired an M1 status indicated by up-regulation of iNOS mRNA, nitric oxide (NO) production, and the co-stimulatory molecule CD86. Conversely, Sp-MΦ exposed to IL-4 exhibited increased levels of mannose receptor (CD 206), arginase-1 (Arg)-1 mRNA expression, and significant urea production typical of M2 cells. At this stage of differentiation, the M2 Sp-MΦ were more efficient at phagocytosis of cell-associated antigens than their M1 counterparts. This polarization was not indefinite as the cells could revert back to their original state upon the removal of stimuli and exhibited flexibility to convert from M2 to M1. Remarkably, both M1 and M2 Sp-MΦ induced more CD4 expression on their cells surface after stimulation. We also demonstrate that adherent macrophages cultured for a short term in IL-4 enhances ARG-1 and YM-1 mRNA along with increasing urea producing capacity: traits indicative of an M2 phenotype. Moreover, in response to *in vivo* virus infection, the adherent macrophages obtained from spleens rapidly express iNOS. These results provide new evidence for the polarization capabilities of Sp-MΦ when exposed to pro-inflammatory or anti-inflammatory cytokines.

Introduction

Macrophages (MΦ) are members of the mononuclear phagocyte system (MPS) critical for bridging innate and adaptive immune responses (hesse Geissmann et al. 2010). MΦ play key roles in the detection of invading pathogens, presenting their antigens in the context of major histocompatibility complex (MHC)-I and activation of cytotoxic effector CD8+ T cells (CTLs) (Pozzi et al. 2005; Asano et al. 2011; Martinez-Pomares and Gordon 2012). MΦ populate various organs and tissue constituting bone marrow (BM)-MΦ, resident spleen MΦ, and alveolar MΦ among others (Gordon and Taylor 2005). Within lymphoid tissues, MΦ have been demonstrated to populate the T cell zone and directly interact with T cells *in vivo* (Asano et al. 2011; Miyake et al. 2007). Spleen-MΦ, such as marginal zone MΦ (MZM) and marginal metallophillic MΦ (MMM) are highly proficient in phagocytic and scavenger activities, and are likely to play an important role in modulating immune responses (Backer et al. 2010; Okamoto et al. 2008; Aichele et al. 2003).

It was previously demonstrated that further differentiation of spleen-derived (Sp)-MΦ *in vitro* can occur in the presence of macrophage colony stimulating factor (MCSF) and that such Sp-MΦ were efficient at antigen cross-presentation (Alatery and Basta 2008; Alatery et al. 2010). Moreover, *in vivo* evidence suggests that splenic MΦ may cross-present antigen during parasite infection (Martinez-Pomares and Gordon 2012; Chtanova et al. 2009). It has also been shown that splenic MΦ can be important in the maintenance of tolerance to self-antigens (McGaha et al. 2011). Altogether, the evidences indicate that splenic MΦ are likely to play an instrumental role in regulating certain parameters in innate and adaptive immunity.

It has been proposed that tissue-resident MΦ are fully differentiated in their respective tissue (Taylor et al. 2005). Despite this dogma, alveolar and peritoneal MΦ displayed remarkable plasticity in response to environmental signals to polarize into pro-inflammatory (M1) or anti-inflammatory (M2) MΦ (Cassol et al. 2010; Mora et al. 2006; Bastos et al. 2002). That said, the capacity, and degree of polarization remains uncharted in many MΦ populations isolated from secondary lymphoid organs. Classically, generation of M1 MΦ requires two signals; priming with IFN-γ followed by LPS stimulation, and M2 MΦ only requires IL-4

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http://dx.doi.org/10.1016/j.imbio.2014.05.005
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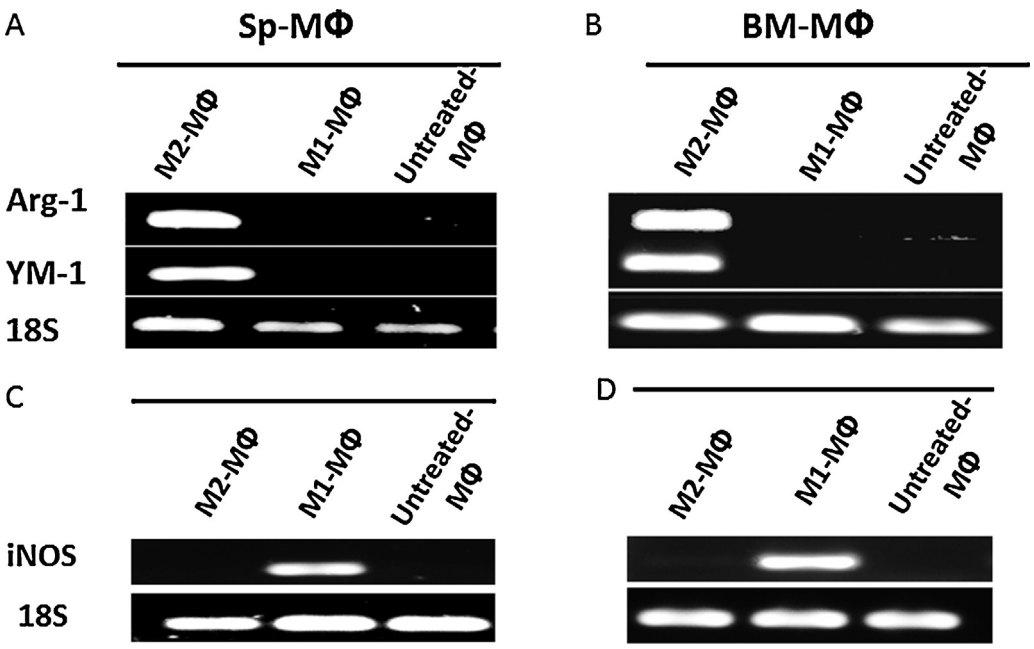

Fig. 1. Gene expression in M1 *vs.* M2 cells from MΦ derived from spleen or bone-marrow. RT-PCR was performed on untreated, M1 and M2 MΦ in both Sp-MΦ and BM-MΦ to examine the expression of Arg-1, YM-1 and iNOS. For M1 treatment, Sp-MΦ and BM-MΦ were primed with IFN-γ overnight and stimulated for 6 h with LPS before RNA isolation. M2 cells were treated overnight with IL-4 while untreated macrophages were left in RPMI before isolation of RNA. RNA was reverse transcribed to cDNA and 18S was used as a loading control. Data for (a) Sp-MΦ, (b) BM-MΦ, (c) Sp-MΦ, and (d) BM-MΦ are representative of two independent trials of cell isolations and culturing at two different occasions.

treatment (Biswas and Mantovani 2010). Much of this M1–M2 paradigm has been mainly established with BM-MΦ. Such cells are derived from precursor cells in the bone marrow that easily differentiate into particular types of the cells in the presence of the appropriate cytokines (Edwards et al. 2006). Since nothing is known about the polarization capacity of Sp-MΦ, we posed the question whether this was possible.

Here, we evaluated Sp-MΦ polarization using biochemical and functional assays and observed that Sp-MΦ are readily polarized into M1 and M2-like states following treatment with IFN-γ + LPS or IL-4, respectively. M1 cells displayed increased iNOS mRNA, NO production, and cell surface expression of CD86 (Edwards et al. 2006). On the other hand, M2 cells had enhanced levels of Arg-1, urea production and total mannose receptor (CD206) (Stein et al. 1992). Interestingly, Sp-MΦ polarization into M1 or M2-like cells resulted in striking differences in the ability of MΦ to phagocytose cell-associated antigen, with M2 exhibiting the higher phagocytic capacity. Using a traditional MΦ enrichment culture technique (Boehmer et al. 2005), we demonstrated the plasticity of adherent MΦ from the spleen can be polarized to M2 status in short term IL-4 culture. Conversely, following *in vivo* virus infection adherent MΦ enriched from the spleen of infected mice had enhanced iNOS expression. This work enhances our understanding of how Sp-MΦ respond to external cues and the effects that they have on their phagocytic function – important in pathogen clearance (Underhill and Goodridge 2012). More so, these results point to the possibility that splenic MΦ may exhibit phenotypic plasticity depending on the type of infection *in vivo*.

Results

IL-4 stimulated Sp-MΦ upregulate expression of gene profiles typical of M2 cell phenotype

To address the plasticity of Sp-MΦ, we first monitored relative mRNA levels of iNOS and arginase-1 by reverse transcriptase polymerase chain reaction (RT-PCR). These two enzymes are central to the metabolism of L-arginine and are reciprocally up regulated following M1 and M2 polarization, respectively (Ho and Sly 2009). As evident in the first columns of Fig. 1A and B, IL-4 treatment of both Sp-MΦ and BM-MΦ (M2-MΦ) induced strong expression of Arg-1 mRNA compared to M1-MΦ and untreated MΦ. We also tested for the expression of the YM-1, a homologue to human eosinophil chemotactic factor shown to be increased in M2 murine MΦ (Raes et al. 2002). YM-1 mRNA expression was induced in IL-4 treated Sp-MΦ and BM-MΦ (M2-MΦ), but was absent in M1 and untreated MΦ indicating that both Sp-MΦ and BM-MΦ upregulate the expression of M2 specific markers following IL-4 treatment. To monitor the M1 status, we evaluated the expression of iNOS (Fig. 1C and D), the enzyme responsible for production of NO (Edwards et al. 2006). In response to IFN-γ + LPS both Sp-MΦ and BM-MΦ (M1-MΦ, middle column) showed a strong induction of iNOS mRNA levels compared to M2 and untreated macrophages MΦ where no iNOS mRNA was detected. These data collectively point towards the ability of Sp-MΦ to be polarized to an M1 and M2 status *in vitro*.

Sp-MΦ upregulate arginase activity following IL-4 treatment

Arginine metabolism has been described as a means of defining MΦ polarization (Hesse et al. 2001). We examined the enzymatic activities of Arg-1 and iNOS by monitoring the levels of urea and nitric oxide (NO) respectively. Urea production is indicative of enhanced Arg-1 activity and an M2 activation profile (Ho and Sly). To test Arg-1 activity, MΦ were stimulated with IL-4 as described in section "Methods", followed by quantification of urea in cell lysate 24 h post stimulation. As shown in Fig. 2A, IL-4 treatment of Sp-MΦ and BM-MΦ (M2) resulted in a significant increase in the capacity to produce urea compared to un-stimulated control. On the other hand, no significant urea production was detected in IFN-γ-primed and LPS-stimulated (M1) compared to un-stimulated. These