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Comparison of the characteristics of macrophages derived from murine spleen, peritoneal cavity, and bone marrow*

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Abstract: Macrophages have a diverse set of functions based upon their activation states. The activation states, including resting (M0) and polarizing (M1 and M2) states, of macrophages derived from the mouse bone marrow, spleen, and peritoneal cavity (BMs, SPMs, and PCMs, respectively) were compared. We evaluated the macrophage yield per mouse and compared the surface markers major histocompatibility complex (MHC) II and CD86 by flow cytometry. The relative mRNA levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, mannose receptor (MR), and Ym1 in the M0, M1, and M2 states were also compared using real-time polymerase chain reaction (PCR) analysis. Bone marrow yielded the most macrophages with the best homogeneity, but they were polarized toward the M2 phenotype. All three types of macrophages had the capacity to polarize into the M1 and M2 states, but SPMs had a stronger capacity to polarize into M1. The three types of macrophages showed no differences in their capacity to polarize into the M2 state. Therefore, the three types of macrophages have distinct characteristics regardless of their resting or polarizing states. Although bone marrow can get large amounts of homogeneous macrophages, the macrophages cannot replace tissue-derived macrophages.

Key words: Macrophage; Murine splenocytes; Murine bone marrow; Peritoneal cavity
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1 Introduction

Macrophages play an important role in the innate immune response and link innate and adaptive immune responses (Gordon and Martinez, 2010; Feng and Mao, 2012). Increasing evidence supports the role of macrophages in promoting inflammatory responses (classical activation or M1 macrophages) and in tissue remodeling, wound healing, and immune regulation (alternate activation or M2 macrophages) (Biswas and Mantovani, 2010; Lee *et al*., 2011; Lu *et al*., 2013; Thornley *et al*., 2014; Das *et al*., 2015). Investigations into the different mechanisms of conversion provide insights into the treatment of diseases of the autoimmune system, regulation of transplantation immunology, and control of tumor infiltration (Lameijer *et al*., 2013; Alagesan and Griffin, 2014; Jiang, 2015; Sica *et al*., 2015).

The bone marrow, spleen, and the peritoneal cavity are common sources of macrophages for research. Yet, the origin of and differences among macrophages from these different sources are controversial. We isolated macrophages from the bone marrow (BMs), spleen (SPMs), and peritoneal cavity (PCMs) of mice. Although macrophages are divided

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into M1 and M2 in vitro according to different stimulations, researchers suggest that in vivo these two types of macrophages represent extreme states and that macrophages in vivo may be in mixed states of M0, M1, and M2 (Duffield, 2010; Lee *et al.*, 2011). Therefore, we evaluated their activation states, including the resting (M0) and polarizing (M1 and M2) states, and analyzed differences in yield.

2 Materials and methods

2.1 Experimental animals

Eight-week-old male BALB/c mice obtained from the Animal Resources Center of Xi'an Jiaotong University (Xi'an, China) were used in this study. All the animals were maintained under constant laboratory conditions at (22±2) °C and (45±5)% humidity, with a 12-h day/night cycle and free access to food and water. The Animal Ethics Committee of Xi'an Jiaotong University approved all procedures.

2.2 Macrophage isolation and polarization

BMs were acquired according to the protocols of Weischenfeldt and Porse (2008). Mouse bone marrow was flushed from the femurs and washed through a 40-μm nylon mesh and red blood cells were lysed in lysis buffer (10 mmol/L $KHCO_3$, 0.15 mmol/L NH_4Cl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA)). The resulting cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), 20 ng/ml macrophage colony-stimulating factor (M-CSF; Peprotech, London, UK), 1% glutamine (Life Technologies, MA, USA), and 1% penicillin/streptomycin (Life Technologies, MA, USA), and grown in 5% CO_2 at 37 °C. After 2 to 3 d, the adherent cells were rinsed and immersed in fresh medium. On Day 7, the adherent cells were cultured with normal medium (DMEM/F12 medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin) for 48 h to obtain M0 (resting) macrophages, stimulated with interferon-γ (IFN-γ; 100 U/ml, Peprotech) and lipopolysaccharide (LPS; 100 ng/ml, Sigma) to obtain M1 macrophages, and stimulated with interleukin-4 (IL-4; 20 ng/ml, Peprotech) to polarize to M2 macrophages.

SPMs were isolated and purified as described previously (Lu *et al.*, 2013). Briefly, mouse spleens were harvested and perfused with ice-cold DMEM/F12 medium to remove blood monocytes, then triturated with sterile syringes. The centrifuged cell pellet was suspended in a lysis buffer (10 mmol/L $KHCO_3$, 0.15 mol/L NH_4Cl, 0.1 mmol/L EDTA) to lyse red blood cells. The resulting cells were filtered through a 40-μm nylon mesh. The cells were cultured under 5% CO_2 at 37 °C. After 40 min, the culture supernatant was discarded and the adherent cells were rinsed three times and cultured as above to obtain M0, M1, or M2 macrophages.

PCMs were isolated and cultured as described by Ray and Dittel (2010). Briefly, the peritoneal cavity was exposed and injected with 5 ml ice-cold DMEM/F12 medium. About 5 to 10 min later, the PCMs were collected in a fluid state and the procedures were repeated. The acquired cells were cultured in 5% CO_2 at 37 °C for 40 min. The adherent cells were rinsed and cultured for 48 h as above to obtain M0, M1, or M2 macrophages. The purity of each of the three types of macrophages was above 95%.

2.3 Flow cytometry

The purity of the isolated macrophages was analyzed by staining the cells with PE-conjugated anti-mouse F4/80 (eBioscience, USA). The macrophage surface markers were analyzed using allophycocyanin (APC)-conjugated anti-mouse major histocompatibility complex (MHC) II, CD86 (BD Biosciences, USA). To eliminate nonspecific staining, isotype control antibody, matched to the surface marker antibody's host species and class, was added to another tube that contained the same number of cells as in the surface markers antibody tube. The following control antibodies were included in the assays: anti-F4/80 rat IgG2a, anti-MHC II rat IgG2b, and anti-CD86 IgG against Armenian hamster. A FACSCalibur cytometer and CellQuest software (BD Biosciences) were used to analyze the percentage of positive cells compared with fluorescence-labeled isotype controls.

2.4 Real-time PCR analysis

Total RNA was isolated and reverse-transcribed with real-time polymerase chain reaction (PCR) using the SYBR Master Mix according to the manufacturer's instructions (Invitrogen) on a ABI 7500 Fast Real-Time PCR System machine. The following primers (forward and reverse) were used: tumor necrosis factor-α (TNF-α), 5'-GCTGAGCTCAAACCCTGG

TA-3', 5'-CGGACTCCGCAAAGTCTAAG-3'; interleukin 1β (IL-1β), 5'-TGTGAAATGCCACCTTTTGA-3', 5'-TGTCCTCATCCTGGAAGGTC-3'; mannose receptor (MR), 5'-CAAGGAAGGTTGGCATTTGT-3', 5'-CCTTTCAGTCCTTTGCAAGC-3'; Ym1, 5'-CAGCTGGGATCTTCCTACCA-3', 5'-ATTCTGCATTCCAGCAAAGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGGCAAAGTGGAGATTGTTGCC-3'; 5'-AAGATGGTGATGGGCTTCCCG-3'. Gene expression was normalized to individual GAPDH. The profile was obtained by plotting gene expression levels relative to those of BMs.

2.5 Statistical analysis

Student's *t*-test was used for statistical analysis. $P<0.05$ was considered to be significantly different.

3 Results

3.1 Macrophage yield

To provide a reference for macrophage research, we compared the macrophage yield per mouse. First, the proportion of macrophages was analyzed in a single-cell suspension from each source. Macrophages accounted for a small proportion (3.5%) of the single-cell suspension from the spleen, resulting in a small yield after purification (Figs. 1a and 1c). In contrast, the peritoneal cavity contained a large proportion (35%) of macrophages. However, the macrophage yield was related to the total single-cell yield per mouse.

We quantified the single-cell differences before and after macrophage purification. We detected the maximum number of single spleen cells and a small yield of macrophages. Bone marrow generated more single cells and macrophages (Figs. 1b and 1c). Although macrophages accounted for a large proportion of the cells from the peritoneal cavity, the total number of single cells retrieved was only 2×10^6–3×10^6, which suggested a low yield (Figs. 1b and 1c). The large yield of BMs was attributed to the division of myeloid progenitors into cells of macrophage/monocyte lineage (Lawrence and Natoli, 2011).

3.2 Macrophage heterogeneity

Macrophages at different locations may represent different states of heterogeneity (Gordon *et al.*, 2014; Komohara *et al.*, 2014). We observed the size

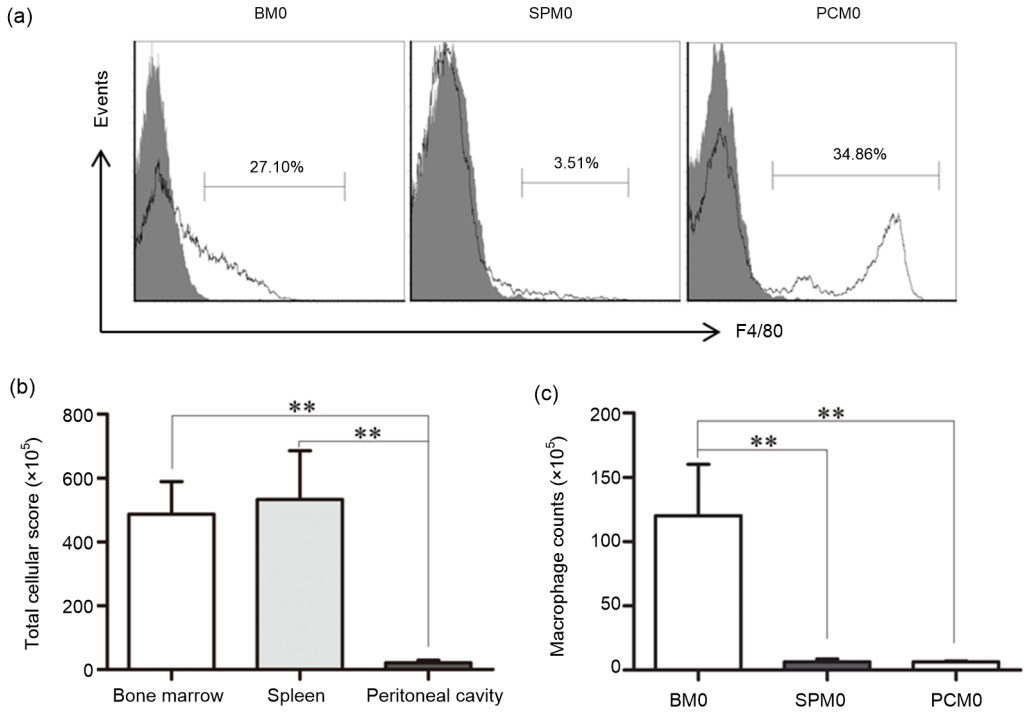

Fig. 1 Comparison of the yield of macrophages from three sources

(a) Macrophages in single-cell suspension (black lines); (b) Total cellular score per mouse before purification; (c) Macrophage yield per mouse after purification. Data are expressed as mean±standard error of the mean (SEM). Results are representative of three independent experiments. ** $P<0.01$

and granularity of each category of macrophages using flow cytometry. We found that BMs were clustered in the forward scatter and side scatter in the flow cytometry data compared to the SPMs and PCMs, which were dispersed among a minimum of two populations (Fig. 2). These results suggest that SPMs and PCMs have distinct heterogeneity. BMs were the most homogeneous due to M-CSF induction.

3.3 BMs were partially polarized to M2 and SPMs showed M1 polarization

Macrophages exist in diverse activation states due to their different physiological roles. We compared their activation states in M0 as reflected by markers or correlation factors expressed and secreted by the macrophages. Initially, we evaluated the expression of the surface markers MHC II and CD86 in the inflammatory microenvironment. We found that BMs expressed low MHC II and CD86 levels (<10%; Figs. 3 and 4) in contrast to SPMs and PCMs (P<0.05), and SPMs expressed high levels of MHC II and CD86 (>15%) compared with PCMs and BMs, although CD86 expression did not differ significantly (Fig. 4a) between SPMs and PCMs. Second, we found that the SPMs showed up-regulated TNF-α (P<0.05) and IL-1β (P<0.01) expression, whereas BMs exhibited up-regulation of Ym1 (also known as chitinase 3-like 3 and eosinophil chemotactic factor-L (ECF-L), and produced primarily by macrophages during inflammation) and MR (P<0.01; Fig. 4b). Compared to BMs, PCMs expressed high MHC II and CD86 and up-regulated IL-1β expression, but showed no change in TNF-α expression (P>0.05; Figs. 4a and 4b). The mRNA levels of TNF-α and IL-1β are up-regulated in M1, while MR and Ym1 are up-regulated in M2 (Raes *et al*., 2002; Lu *et al.*, 2013). A comprehensive analysis of these results suggested that BMs were partially polarized to the M2 phenotype and SPMs manifested M1 features. SPMs showed M1 polarization and PCMs manifested some M1 features.

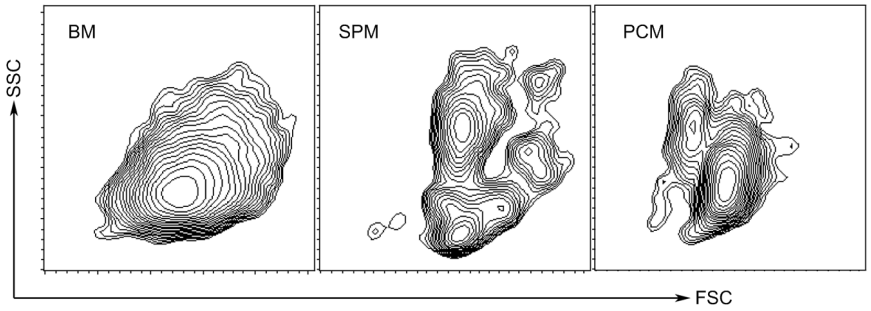

Fig. 2 Comparison of the size and granularity of macrophages from different sources
SSC: side scatter; FSC: forward scatter

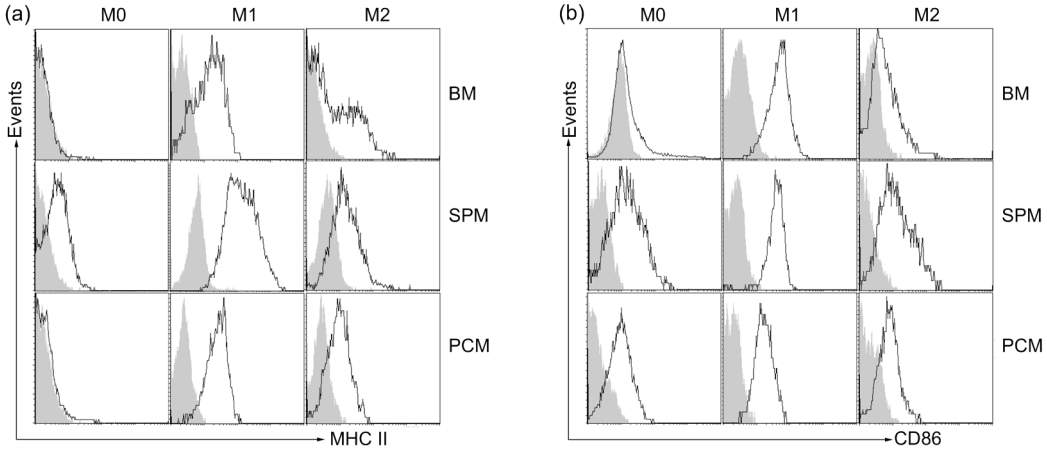

Fig. 3 Representative flow cytometry histograms
MHC II (a) or CD86 (b) (black lines) and relevant isotype controls (grey-filled histograms) were stained on the surfaces of the three types of macrophage in M0, M1, and M2 states

3.4 All three types of macrophages showed the capacity to polarize into M1 and M2 phenotypes

The three types of macrophages showed different activation states in the M0 state. We investigated their potential to polarize following in vitro stimulation. We assessed the surface markers MHC II and CD86 and relative mRNA levels of TNF-α, IL-1β, MR, and Ym1 when the macrophages were stimulated with IFN-γ and LPS for 48 h to differentiate M1, or treated with IL-4 for 48 h to induce the M2 state. In the

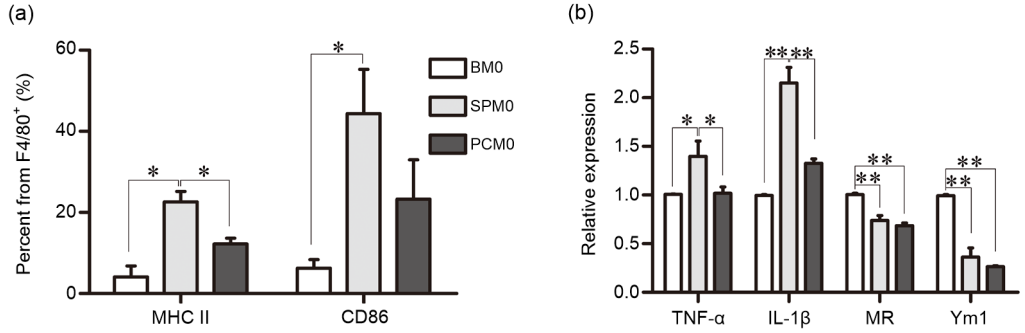

Fig. 4 Macrophage states in M0 analyzed by flow cytometry and real-time PCR

MHC II and CD86 expression (a) and the GAPDH-normalized mRNA expression (b) of TNF-α, IL-1β, MR, and Ym1. Data (mean±SEM) represent three separate experiments. * P<0.05, ** P<0.01

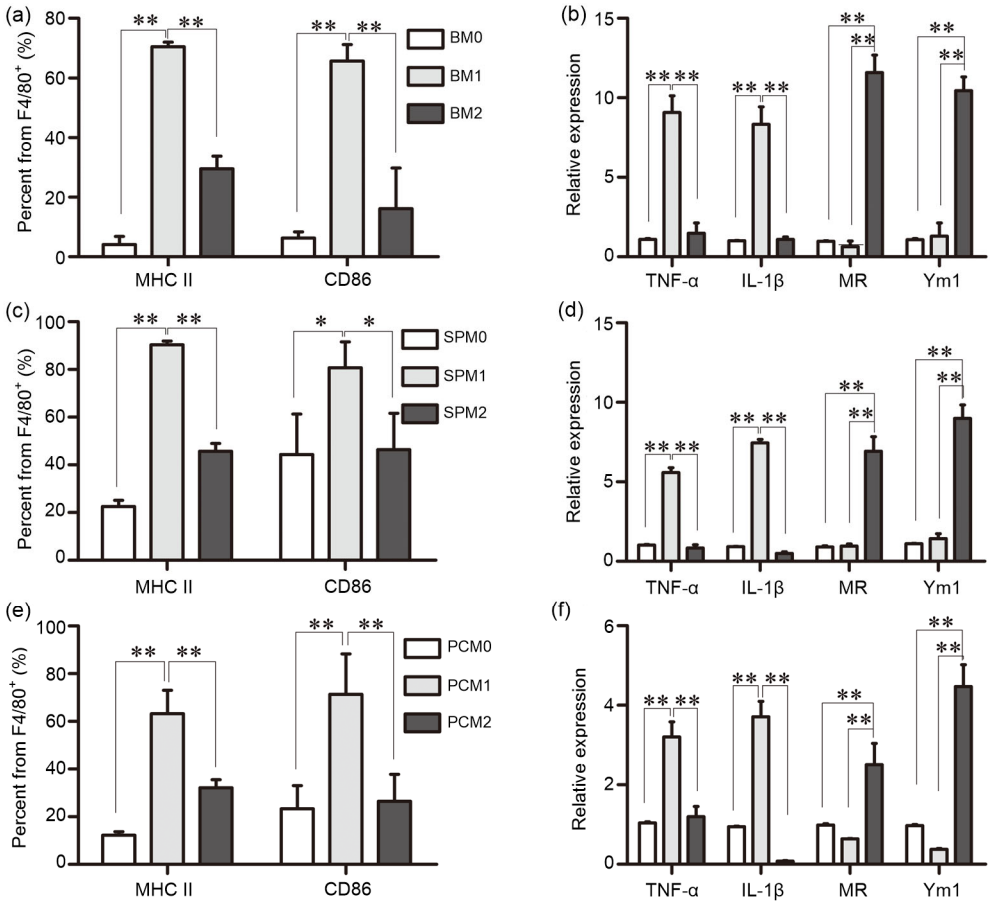

Fig. 5 Three kinds of macrophages polarized to the M1 or M2 phenotype

MHC II and CD86 expression of BM (a), SPM (c) and PCM (e) was measured in M0, M1, and M2 states. GAPDH-normalized mRNA expression of TNF-α, IL-1β, MR, and Ym1 was assessed in M0 (b), M1 (d), and M2 (f) states. Data (mean±SEM) are representative of three separate experiments. * P<0.05, ** P<0.01

macrophages from all sources, IFN-γ and LPS induced MHC II and elevated CD86 expression (P<0.05), up-regulated TNF-α and IL-1β expression in contrast to M0 and M2 (P<0.01), but down-regulated Ym1 and MR expression compared with M2 (P<0.01; Fig. 5). Nevertheless, the expression pattern of M2 polarized macrophages differed from that of M1 macrophages. Therefore, we concluded that the three types of macrophages had the capacity to polarize into M1 and M2 phenotypes.

3.5 All three types of macrophages showed the capacity to polarize into M1 and M2 phenotypes

The three types of macrophages showed a capacity to polarize to the M1 and M2 phenotypes. The differences between the M1 and M2 states were analyzed for the three sources. The comparative data displayed in Fig. 6 show that SPM1 expressed higher MHC II and CD86, and higher mRNA levels of TNF-α and IL-1β under the M1 state (P<0.01). Compared with the M2 state, IL-1β and MR levels were higher, and the Ym1 level was lower in PCMs (P<0.05), suggesting that SPMs had a stronger capacity to polarize to M1 but the three macrophages showed no difference in their capacity to polarize to M2.

4 Discussion

Although macrophages have not been viewed as terminally differentiated cells in recent years and are distributed in many tissues (Davies and Taylor, 2015; Lavin *et al.*, 2015), isolation of vast numbers of mature macrophages is still difficult. The peritoneal cavity is a common source of primary mouse macrophages. Diverse immune cell types occur in the peritoneal cavity, including B- and T-cells, macrophages, and natural killer (NK) cells. We obtained primary macrophages using peritoneal lavage with culture medium to avoid the polarization or conversion of macrophage states associated with the use of Brewer's thioglycollate broth or proteose peptone (Zhang *et al.*, 2008). The yield, however, was typically only 2 to 3 million peritoneal cells from an unmanipulated mouse. Among the cell types in the peritoneal cavity, 35% were macrophages. Brewer's thioglycollate broth and proteose peptone increase the yield of macrophages, but alter the physiological characteristics of the cells (Hoover and Nacy, 1984). The spleen, as the major organ for immunity, contains large numbers of immune cells as confirmed experimentally, which are readily available as a single-cell suspension for purification by adhesion to obtain

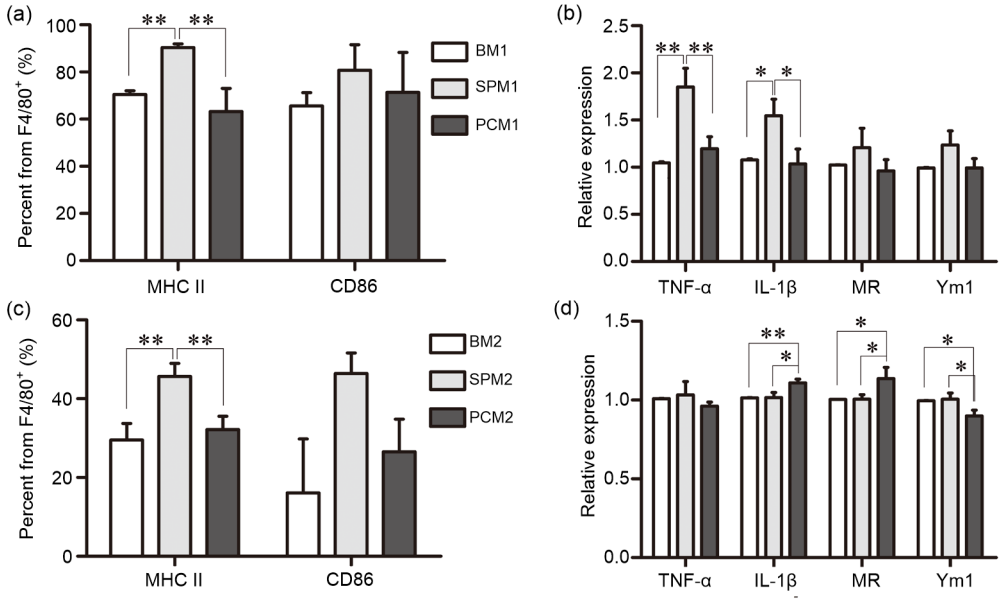

Fig. 6 Comparative ability of the three types of macrophages to polarize to M1 or M2

MHC II and CD86 expression of these macrophages was measured in M1 (a) and M2 (c) states. The GAPDH-normalized mRNA expression of TNF-α, IL-1β, MR, and Ym1 was assessed in M1 (b) and M2 (d) states. Data (mean±SEM) are representative of three separate experiments. * P<0.05, ** P<0.01

macrophages. The yields are minimal and the purity is variable. Prolonging the adhesion time decreases the purification, but increases the yield. The macrophages need to be purified via flow cytometry or magnetic bead separation. A duration of 40 to 60 min is appropriate for purification by adhesion. A large number of macrophages can be obtained only by increasing the number of mice. Bone marrow is a common source for acquiring large amounts of purified macrophages (Weischenfeldt and Porse, 2008; Parsa *et al.*, 2012). Inconvenient factors include the enormous cost of stimulating factors and several days of cultivation. However, their characterization requires investigation.

Tissue macrophages are derived mainly from the infiltration of monocytes from a macrophage and dendritic cell progenitor-derived pro-monocyte precursor in the bone marrow (Gordon and Taylor, 2005; Lawrence and Natoli, 2011), which enables the differentiation of the precursors into macrophages in vitro. However, macrophage differentiation requires specific growth factors including lineage-specific M-CSF to induce the proliferation and differentiation of committed myeloid progenitors to differentiate into macrophages. The myeloid progenitors cultured in vitro show relatively stable differentiation into macrophages, which suggests better homogeneity compared with SPMs and PCMs. In the spleen or peritoneal cavity, the presence of various immune cells and antigen factors enables the interaction of macrophages and results in heterogeneity. Although induced BMs are homogeneous, they cannot replace tissue-derived macrophages. Homogeneity does not translate to an adequate primary response. We found that BMs were partially polarized to M2 based on flow cytometry and PCR, suggesting that M-CSF polarized macrophages generate an increased number of anti-inflammatory factors. Granulocyte-macrophage (GM)-CSF induces macrophages to produce higher amounts of inflammatory factors (Chung *et al.*, 2015).

Macrophages express MHC II and CD86, a co-stimulatory molecule, to variable degrees depending on their activation states. They are highly expressed in inflammation circumstances and considered a biological marker of M1 (Wilson *et al.*, 2004). Although IL-4 can also induce MHC II expression, it does so in restricted macrophage populations and only HLA-DR and HLA-DP MHC class II molecules are induced (Gerrard *et al.*, 1990). In contrast, IFN-γ and LPS provide double signals to polarize macrophages and induce all three class II molecules on most macrophages (Cao *et al.*, 1989). Thus, the high expression of MHC II and CD86 induced by IFN-γ and LPS in contrast to the expression induced by IL-4 may reflect M1 activation. MR, a type of pattern recognition receptor, mediates the binding and ingestion of micro-organisms and its expression can be increased by IL-4. Ym1 is a novel mammalian lectin and can be synthesized by IL-4-activated macrophages (Raes *et al.*, 2002). Therefore, we analyzed MR and Ym1 expression for different states of macrophages to reflect the M2 state. TNF-α and IL-1β, proinflammatory factors, can represent the state of M1 (Mantovani *et al.*, 2004). Analysis of these indexes (MHC II, CD86, TNF-α, IL-1β, MR, and Ym1) should reflect the states of the macrophages, but this may also be limited and more research is needed.

The three types of macrophages show different states. Our experiment demonstrated that they all had the potential to polarize into M1 and M2 phenotypes, which further indicated that macrophage plasticity can enable disease control or treatment. However, differences in polarization strength may relate to different microenvironments. Strong M1 polarizing ability does not suggest weak M2 functions (tissue remodeling, wound healing, and immune regulation capacity) in vivo. Despite a strong capacity to polarize to M1, SPMs manifested stronger wound healing and tissue remodeling abilities under the M2 state, compared with BMs that failed renoprotection due to a proliferation-dependent phenotypic switch in vivo (Cao *et al.*, 2014). Therefore, the three types of macrophages have distinct characteristics regardless of their resting or polarizing states. Although the induced BMs are homogeneous, they cannot replace tissue-derived macrophages.

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Compliance with ethics guidelines

Yan-long ZHAO, Pu-xun TIAN, Feng HAN, Jin ZHENG, Xin-xin XIA, Wu-jun XUE, Xiao-ming DING, and Chen-guang DING declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：小鼠脾脏、腹腔和骨髓源性巨噬细胞特征比较

目　的：对比分析脾脏、腹腔和骨髓源性巨噬细胞（SPMs、PCMs 和 BMs）在安静及极化状态下的差异。

创新点：首次对比分析脾脏、腹腔和骨髓源性巨噬细胞在安静（M0）及极化状态（M1 和 M2）下的特征。

方　法：通过小鼠脾脏研磨及单细胞贴壁获得脾源性巨噬细胞；腹腔灌洗及细胞贴壁获得腹腔源性巨噬细胞；骨髓贴壁细胞在巨噬细胞集落刺激因子体外刺激下培养 7 天获得骨髓源性巨噬细胞。三种细胞即为 M0 型巨噬细胞，M0 在干扰素及脂多糖刺激下获得 M1 型巨噬细胞，M0 在白介素 4（IL-4）刺激下获得 M2 型巨噬细胞。通过流式细胞仪分析三种类型巨噬细胞在三种状态下的 II 类主要组织相容性复合体（MHC II）和 CD86 表达差异。通过实时荧光定量聚合酶链式反应（qPCR）检测肿瘤坏死因子 α（TNF-α）、白介素 1β（IL-1β）、甘露糖受体（MR）和类几丁质酶 3 样分子（Ym1）的表达变化。

结　论：骨髓贴壁细胞培养能获得最大量的同源巨噬细胞（图 1 和 2），但表型偏向于 M2 型巨噬细胞（图 3 和 4）。三种巨噬细胞均能极化为 M1 和 M2 型巨噬细胞（图 5），其中 SPMs 具有更强的 M1 型极化能力，而 M2 型极化能力之间未见明显差异（图 6）。综上所述，三种细胞无论在安静及极化状态下均具有不同的特征，骨髓可以获得大量同源性巨噬细胞，但性质不同于组织源性的脾脏和腹腔巨噬细胞。

关键词：巨噬细胞；脾脏；腹腔；骨髓