The Macrophage Heterogeneity: Difference Between Mouse Peritoneal Exudate and Splenic F4/80+ Macrophages

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Macrophages isolated from various tissues manifest differences in cell shape, the expression of surface markers, as well as metabolic and functional activities. However, the heterogeneity of macrophages expressing the same marker in different tissues has not been fully addressed. In the present study, mouse F4/80+ peritoneal exudate macrophages (PEMs) and splenic macrophages (SPMs) appeared similar in most respects. But the percentages of cells expressing CD80, CD40, MHC-II, TLR2, or TLR4, but not CD11c, CD54, or CD23, in freshly isolated F4/80+ SPMs were significantly higher than those in PEMs, whereas the levels of CD86+ cells in F4/80+ SPMs were markedly lower than those in PEMs. After lipopolysaccharide (LPS) stimulation, F4/80+ SPMs expressed significantly higher levels of CD86, CD40, or MHC-II than F4/80+ PEMs, but not CD11c, CD80, CD54, or CD23. F4/80+ SPMs had remarkably lower non-opsonic phagocytotic capacity against chicken RBCs or allo-T cells than PEMs as determined by two-photon microscopes and flow cytometry. SPMs produced markedly more NO than PEMs when cultured with LPS or allo-T cells. Furthermore, SPMs exhibited stronger immunogenicity than PEMs, as determined by the ability to stimulate T cell proliferation, delayed type hypersensitivity, and IFN-γ production. The data showed the differences between SPMs and PEMs with regard to the phenotypes, phagocytosis, and immunogenicity, which may offer important information for us to better understand the distinguished immune responses of macrophages in spleens and the peritoneal cavity.   J. Cell. Physiol. 209: 341–352, 2006. © 2006 Wiley-Liss, Inc.

Macrophages, as part of the first defense line (innate immunity) against invading microorganisms, viruses and transformed cells, are one of the critical initiators and regulators on immune responses in organisms (Gordon, 2003). Importantly, by releasing cytokines, presenting antigens or phagocytosis effects, macrophages also function in the secondary defense line, both humoral and cell-mediated immunity (Esashi et al., 2003). A common progenitor gives rise to tissue macrophages, dendritic cells (DCs) and osteoclasts, which are distinct, irreversibly differentiated sublineages. Once distributed through the blood stream, monocytes constitutively enter all tissue compartments of the body, including the peritoneal cavity, to differentiate into macrophages (Gordon, 2004). Resident macrophage populations in different organs, such as Kupffer cells in the liver, alveolar macrophages in the lungs, microglia in the central nervous system, and macrophages in spleens and the peritoneal cavity, adapt to their local microenvironment (Barrington et al., 2001; Gordon, 2003). In addition, recruited macrophages exhibit many phenotypic differences from resident tissue macrophages.

Different macrophage subtypes in various or same locations were referred to as macrophage heterogeneity (Shortman and Wu, 2004; Liu et al., 2005a). Macrophages in a different microenvironment display a wide diversity in terms of their function and morphology (Minto et al., 2003; Liu et al., 2005a). The heterogeneity of macrophages may be important for the diversity, flexibility and validity of innate and adaptive immune responses to all kinds of stimuli (Kaufmann and Schaible, 2005). Macrophage heterogeneity could conceivably originate through a variety of pathways.

Because macrophages only express germline-encoded and non-clonal receptors that can be modulated markedly in cell populations, their heterogeneity is

fundamentally and conceptually different from that of T and B cells, which express somatic-recombinant antigen-specific receptors (Gordon, 2003).

The peritoneal exudate macrophages (PEMs) are the most studied primary macrophages in mice because they are easily isolated by peritoneal lavage (Cailhier et al., 2005; Kaufmann and Schaible, 2005). Because the peritoneal environment, with a very low organ tension, is quite unusual, PEMs may have some unique properties. Given some inducing factor/factors, PEMs can differentiate into DCs. Some researchers even recognize PEMs as precursors of DCs, thus the idea that DCs and PEMs is in the end stage of cell differentiation was recently challenged (Monney et al., 2002; Esashi et al., 2003; Gordon, 2003; Lee et al., 2005b). On the other hand, because the spleen, with a rich blood supply, is responsible for filtering and clearing blood borne particles as well as one of the important peripheral immune organs, splenic macrophages (SPMs) are a widely heterogeneous population of cells and have important classical immunomodulatory effects. SPMs are an important component of the innate immune system, as evidenced by the incidence of septicemia following splenectomy (Sodhi et al., 2005). Interestingly, SPMs and PEMs may exhibit distinct alterations when they are exposed to certain chemicals or stimulations (Shortman and Wu, 2004; Stout and Suttles, 2005; Olsson and Sundler, 2006). It has been reported that in tumor-bearing mice, the cytotoxicity was significantly decreased in PEMs but markedly increased in SPMs (Gordon, 2004; Morrison et al., 2004). Although we have been aware of the macrophage diversity for quite a while, the differences between peritoneal and SPMs have not been fully addressed. To determine the basic physiological properties of PEMs and SPMs is essential for us to understand the different responses against the same stimulation of these two local macrophages. In the present study, in order to achieve a more detailed understanding of the unique properties of PEMs and the differences between PEMs and macrophages in other tissues, we have undertaken a comparing study on mouse $F4/80^+$ PEMs and SPMs with regard to their morphology and physiological functions, including cell size, microscope morphology, cellular phenotype, phagocytosis, immunogenicity, as well as cytokine and nitric oxide (NO) productions (Chomarat et al., 2003; Sodhi et al., 2005). This study may offer the basic information for us to better understand the different immune responses in various locations in organisms in mice.

MATERIALS AND METHODS
Animals

Five- to seven- week-old C57BL/6 (B6) (H-2^b), Balb/c (H-2^d), and C3H (H-2^k) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Monoclonal antibodies (mAbs)

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD40 mAb (3/32; IgG2a), FITC-labeled hamster anti-mouse CD54 (ICAM-1) mAb (3E2; IgG2a), FITC-labeled hamster anti-mouse CD80 (B7-1) mAb (16-10A1; IgG2b), FITC-conjugated rat anti-mouse CD86 (B7-2) mAb (GL1, IgG2a), FITC-labeled mouse anti-mouse H-

$2K^b$ mAb (AF6-88.5; IgG2a), FITC-labeled mouse anti-mouse I-A^b mAb (AF6-120.1; IgG2a), FITC-labeled hamster anti-mouse TCR β-chain mAb (H57-597, IgG2a), phycoerythrin (PE)-labeled rat anti-mouse CD4 mAb (RM4-5; IgG2a), PE-labeled rat anti-mouse CD8α mAb (53-6.7; IgG2a). FITC-conjugated anti-mouse F4/80 mAb (BM8), FITC-conjugated anti-mouse I-A^d mAb (39-10-8), FITC-conjugated anti-mouse CD11c mAb (HL3), FITC-conjugated anti-mouse CD23 mAb (B3B4), FITC-conjugated anti-mouse toll-like receptor 2 mAb (TLR2, clone mT2.7), FITC-conjugated anti-mouse TLR4 mAb (UT41), FITC-conjugated anti-mouse IFN-γ mAb (GIR-208). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory.

Preparation of PEMs and SPMs

Mouse peritoneal exudate cells were obtained from the peritoneal exudates of mice. Mouse splenocytes were prepared, and red blood cells were lysed with ACK Lysis buffer (Invitrogen, San Diego, CA) as described before (Yan et al., 2002). After the cells were washed twice with cold Hanks' solution, these cells were adjusted to 5×10^6 cells/ml in RPMI1640 medium (Gibco BRL, Grand Island, NY) and cultured in 2% gelatin (Sigma)-pretreated 6-well plates (Costar, Cambridge, MA) for 3–4 h at 37°C and 5% CO_2. The non-adherent cells were removed by washing them with warm RPMI1640 medium. The adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and readjusted to 1×10^6 cells/ml. The cell viability was usually more than 95% as determined by trypan blue exclusion. The macrophage purity was analyzed by a two-proton microscope LSM510 (Zeiss, Wetzlar, Germany) and flow cytometry (Becton Dickinson, Mountain View, CA), using macrophage marker F4/80. The adherent cells constituted more than 90% of $F4/80^+$ macrophages, as reported before (Delneste et al., 2003).

Morphology observation

Either Balb/c or C57BL/6 $F4/80^+$ PEMs and SPMs were cultured in RPMI1640 medium containing 2.0 mM L-glutamine (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin for 2 h so that they were adherent to the sterilized glass slides. For microscopic studies, the adherent cells were stained by Giemsa and Wright staining solution (Sigma) after using methanol to fix, as reported before (Ammon et al., 2000; Esashi et al., 2003).

Immunofluorescence staining and flow cytometry (FCM)

5×10^5 Balb/c or C57BL/6 mouse PEMs and SPMs were washed once with FACS buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% bovine serum albumin (BSA)). For two-color staining, cells were stained with PE-labeled anti-mouse F4/80 mAb versus FITC-labeled anti-I-A^d (39-10-8), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), CD54 (3E2), CD23 (B3B4), toll-like receptor 2 (TLR2, clone mT2.7), or TLR4 (UT41) mAb or the non-specific staining control mAb, respectively. Non-specific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least 10,000 cells were assayed by two-color FCM using a FASCalibur flow cytometry (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson). Non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained non-specifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs. Certain molecule expression levels were determined as the median fluorescence intensity (MFI) of the cells positively stained with the specific mAb. The cell size of F4/80 positive cells was determined by the analysis of the forward scatter side with gating on $F4/80^+$ cells.

The IFN-γ production in $CD4^+$ T cells stimulated by allogeneic PEMs and SPMs was detected using BD cytofix/cytoperm plus (with Golgi Plug^{TM}) intracellular staining kits (BD Biosciences PharMingen). The non-adherent splenocytes (2×10^6 cells/well) were co-cultured with allogeneic or syngeneic PEMs or SPMs (1×10^6 cells/well) in 6-well plates for 48 h. Cells were then pulsed with 1.0 μl/ml Brefeldin A (BD Golgi Plug; BD Biosciences PharMingen) for the last 8 h of culture. Suspended cells were collected and washed once with

FACS buffer. After incubation with FcR Blockade (2.4G2) and FITC-conjugated anti-mouse CD4 mAb (SK3; BD Biosciences PharMingen) in the dark at room temperature (RT) for 15 min, cells were washed once with staining buffer, then fixed and permeabilized with 500 µl of BD Cytofix/Cytoperm solution at RT in the dark for 20 min, according to the instruction offered by the manufacturer. After they were stained with 0.25 µg of anti-mouse IFN-γ mAb (GIR-208; BD Biosciences PharMingen) for 30 min at RT in the dark and washed three times, 10,000 CD4$^+$ cells were then analyzed by FCM (Morrison et al., 2004).

Allogeneic mixed leukocyte reactions (MLR)

Murine splenocytes were prepared using the sterile technique as described before (Zhao et al., 2003a). CD4$^+$ T cells were purified by negative selection of mouse splenocytes using mouse CD4$^+$ T lymphocyte enrichment set-DM (BD Biosciences PharMingen). Cells were suspended in RPMI1640 medium supplemented with 10% (vol/vol) mouse serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids (GibcolBrl), 1 mM sodium pyruvate, 10 U/ml penicillin and 10 µg/ml streptomycin, 10 mM HEPES buffer (GibcolBrl), and 10 µM 2-mercaptoethanol (Sun et al., 2006). Triplicate wells containing 2×10^5 responders with 1×10^5 or with the indicated doses of allogeneic macrophage stimulators (pretreated with 50 µg/ml mitomycin C) in a total volume of 0.2 ml of medium were incubated in U-bottomed 96-well microplates (Costar) at 37°C in 5% CO$_2$. Duplicate plates were pulsed with 1 µCi of ^{3}H-labeled thymidine (radioactivity, 185 GBq/mmol; Atomic Energy Research Establishment, China) per well on days 3 and 4 and, after 18 h further incubation, were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a Liquid Scintillation Analyzer (Beckmon, Fullerton, CA). Values are expressed as counts per minute (cpm) from triplicate wells and are the results after subtracting cpms from wells in the absence of stimulator cells (Zhao et al., 2003b).

The phagocytosis of chicken red blood cells (cRBC) or allogeneic T cells by macrophage

A single-cell suspension of cRBCs were obtained freshly. CD3$^+$ T cells were purified by negative selection of mouse spleens of Balb/c and C57BL/6 mice using mouse CD3$^+$T lymphocyte enrichment set-DM (BD Biosciences PharMingen). Following two washes with PBS, 1×10^7 cells/ml cRBC or CD3$^+$T, cells in PBS were labeled with 5.0 µM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C. These cells were then washed thoroughly and re-suspended at a concentration of 1×10^7 cells/ml. Cell viability was determined by trypan blue exclusion. Cell viability was usually more than 95%. 1×10^6 macrophages (F4/80$^+$ PEMs or SPMs) were co-incubated with 2×10^6 CFSE-labeled cRBC or CD3$^+$ T cell in 6-well plates (Costar) that had been preset with cover glass at 37°C and 5% CO$_2$ for 3–4 h. The cover glasses were washed once and adherent cells were blocked with anti-mouse FcγR mAb (clone 2.4G2) and stained with PE-conjugated anti-F4/80 mAb (BM8, eBioscience, San Diego, CA). Three-channel images were taken with a two-photon laser scanning microscope (LSM510, Zeiss). Individual macrophages were isolated from Z stacks with the extract region feature and further analyzed using the ortho and gallery displays of the LSM510 imaging software (Lee et al., 2005b).

The response of mouse F4/80$^+$ SPMs and PEMs to LPS

Mouse F4/80$^+$ PEMs and SPMs at 1×10^6 cells/ml were stimulated with 0.5 µg/ml lipopolysaccharide (LPS, *E. coli* III: B4; Sigma, St. Louis, MO) in 24-well plates (Costar) for 24 h at 37°C and 5% CO$_2$, as described before (Swirski et al., 2004).

Detection of nitric oxide (NO) production

NO production by macrophages was determined by the measurement of the nitrite concentration with Griess assay. Supernatants (100 µl) were added to 100 µl of a 1:1 mixture of 1% sulfanilamide dihydrochloride (Sigma) and 0.1% naphthyl

ethylenediamine dihydrochloride (Sigma) in 2.5% H$_3$PO$_4$. Plates were incubated at 37°C for 10 min, and the absorbance at 550 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentration was calculated with a sodium nitrite standard curve as reported before (Monney et al., 2002).

Delayed type hypersensitivity (DTH)

Sensitized effector T cells were generated by immunizing Balb/c mice with allogeneic C57BL/6 splenocytes. Ten days after immunization, Balb/c CD4$^+$ T cells were enriched using the negative selecting MACS kit for CD4$^+$ T lymphocytes (BD Biosciences PharMingen). C57BL/6 F4/80$^+$ PEMs or SPMs were used as stimulator cells. Sensitized Balb/c effector CD4$^+$ T cells and allogeneic or syngeneic macrophage stimulators (5×10^5 cells/each) in 10 µl RPMI1640 medium were injected intradermally into the pinnate of naïve Balb/c mice. The changes in ear thickness were measured using an engineer's micrometer at 24 or 48 h after challenge (Lee et al., 2005a). The ear thickness change was calculated by subtracting the thickness of the same ear before injection from the thickness of the ear after injection.

Statistical analysis

All data are presented as the mean ± SD. Student's unpaired t test for comparison of means was used to compare groups. A P value less than 0.05 was considered to be statistically significant.

RESULTS
Cellular morphology of mouse F4/80$^+$ PEMs and SPMs

First of all, we determined the purity of the separated mouse PEMs and SPMs by a two-photon laser scanning microscope and FCM. Splenic and peritoneal exudate adherent cells constituted more than 90% of F4/80$^+$ macrophages (data not shown), as reported before (Lee et al., 2005a; Lin et al., 2005). In general, $4\text{-}6 \times 10^6$ F4/80$^+$ PEMs or SPMs from each Balb/c or C57BL/6 mouse were harvested (data not shown). The purity of F4/80$^+$ PEMs or SPMs in each experiment was routinely determined by FCM.

As is shown in Figure 1, both freshly isolated Balb/c F4/80$^+$ PEMs and SPMs showed that these cells had typical globular characteristics in shape, their nuclei were usually eccentric, irregular and deeply stained with Giemsa, the cytoplasm showed scarce elements and the karyoplasmic ratio was high, as reported before (Lin et al., 2005; Stout et al., 2005). However, the cell sizes of Balb/c F4/80$^+$ PEMs were significantly smaller than that of F4/80$^+$ SPMs ($P < 0.01$, Fig. 1B,C). After stimulation with LPS, both Balb/c F4/80$^+$ PEMs and SPMs became significantly larger in size with much more irregular nuclei, small pseudopods and fewer of granularity and cavitation than the unstimulated control cells did (Fig. 1A–C). However, no significant differences for LPS-stimulated Balb/c F4/80$^+$ PEMs and SPMs were observed in these respects ($P > 0.01$, Fig. 1C), although F4/80$^+$ PEMs were markedly smaller than those of F4/80$^+$ SPMs before activation. Identical observation was gained with C57BL/6 F4/80$^+$ PEMs and SPMs before and after LPS stimulation, respectively (data not shown).

The phenotypes of mouse F4/80$^+$ PEMs and SPMs

One of the most important functions for macrophages is to present antigens to surrounding T cells through their cell-surface interactions. The expression of co-stimulatory molecules on antigen presenting cells (APCs) is crucial in determining the nature and extent
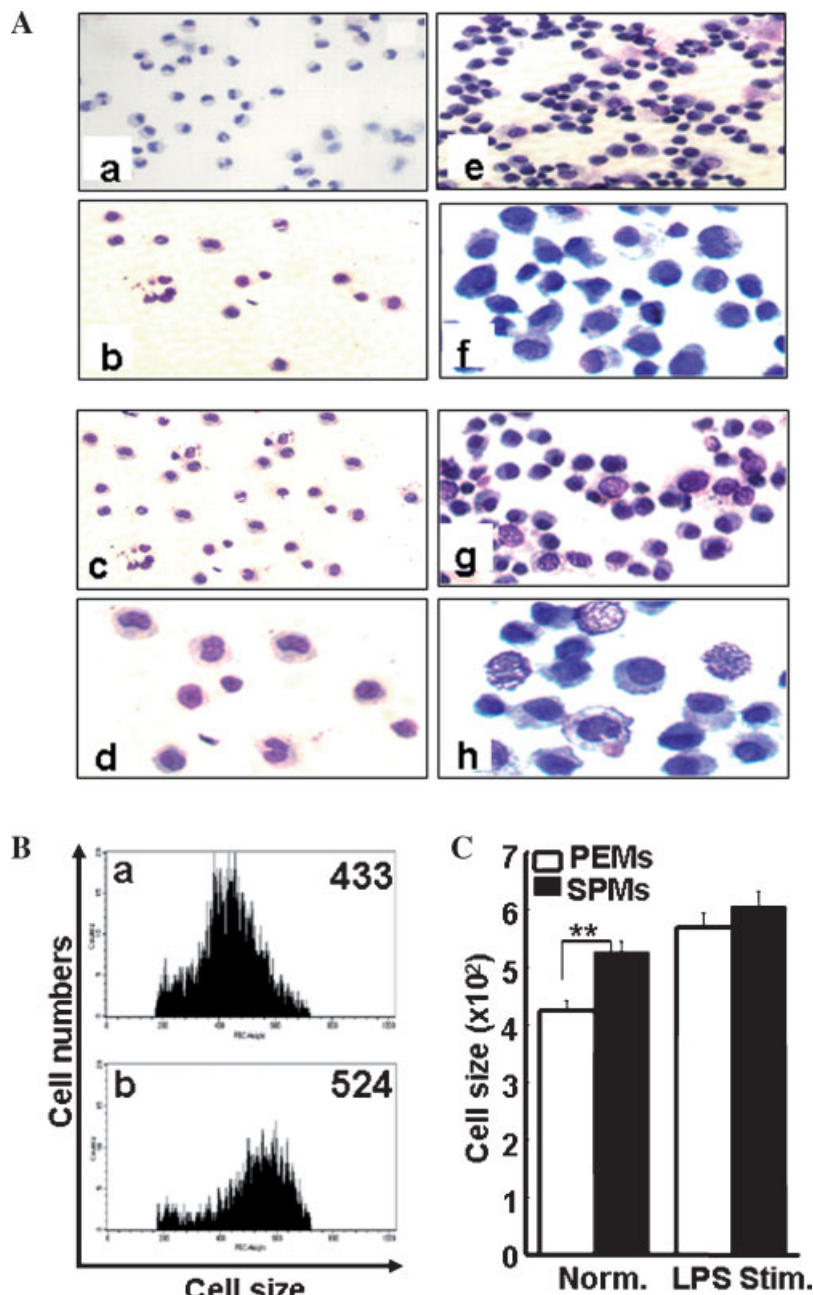
Fig. 1. Morphology of Balb/c F4/80+ PEMs and SPMs assayed by Giemsa staining and FCM. **A**: Morphology of Balb/c PEMs and SPMs with or without LPS stimulation. a, b, e, and f: Freshly isolated Balb/c PEMs (a and b) and SPMs (e and f) (magnification: a and e, 200×; b and f, 400×); c, d, g, and h, LPS-treated Balb/c SPMs (g and h) and PEMs (c and d) (magnification: c and g, 200×; d and h, 400×); macrophages were treated with 0.5 µg/ml LPS for 24 h. **B**: Cell size of Balb/c PEMs and SPMs determined by FCM. **C**: Cell size comparison of Balb/c PEMs and SPM with or without LPS stimulation as detected by FCM. **P < 0.01 between the indicated groups. One representative of four independent experiments with identical results was shown.

of the immune response. To investigate the potential differences for mouse F4/80+ PEMs and SPMs in respect of antigen presenting ability, we have studied the expressions of MHC-II molecules and co-stimulatory molecules on Balb/c F4/80+ PEMs and SPMs. Without LPS stimulation, both Balb/c F4/80+ PEMs and SPMs express relatively low levels of MHC-II and co-stimulatory molecules including CD80, CD86, CD40, and CD54 (Fig. 2). However, more Balb/c F4/80+ SPMs express markedly higher levels of CD86, CD40, and MHC-II molecules, as well as lower levels of CD80 molecules, than those of Balb/c F4/80+ PEMs ($P < 0.05$ or $P < 0.01$, respectively). After the treatment with LPS

in vitro for 24 h, both Balb/c F4/80+ PEMs and SPMs expressed very high levels of MHC-II and co-stimulatory molecules CD80, CD86, CD40, CD54, and CD23 molecules, as reported previously (Lee et al., 2005b). However, significantly higher levels of I-A, CD86, and CD40 molecules, but not CD11c, CD80, CD54, and CD23 molecules, expressed on Balb/c F4/80+ SPMs than those on PEMs were detected after stimulation with LPS in vitro ($P < 0.01$ or $P < 0.001$, respectively; Fig. 2B,C). Similar changes of the cell phenotypes were observed using C57BL/6 F4/80+ PEMs and SPMs, instead of Balb/c F4/80+ PEMs and SPMs, treated with or without LPS (data not shown).
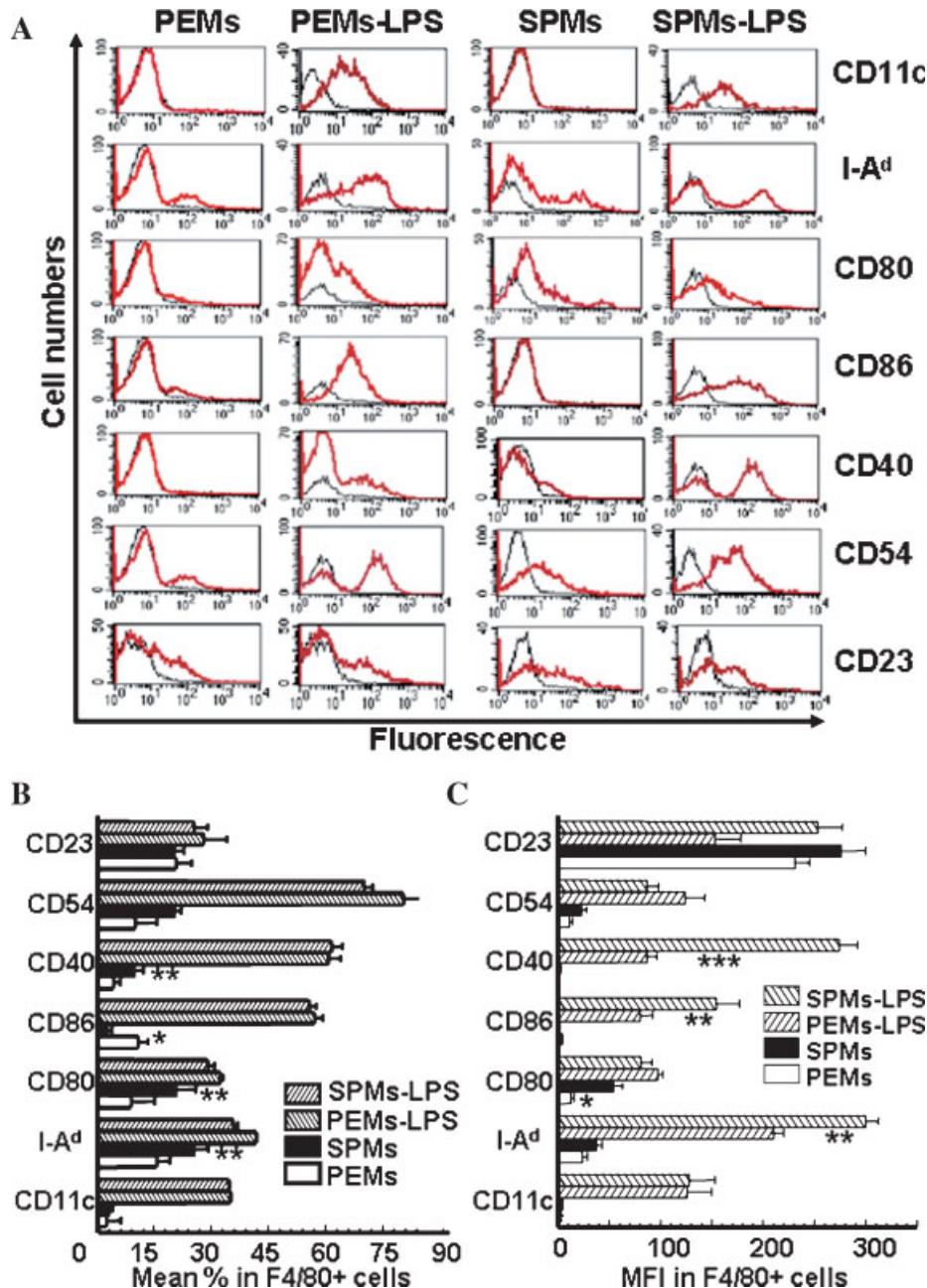
Fig. 2. Phenotype characteristics of Balb/c F4/80$^+$ PEMs and SPMs. Balb/c macrophages were cultured with or without the stimulation with 0.5 µg/ml LPS for 24 h, and then stained with PE-labeled anti-F4/80 mAb versus FITC-labeled anti-CD11c, I-A^d, CD80, CD86, CD40, CD54, or CD23 mAb. Ten thousand F4/80$^+$ cells were analyzed by FCM. **A**: A representative of the phenotypes of Balb/c F4/80$^+$ PEMs and SPMs assessed by FCM. The black lines represent the non-specific mAb staining and red lines were the indicated mAb staining. **B**: The percentages of CD11c, I-A^d, CD80, CD86, CD40, CD54, and CD23-positive cells in Balb/c F4/80$^+$ SPMs and PEMs stimulated with or without LPS. **C**: The levels of CD11c, I-A^d, CD80, CD86, CD40, CD54, and CD23 molecules in Balb/c F4/80$^+$ SPMs and PEMs stimulated with or without LPS, as determined by two-color FCM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the corresponding groups. More than eight mice in each group were examined.

TLRs mediate recognition of several microbial products. Accumulating evidences have demonstrated that TLRs are capable of inducing distinct responses in macrophages and other types of APCs, and subsequently direct T-helper cell differentiation in different directions (Liu et al., 2005b, 2006; Stout et al., 2005). Thus, the levels of TLR expressions on the unstimulated Balb/c or C57BL/6 F4/80$^+$ PEMs and SPMs were directly determined by FCM in this study, as described in Materials and Methods. As is shown in Figure 3A,B, significantly more Balb/c or C57BL/6 F4/80$^+$ PEMs

expressed TLR2 or TLR4 than F4/80$^+$ SPMs, respectively ($P < 0.01$, compared with the identical groups). In addition, both Balb/c and C57BL/6 F4/80$^+$ PEMs expressed significantly higher levels of TLR2 and TLR4 than F4/80$^+$ SPMs, respectively ($P < 0.01$, compared with the identical groups, Fig. 3C,D).

The phagocytosis of allogeneic and xenogeneic cells by mouse F4/80$^+$ PEMs and SPMs

Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens.
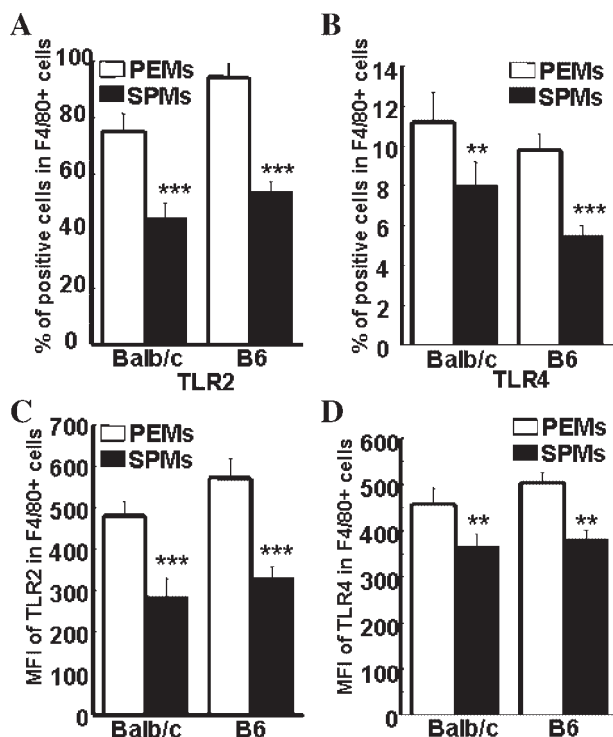
Fig. 3. The expression TLR2 and TLR4 on Balb/c or C57BL/6 F4/80$^+$ SPMs and PEMs. The percentages of TLR2$^+$ (**A**) and TLR4$^+$ (**B**) cells in Balb/c and C57BL/6 F4/80$^+$ SPMs and PEMs were determined by two-color FCM. The levels of TLR2 (**C**) or TLR4 (**D**) expression on F4/80$^+$ SPMs and PEMs are shown. *$P < 0.05$; **$P < 0.01$ compared with the corresponding PEMs. More than five mice in each group were assessed. Data was one representative of three independent experiments.

Balb/c F4/80$^+$ PEMs or SPMs co-cultured with CFSE-labeled cRBCs for 4 h and the phagocytic ability of macrophages was investigated using a two-photon microscope and FCM. As is shown in Figure 4, both Balb/c F4/80$^+$ PEMs and SPMs have the ability to phagocytize cRBCs efficiently. However, Balb/c F4/80$^+$ PEMs exhibited significantly higher phagocytosis capacity against xenogeneic target cells cRBCs than those of F4/80$^+$ SPMs ($P < 0.05$, Fig. 4A,B).

In addition, the macrophage phagocytic capacity against allogeneic T cells of mouse F4/80$^+$ PEMs and SPMs was assessed using a two-photon microscope and FCM. As is shown in Figure 5, both Balb/c F4/80$^+$ PEMs and SPMs have the ability to engulf allogeneic C57BL/6 T lymphocytes but not syngeneic T cells as detected with a two-photon microscope. When the phagocytosis of macrophages was quantitatively assayed using FCM, Balb/c F4/80$^+$ PEMs had a significantly higher phagocytic rate to allogeneic C57BL/6 T lymphocytes than those of F4/80$^+$ SPMs ($P < 0.001$, Fig. 5C). Furthermore, C57BL/6 F4/80$^+$ SPMs and PEMs showed similar patterns for their phagocytosis when studied using Balb/c T cells as target cells in vitro (data not shown).

NO secretion of F4/80$^+$ PEMs and SPMs stimulated with or without LPS and cultured with allogeneic T cells

NO is one of the important mediators for macrophage functions (Ammon et al., 2000). Without LPS stimulation, both mouse F4/80$^+$ SPMs and PEMs spontaneously produced detectable levels of NO (Fig. 6A). However, F4/80$^+$ SPMs secreted significantly higher levels of NO than that of F4/80$^+$ PEMs in either C57BL/c or Balb/c

mice ($P < 0.01$). After the treatment with LPS for 24 h or cultured with allogeneic T cells for 48 h, both F4/80$^+$ SPMs and PEMs produced a large amount of NO ($P < 0.01$, compared with the untreated identical control group; $P < 0.05$, compared with cultured with syngeneic T cell group), but F4/80$^+$ SPMs produced markedly more NO than that of F4/80$^+$ PEMs ($P < 0.001$, Fig. 6). These differences between F4/80$^+$ SPMs and PEMs in both Balb/c and C57BL mice were observed similarly, indicating that these differences may be unrelated with the mouse stains.

Immunogenicity of F4/80$^+$ PEMs and SPMs to naïve allogeneic CD4$^+$ T cells in vitro and in vivo

Macrophages, as an important APC, play a critical role in the initiation of adoptive immune responses. The immunogenicity of mouse F4/80$^+$ PEMs and SPMs to the allogeneic T cells was compared in the present study. The in vitro results have shown that both F4/80$^+$ SPMs and PEMs could efficiently induce the proliferative reaction of allogeneic C57BL/6 or C3H T cells in MLR assays (Fig. 7A). However, Balb/c F4/80$^+$ SPMs stimulated the proliferation of allogeneic C57BL/6 or C3H CD4$^+$ T lymphocytes more efficiently than did Balb/c F4/80$^+$ PEMs ($P < 0.01$). Consistently, the proliferation of allogeneic Balb/c or C3H CD4$^+$ T lymphocytes induced by C57BL/6 F4/80$^+$ SPMs was significantly stronger than those induced by C57BL/6 PEMs in MLR ($P < 0.001$, Fig. 7B).

To further determine the difference for the immunogenicity of mouse F4/80$^+$ PEMs and SPMs, the in vivo assay, DTH, induced by allogeneic F4/80$^+$ PEMs or SPMs was performed. Sensitized CD4$^+$ T lymphocytes were co-injected intradermally with allogeneic F4/80$^+$ PEMs or SPMs, respectively, into the pinnate of naïve Balb/c mice. The changes in ear thickness were measured at 24 h after challenge. As is shown in Figure 7C, significant DTH responses were observed if sensitized C57BL/6 T cells were stimulated by allogeneic Balb/c F4/80$^+$ PEMs or SPMs, whereas there were no significant DTH responses if unsensitized C57BL/6 T cells were stimulated by allogeneic Balb/c F4/80$^+$ PEMs or SPMs, as reported. However, Balb/c F4/80$^+$ SPMs induced significantly higher DTH responses of sensitized C57BL/6 T cells than Balb/c F4/80$^+$ PEMs did ($P < 0.05$). Similarly, C57BL/6 F4/80$^+$ SPMs induced significantly higher DTH responses of sensitized Balb/c T cells than C57BL/6 F4/80$^+$ PEMs did ($P < 0.05$, Fig. 7D).

IFN-γ production of CD4$^+$ T cells stimulated by allogeneic F4/80$^+$ PEMs and SPMs in vitro

After Balb/c CD4$^+$ T cells co-cultured with allogeneic C57BL/6 F4/80$^+$ SPMs or PEMs, respectively, for 4 days, the levels of IFN-γ$^+$ cells in CD4$^+$ T lymphocytes were determined by the intracellular staining method. As is shown in Figure 8, the percentages of IFN-γ$^+$ cells in Balb/c CD4$^+$ T lymphocytes cultured with allogeneic C57BL/6 F4/80$^+$ SPMs or PEMs were significantly higher than those cultured with syngeneic Balb/c F4/80$^+$ SPMs or PEMs, respectively, as expected ($P < 0.001$). In consistency with the high immunogenicity of F4/80$^+$ SPMs to allogeneic T cells as indicated by the in vitro and in vivo assays mentioned above, Balb/c F4/80$^+$ SPMs-stimulated allogeneic C57BL/6 CD4$^+$ T cells to produce significantly higher levels of IFN-γ than Balb/c F4/80$^+$ PEMs did ($P < 0.01$). Identical results were obtained when C57BL/6 F4/80$^+$ PEMs and SPMs were studied in vitro (data not shown).
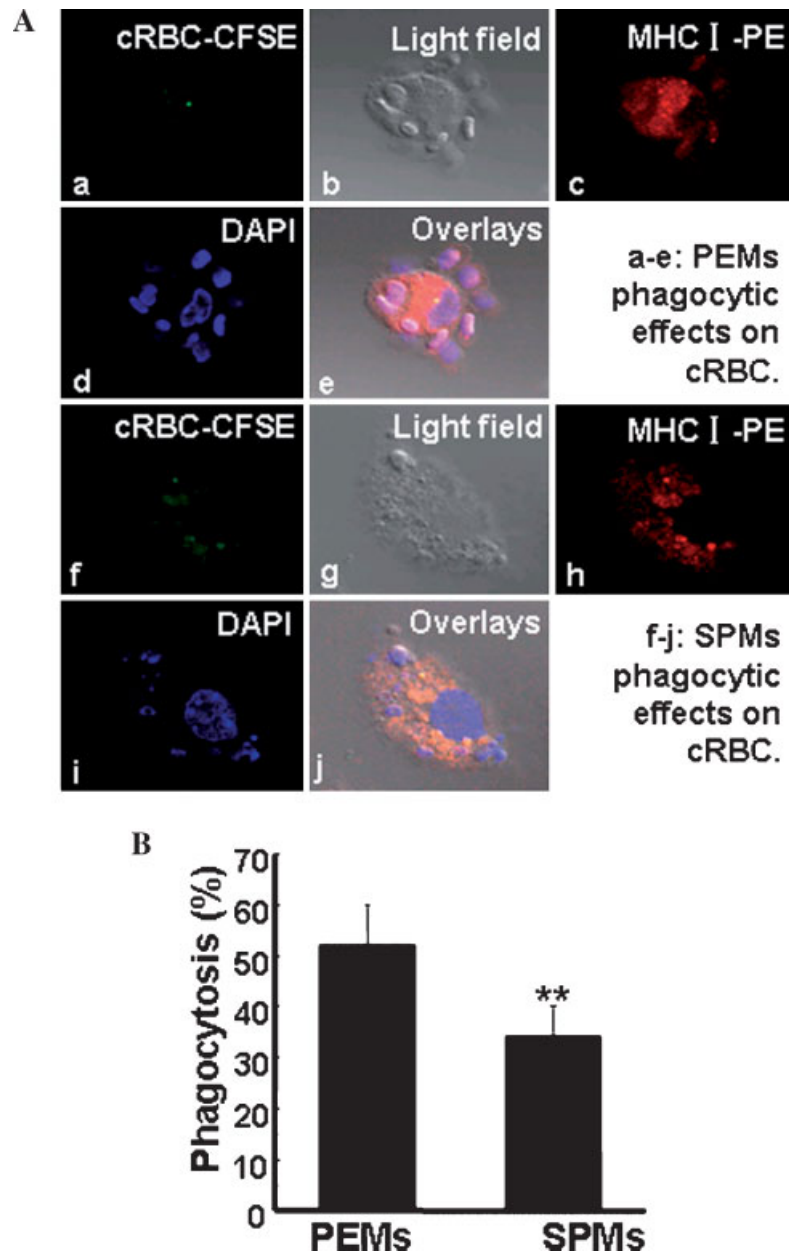
Fig. 4. Phagocytosis against cRBCs by Balb/c PEMs and SPMs as detected by two-photon microscopy. **A**: A representative of PEMs and SPMs phagocytosis to cRBCs. **a–e**: PEMs (630×; **f–j**: SPMs 630×). One representative of five independent experiments with similar results was shown. **B**: The phagocytosis percentages of cRBCs by Balb/c PEMs and SPMs were summarized. **P < 0.01 versus corresponding PEMs. Six mice in each group were assayed and three independent experiments were performed.

DISCUSSION

Macrophages are a highly heterogeneous population of cells and specialized subpopulations of macrophages occupy distinct anatomical compartments in the body (Delneste et al., 2003). Resident macrophages are generally considered to be derived from circulating monocytes (Belardelli and Ferrantini, 2002; Kaufmann and Schaible, 2005). In the present study, the significant morphology and functional differences between mouse F4/80+ PEMs and SPMs were observed, although mouse F4/80+ SPMs and PEMs, indeed, exhibited some similarities in most respects. In consistent with the previous studies (Kaufmann and Schaible, 2005), mouse F4/80+ PEMs, with typical sphere characteristics including large karyoplasmic ratio and deeply stained nuclei with Giemsa staining, are significantly smaller than F4/80+ SPMs. However, after the treatment with LPS, mouse F4/80+ PEMs acquired markedly larger size and their nuclei appeared much more irregular, which showed no detectable difference with mouse F4/80+ SPMs stimulated with LPS. These data indicate that F4/80+ PEMs might have great potential ability for the enlarging cell size after stimulation.

It is well known that macrophages can present information concerning antigens to surrounding T cells through cell-surface interactions (Chomarat et al., 2003). We have investigated the potential antigen presenting ability of mouse macrophages by detecting the expressions of MHC-II and co-stimulatory molecules
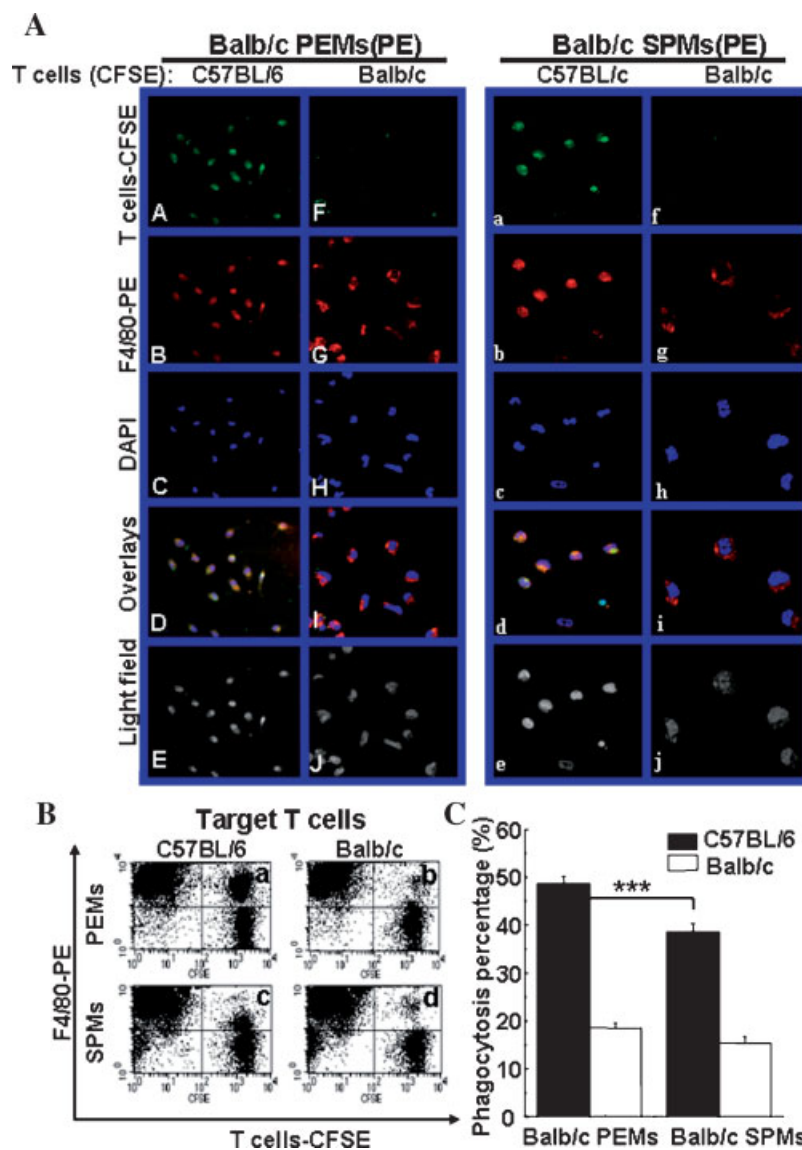
Fig. 5. Phagocytosis of allogeneic CD3$^+$ T cells by mouse PEMs and SPMs. **A**: Mouse PEMs and SPMs phagocyted allogeneic CD3$^+$ T cells as determined by two-photon microscope (magnification, 400×). Macrophages were stained by PE-labeled anti-H-2^d mAb, and target T cells were labeled with CFSE. Balb/c PEMs were co-cultured with C57BL/6 (**A–E**) or Balb/c T cells (**F–J**), Balb/c SPMs were co-cultured with C57BL/6 (**a–e**) or Balb/c T cells (**f–j**). Data were one representative of six independent experiments showing similar results.

B and **C**: Mouse PEMs and SPMs phagocyted allogeneic CD3$^+$ T lymphocytes as determined by FCM. Balb/c PEMs or SPMs were stained with PE-labeled anti-F4/80 mAb, whereas C57BL/6 and Balb/c T cells were labeled with CFSE. Balb/c PEMs were co-cultured with C57BL/6 (a) or Balb/c T cells (b), Balb/c SPMs were co-cultured with C57BL/6 (c) or Balb/c T cells (d). ***$P < 0.001$ versus the corresponding PEMs. Six mice in each group were used.

on mouse F4/80$^+$ PEMs and SPMs with or without LPS stimulation with gating on F4/80$^+$ cells by two-color staining FCM. The F4/80 mAb, recognizing a membrane of the epidermal growth factor-transmembrane 7 families, has been used widely as a marker for mouse macrophages. However, it has been demonstrated that marginal zone macrophages appear F4/80$^-$. Thus, the marginal zone macrophages may be neglected in this study. Both mouse F4/80$^+$ PEMs and SPMs expressed low levels of MHC-II and co-stimulatory molecules such as CD80, CD86, CD40, and CD54 molecules. However, the percentages of cells expressing CD80 or CD40 molecules in F4/80$^+$ SPMs were markedly higher than those in mouse F4/80$^+$ PEMs, respectively, as determined by FCM, whereas the percentage of cells expressing CD86 molecules in F4/80$^+$ SPMs was significantly lower than those in mouse F4/80$^+$ PEMs. The relative

lack of co-stimulatory molecules such as CD80 or CD40 on mouse F4/80$^+$ PEMs may be related to their poor immunogenicity that was confirmed by our studies in vitro and in vivo (Barrington et al., 2001; Wysocka et al., 2001; Morrison et al., 2004).

After the treatment with LPS, more F4/80$^+$ PEMs and SPMs expressed markedly high levels of MHC-II and co-stimulatory molecules, compared with the unstimulated cells. Unexpectedly, LPS-stimulated F4/80$^+$ SPMs expressed significantly higher levels of CD86, CD40, and I-A molecules than LPS-stimulated F4/80$^+$ PEMs, while no significant differences were observed on the levels of CD23, CD54, CD80, and CD11c molecules expressed on both LPS-stimulated SPMs and PEMs. This data shows that, even after full activation, F4/80$^+$ SPMs and PEMs exhibited distinguished phenotypes, excluding the possibility that the heterogeneity of
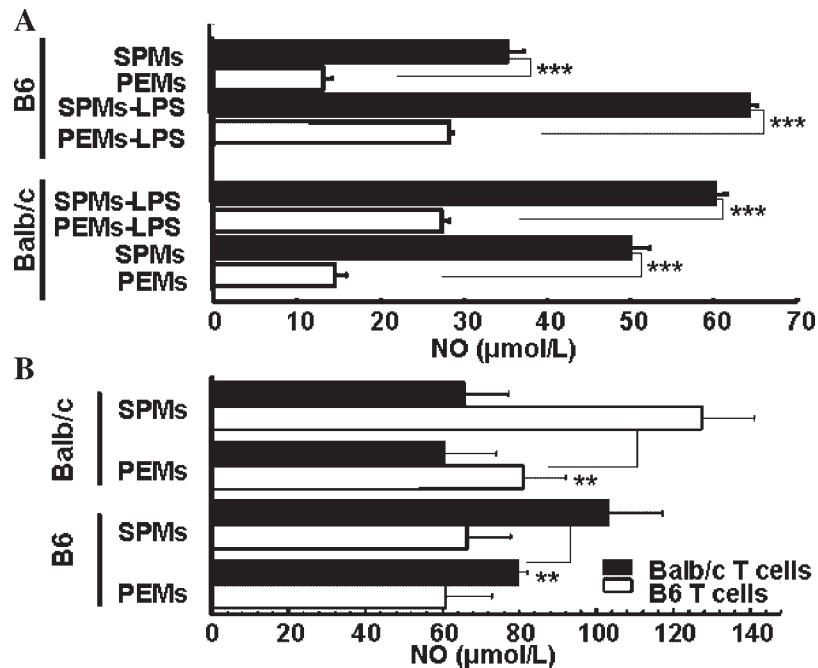
Fig. 6. NO secretion of mouse PEMs and SPMs stimulated with or without LPS and cultured with allogeneic T cells. Cells were cultured with 0.5 µg/ml LPS for 24 h (**A**) or with allogeneic T cells for 48 h (**B**). The levels of NO in the supernatant were measured by Griess assay. Data was shown as mean $\pm$ SD. **$P < 0.01$ and ***$P < 0.001$ versus corresponding PEMs. Data was one representative of four independent experiments with similar data.

freshly isolated F4/80$^+$ SPMs and PEMs may be due to these cells in different development or activation states. The different responses, including cell phenotype and NO production by mouse F4/80$^+$ SPMs and PEMs after LPS or allogeneic stimulation, suggested that these cells might be functionally distinctive subpopulations.

It is very striking that freshly isolated F4/80$^+$ PEMs expressed higher levels of CD86 than fresh F4/80$^+$ SPMs, whereas after LPS stimulation F4/80$^+$ PEMs expressed significantly lower levels of CD86 than F4/80$^+$ SPMs. F4/80$^+$ SPMs always expressed higher levels of CD80 than F4/80$^+$ PEMs regardless of LPS stimulation. The co-stimulatory signals to T cells provided by CD80 and CD86 expressed on APCs have been demonstrated. Interaction between these ligands and CD28 or CTLA-4 on T cells either enhances or downregulates T cell responses, respectively. In addition, the individual roles of CD80 and CD86 may be distinct or overlapping during an adaptive immune response depending on the disease model studied and the immune mechanisms involved. It has been reported that CD80 is pathogenic in crescentic glomerulonephritis by enhancing survival and proliferation of CD4$^+$ T cells, whereas CD86 is protective by enhancing Th2 and attenuating Th1 responses (Troelstra et al., 1999; Raychaudhuri et al., 2000). The remarkable heterogeneity of F4/80$^+$ SPMs and PEMs in terms of CD80 and CD86 expressions observed in the present study may contribute to their distinct functions. Furthermore, F4/80$^+$ PEMs exhibited significantly higher levels of TLR2 and TLR4 than F4/80$^+$ SPMs, indicating that mouse F4/80$^+$ PEMs might have greater capacity on recognition and clearness of pathogens or other foreign stimuli, which are recognized by certain TLRs, than F4/80$^+$ SPMs. This expectation was somehow supported by our studies showing that mouse PEMs had stronger non-opsonic phagocytosis against allo-

geneic and xenogeneic target cells as detected by two-photon microscopes and FCM.

After the treatment with LPS and culture with allogeneic T cells in vitro, mouse SPMs and PEMs secreted strikingly larger amounts of NO, but SPMs produced significantly more NO than PEMs. Significantly larger amount of NO production by SPMs than by PEMs was observed even after subtracting the spontaneous NO from the stimulated groups. NO, as an important factor of macrophages, influences a great variety of biological processes in the organisms (Kaufmann and Schaible, 2005). It has been reported that SPMs produced more IL-1 and IL-12, less IL-6, and PGE2 as well as similar levels of tumor necroses factor-α than PEMs or macrophages in other tissues such as the liver and lungs (Wu et al., 1993; Ogle et al., 1994). TLR4 was identified as the signaling receptor for LPS, a Gram-negative bacterial wall component (Yan et al., 2002; Liu et al., 2005b). The F4/80$^+$ SPMs expressed significantly lower levels of TLR4 than F4/80$^+$ PEMs in Balb/c and C57BL/6 mice, as determined by FCM. The inconsistency between NO products and TLR4 expression on PEMs and SPMs was unexplained. The higher levels of NO produced by SPMs might due to other factors or pathways in cells related to NO production (Kaufmann and Schaible, 2005). In addition, macrophages stimulated with allogeneic T cells produced more NO compared with macrophages stimulated with LPS. Our present data could not offer explanation for it. Allogeneic T cells may offer strong signals such as cytokines and ligands for macrophages to produce NO. Nevertheless, these results have clearly shown that the heterogeneity of SPMs and PEMs also present with regard to the NO production.

In order to gain more insight into the functional heterogeneity of macrophages, we have studied their ability to stimulate the immune responses of allogeneic
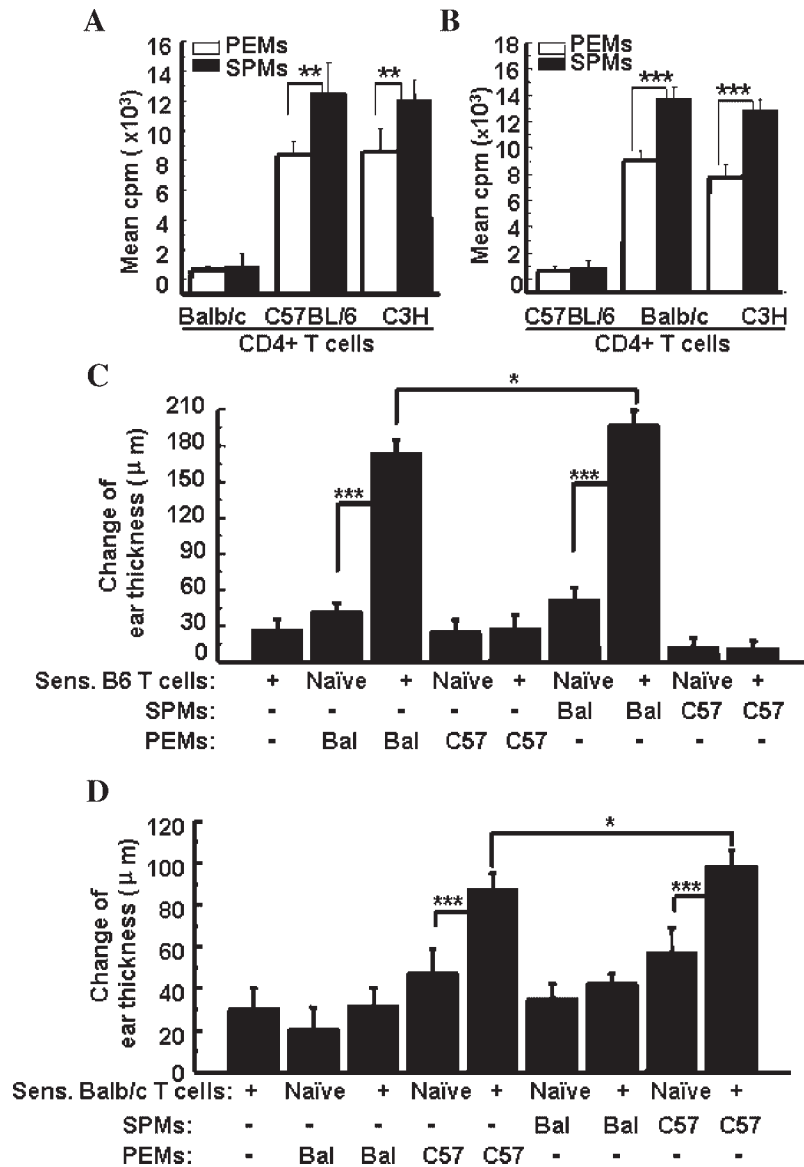
Fig. 7. The immune responses of CD4^{+} T cells stimulated by allogeneic PEMs or SPMs. The proliferation of CD4^{+} T cells induced by allogeneic Balb/c (**A**) or C57BL/6 (**B**) PEMs and SPMs in vitro. Data were presented as mean ± SD of triplicate wells. One representative of four independent experiments with similar data was shown (**C**), DTH responses induced by allogeneic PEMs or SPMs, respectively, in vivo. More than nine mice in each group were assayed. Data was a summary of seven independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the corresponding PEMs group.

T cells and their phagocytosis on xenogeneic and allogeneic target cells. The ability to stimulate the proliferation and IFN-γ production of allogeneic CD4^{+} T cells by F4/80^{+} SPMs was significantly higher than those of F4/80^{+} PEMs, whereas both F4/80^{+} SPMs and F4/80^{+} PEMs had no effects on stimulating syngeneic CD4^{+} T lymphocytes. Furthermore, mouse F4/80^{+} SPMs induced significantly stronger allogeneic DTH responses than F4/80^{+} PEMs. All these coincident results in vitro and in vivo suggested that mouse F4/80[+] SPMs have stronger immunogenicity than mouse F4/80[+] SPMs. Their high levels of MHC-II and co-stimulatory molecule expressions as well as the efficient production of NO may explain the significantly higher immunogenicity of SPMs. In addition, mouse PEMs have significantly higher phagocytosis capacity against cRBC or allogeneic T cells, respectively, than SPMs.

This was reversely related to the levels of MHC-II and co-stimulatory molecules expressed on macrophages and the immunogenicity to allogeneic T cells. It is known that once peritoneal organs suffered bacterial infection and injury, a great quantity of peritoneal exudates including macrophages were secreted (Millard et al., 2002). Macrophages are attracted to the peritoneal environment more abundantly than any other cell types (Anderson and Mosser, 2002). So, the results showing that F4/80[+] PEMs were significantly different from SPMs may have important clinical significance to explain different macrophage responses in different body locations.

The difference between mouse PEMs and SPMs with respect to cellular phenotype and function might contribute to understand the unique properties of PEMs and the immune functional heterogeneity of
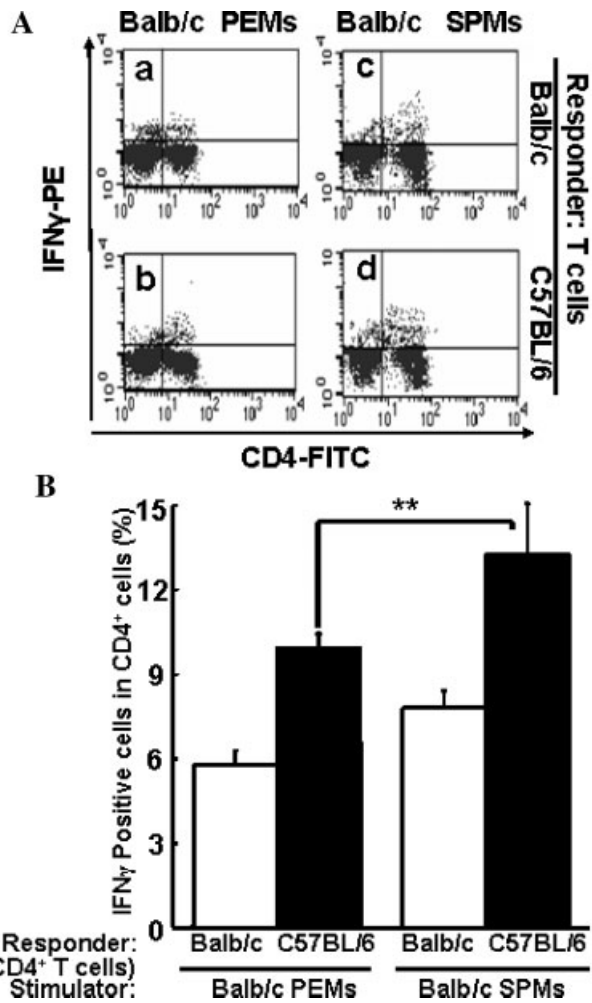
Fig. 8. The percentages of IFN-γ^+ cells in CD4$^+$ T cells stimulated by allogeneic PEMs and SPMs. IFN-γ production by CD4$^+$ T cells stimulated by allogeneic F4/80$^+$ PEMs or SPMs was determined by two-color intracellular staining FCM. **A**: One representative of IFN-γ staining was shown. Balb/c PEMs (**a** and **b**) or SPMs (**c** and **d**) were co-cultured with Balb/c (a) or C57BL/6 T cells (b). **B**: Data were presented as mean $\pm$ SD of triplicate wells. F4/80$^+$ PEMs or SPMs from four mice in each group were pulled together to get enough cells. Data was one representative of three independent experiments. **$P < 0.01$ versus corresponding PEMs.

macrophages in clinical and basic immunology. It is still quite confusing in our understanding of macrophage heterogeneity (Ravasi et al., 2002). The macrophage heterogeneity presents in different organs or tissues, as well as even in a single organ (Monney et al., 2002). Macrophage heterogeneity could originate through a variety of pathways (Shortman and Wu, 2004; Stout and Suttles, 2005). Recent studies have shown that macrophage phenotypic and functional heterogeneity might be related to a wide diversity of factors, including different precursor cells, different differentiation stages, different microenvironments, or even different stimulatory factors (Chakraborty et al., 2005). PEMs might have different precursor cells from spleen or other tissue macrophages (Gordon, 2004). Some researchers believe that PEMs may be at the early stages during macrophage development and differentiation (Shortman and Wu, 2004; Stout and Suttles, 2005).

In summary, with significant phenotype difference between SPMs and PEMs before and after activation, F4/80$^+$ SPMs had remarkably lower non-opsonic pha-

gocytotic capacity against chicken RBCs or allo-T cells. SPMs produced markedly more NO than PEMs when cultured with LPS or allo-T cells. Furthermore, SPMs exhibited stronger immunogenicity than PEMs, as determined by allogeneic cell proliferation, DTH, and IFN-γ production.

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