

# Aspirin significantly decreases the nonopsonic phagocytosis and immunogenicity of macrophages in mice

Aqeel Javeed · Yuzhu Hou · Kaizhong Duan · Baojun Zhang · Hong Shen · Yuhong Cao · Yong Zhao

Received: 9 July 2010/Revised: 7 November 2010/Accepted: 12 November 2010/Published online: 1 December 2010  
© Springer Basel AG 2010

## Abstract

**Objective and design** The effect of acetylsalicylic acid (ASA) on the phagocytosis and immunogenicity of macrophages remains to be determined.

**Material or subjects** BALB/c mice and peritoneal macrophages were used.

**Treatment** BALB/c mice were treated with ASA (0, 6, or 60 mg/kg/day) intraperitoneally for 1 week.

**Methods** Flow cytometry and phagocytosis assay were employed.

**Results** ASA significantly decreased cell numbers, non-opsonic phagocytosis, and immunogenicity as well as changing the phenotypes of peritoneal macrophages in vivo. After in vitro treatment with ASA, macrophages expressed low levels of MHC-II, CD80, and CD47 molecules, and showed significantly decreased phagocytosis. Importantly, ASA blocked the differentiation of macrophages from bone marrow cells in vitro as indicated by

decreased macrophage cell numbers and phenotype alteration.

**Conclusions** The present study provides basic information on the direct effect of ASA on macrophages, supporting the potential application of ASA in patients with allo-grafts or autoimmune diseases.

**Keywords** Aspirin · Macrophages · Phagocytosis · Immunogenicity

## Abbreviations

ASA	Aspirin
BBMs	Bone marrow derived macrophages
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CPM	Counts per minute
cRBCs	Chicken red blood cells
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
NSAIDs	Nonsteroidal anti-inflammatory drugs
PE	Phycoerythrin
PEMs	Peritoneal macrophages
PGs	Prostaglandins
PI	Propidium iodide
TPM	Two-photon microscopy

Responsible Editor: Graham Wallace.

A. Javeed · Y. Hou · K. Duan · B. Zhang · H. Shen · Y. Cao · Y. Zhao (✉)  
Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Datun Road, Beijing 100101, China  
e-mail: zhaoy@ioz.ac.cn

A. Javeed · Y. Hou · K. Duan · B. Zhang · H. Shen · Y. Cao · Y. Zhao  
China–U.S. Research Center for Life Sciences, Chinese Academy of Sciences, Beijing, China

H. Shen  
Department of Animal Science and Technology, Beijing University of Agriculture, Beijing, China

## Introduction

Acetylsalicylic acid (ASA), also called aspirin, is an established and widely used drug, mainly due to its broad therapeutic spectrum with anti-inflammatory, antipyretic,

and analgesic effects by predominantly inhibiting cyclooxygenase (COX) activity, which contributes to the conversion of arachidonic acid to prostaglandins (PGs). This suppression of PG synthesis by COX inhibition is the major effect of ASA and its metabolite, salicylate [1, 2]. Recently, ASA has also been shown to protect allograft function and survival as well as to reduce complications in transplant recipients [3–5], but the exact mechanism is not clear.

The acute and chronic rejection of allografts and xenografts is closely related to innate immune processes, including those mediated by granulocytes and macrophages [6, 7]. In addition to their phagocytotic, killing, and pro- and/or anti-inflammatory activities, macrophages signal adaptive responses through a variety of cytokines, costimulatory molecules, and antigen presentation [8, 9]. Macrophages can perform nonopsonic and Fc $\gamma$ R- and CR3-mediated opsonic phagocytosis to efficiently eradicate pathogens [6]. Moreover, the nonopsonic phagocytosis of macrophages plays a major role in the rejection response to allografts [10]. Some reports described the effect of ASA on the functions of macrophages, e.g. opsonic phagocytosis [11] and nitric oxide [12]. However, the effect of ASA on the nonopsonic phagocytosis and immunogenicity of macrophages has not yet been studied. The present study, therefore, aimed to investigate the effect of ASA on the cell numbers, phenotype, nonopsonic phagocytosis, and immunogenicity of macrophages. The results of the present study showed that ASA significantly decreased cell numbers, nonopsonic phagocytosis, and immunogenicity as well as changing the phenotype of macrophages. Our data offered basic information which may provide new insights for the application of ASA in patients with allografts or autoimmune diseases.

## Materials and methods

### Animals and reagents

Eight-to-ten-week-old BALB/c (H-2<sup>d</sup>) and C57BL/6 (B6; H-2<sup>b</sup>) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). All mice were maintained in a 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.

The culture medium used in the present study was RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 50  $\mu$ M 2-ME (Sigma, St. Louis, MO, USA). ASA was

purchased from Sigma Chemical Co (St Louis, USA). Mitomycin C (C<sub>15</sub>H<sub>18</sub>N<sub>4</sub>O<sub>5</sub>) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). [<sup>3</sup>H]Thymidine was purchased from the China Institute of Atomic Energy (Beijing, China).

The following monoclonal antibodies (mAbs) were purchased from BD Biosciences Pharmingen (San Diego, CA, USA): phycoerythrin (PE)-conjugated anti-mouse F4/80 mAb (BM8), fluorescein isothiocyanate (FITC)-conjugated anti-CD80 mAb (16-10A1; IgG2b), anti-CD47 mAb (MIAP301), and anti-I-A<sup>d</sup> mAb (39-10-8). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, MD, USA) in our laboratory.

### ASA treatment

BALB/c mice were divided into three groups for in vivo studies: control group, low dose ASA-treated group (6 mg/kg), and high dose ASA-treated group (60 mg/kg). ASA (Sigma) was dissolved in DMSO (5 M) and then diluted with normal saline (NS) and administered intraperitoneally at the dose of 6 or 60 mg/kg/day for 1 week. Control group mice were injected with NS (with <0.4% DMSO as the experiment group) only. For in vitro studies, macrophages were treated with different doses of ASA (0, 0.5, 1, 2, or 5 mM) and the control buffer (with 0.4% DMSO) as indicated in the figure legends.

### Preparation of peritoneal exudate macrophages (PEMs)

Resident peritoneal cells were obtained from the peritoneal exudates of mice as described previously [13, 14]. Briefly, the peritoneal exudate cells were washed twice with cold Hanks' solution, adjusted to  $5 \times 10^6$  cells/ml in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and cultured in 6-well plates (Costar, Cambridge, MA, USA) pretreated with 2% gelatin (Sigma) for 3–4 h at 37°C and 5% CO<sub>2</sub>. The non-adherent cells were removed by washing with warm RPMI1640 medium. The adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and re-adjusted to  $3 \times 10^5$  cells/ml per well into 24-well plates. The cell viability was determined by trypan blue exclusion. The macrophage purity was analyzed by a two-photon microscope (TPM) LSM510 (Zeiss, Wetzlar, Germany) and flow cytometry (FCM) (Becton–Dickinson, Mountain View, CA, USA), using a mouse macrophage marker F4/80, as reported previously [14, 15].

### Preparation of bone-marrow-derived macrophages (BMMs)

BMMs were prepared as described previously [16], whereby these macrophages were produced by M-CSF

stimulation of bone marrow cells for a total of 7 days. Briefly, BMM were obtained from specific-pathogen-free BALB/c mice (8–10 weeks old). Mice were killed by cervical dislocation and both femurs were dissected free of adherent tissue. The bones were trimmed at each end, and the marrow was flushed out with RPMI 1640 medium. Cells were suspended by pipetting and washed by centrifugation. Red blood cells were lysed and the cell suspension was washed and filtered to remove debris. The initial adherence step in petri dishes removed bone marrow stromal cells and any mature resident bone marrow macrophages. Removal of non-adherent cells at this point allowed progenitor cells to expand and differentiate under the influence of M-CSF. Cell suspensions were then washed and plated in 24-well tissue culture plates at a concentration of  $10^6$  cells per well in complete RPMI 1640 medium (10% heat-inactivated FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 50  $\mu$ M 2-ME) containing the indicated concentrations of ASA in the presence of M-CSF (100 ng/ml). Each concentration of drug was tested in triplicate wells. The cell monolayer was then incubated at 37°C in 5% CO<sub>2</sub> for 7 days, with the medium changed on day 4. BMMs were obtained after 7 days in culture in the presence of M-CSF. These BMMs could be detected by the expression of F4/80 molecules, and were used for later functional or phenotypic assays.

#### Immunofluorescence staining and FCM

Aliquots of  $2 \times 10^5$  mouse macrophages (PEMs or BMMs) were washed once with FACS buffer [PBS, pH 7.2, containing 0.1% NaN<sub>3</sub> 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 CD80 (16-10A1), I-A<sup>d</sup> (39-10-8), CD86 (GL1), CD47 (MIAP301), or the non-specific staining control mAb, respectively [17]. Non-specific FcR binding was blocked by anti-mouse FcRmAb (clone 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 isotype control mAb from the percentage stained 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<sup>+</sup> cells was determined by the analysis of the forward scatter side with gating on F4/80<sup>+</sup> cells.

#### Phagocytosis of xenogeneic chicken red blood cells (cRBCs) by mouse macrophages

A single-cell suspension of cRBCs was freshly obtained. Following two washes with PBS,  $1 \times 10^7$  cells/ml cRBCs in PBS were labeled with 5.0  $\mu$ M 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR, USA) for 15 min at 37°C [18]. These cells were then washed thoroughly and suspended in RPMI 1640 without serum at the required concentration [6]. Cell viability was usually more than 95% as determined by trypan blue exclusion. PEMs treated in vivo were obtained from mice administered intraperitoneally with or without ASA for 1 week. In vitro treated macrophages (PEMs or BMMs) were pre-incubated with or without ASA at the concentrations and times indicated in the figure legends. These ASA-treated PEMs treated both in vivo and in vitro were co-incubated with CFSE-labeled cRBCs at a ratio of 1:10 for 2–2.5 h at 37°C and 5% CO<sub>2</sub> in 24-well plates (Costar) that had been pre-treated with 2% gelatin (Sigma) [19]. After 2–2.5 h, un-ingested cRBCs were removed by aspirating supernatants and adding cold PBS to stop phagocytosis. Cells were then gently washed three times with cold PBS. Adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and re-adjusted. Cells were blocked with anti-mouse Fc $\gamma$ R mAb (clone 2.4G2) and stained with PE-conjugated anti-F4/80 mAb (BM8; eBioscience, San Diego, CA, USA). After washing with cold FACS buffer three times, the phagocytosis percentages of F4/80<sup>+</sup> gate cells were determined using FCM (BD Biosciences, San Diego, CA, USA). Forward and side scatter gates were set to exclude debris. Data were analyzed using Cell Quest software (BD Biosciences) [20]. Meanwhile, for the phagocytosis assay by TPM, 24-well plates (Costar) had been preset with cover glasses [17]. The cover glasses were washed once and adherent cells were blocked with anti-mouse Fc $\gamma$ R mAb (clone 2.4G2) and stained with PE-conjugated anti-F4/80 mAb (BM8). 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.

#### Delayed-type hypersensitivity (DTH)

Sensitized C57BL/6 effector T cells were generated by immunization with allogeneic BALB/c splenocytes. Ten days after immunization, C57BL/6 CD4<sup>+</sup> T cells were enriched using the negative selecting MACS kit for CD4<sup>+</sup> T lymphocytes (BD Biosciences Pharmingen). BALB/c PEMs were used as stimulator cells. Sensitized C57BL/6

effector CD4<sup>+</sup> T cells and allogeneic macrophage stimulators ( $5 \times 10^5$  cells each) treated with or without ASA both in vivo and in vitro were co-injected intradermally into the pinna of naïve C57BL/6 mice. The changes in ear thickness were measured using an engineer's micrometer at 24 or 48 h after challenge [21]. The ear thickness change was calculated by subtracting the thickness of the ear before injection from the thickness of the same ear after injection.

#### Allogeneic mixed leukocyte reactions (MLRs)

Murine splenocytes were prepared using the sterile technique as described previously [22]. Cells were suspended in RPMI 1640 medium supplemented with 10% (v/v) mouse serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids (GibcoBrl), 1 mM sodium pyruvate, 10 U/ml penicillin and 10 µg/ml streptomycin, 10 mM HEPES buffer (GibcoBrl), and 10 µM 2-mercaptoethanol [23]. Triplicate wells containing  $1 \times 10^5$  responders with  $1 \times 10^5$  of allogeneic macrophage stimulators (PEMs 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<sub>2</sub>. Cells were cultured at 37°C and 5% CO<sub>2</sub> for 4 days. [<sup>3</sup>H]Thymidine (0.5 µCi; radioactivity 185 GBq/mmol; Atomic Energy Research Establishment, China) was added for the last 18 h [24]. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a Liquid Scintillation Analyzer (Beckman, Fullerton, CA, USA). Values are expressed as counts per minute (cpm) from triplicate wells and are the results after subtracting cpm from wells in the absence of stimulator cells [25].

#### Cell viability analysis (MTT assay)

MTT assay was also used to measure the number of viable cells. After incubation of murine PEMs with different concentrations (0.5, 1, 2, and 5 mM) of ASA, cells were incubated with 20 µl MTT solution (5 mg/ml) per well for another 4 h at 37°C. After removal of the supernatant, DMSO 150 µl was added and shaken for 5 min until the crystals were dissolved. The optical density (OD) value of each well was recorded using spectrophotometry with a microplate reader (Bio-Rad Smartspec, Model 450, USA) at 490 nm. The reading is directly proportional to cell viability and results are expressed as percentage of cell viability with respect to control absorbance (100% cell viability). The same procedure was repeated three times and the OD value was calculated for statistical analysis.

#### Statistical analysis

All experiments were performed at least three times and in triplicate. The data are given as the mean  $\pm$  SD. Data were analyzed using Student's unpaired *t* test.  $P < 0.05$  was considered significant.

## Results

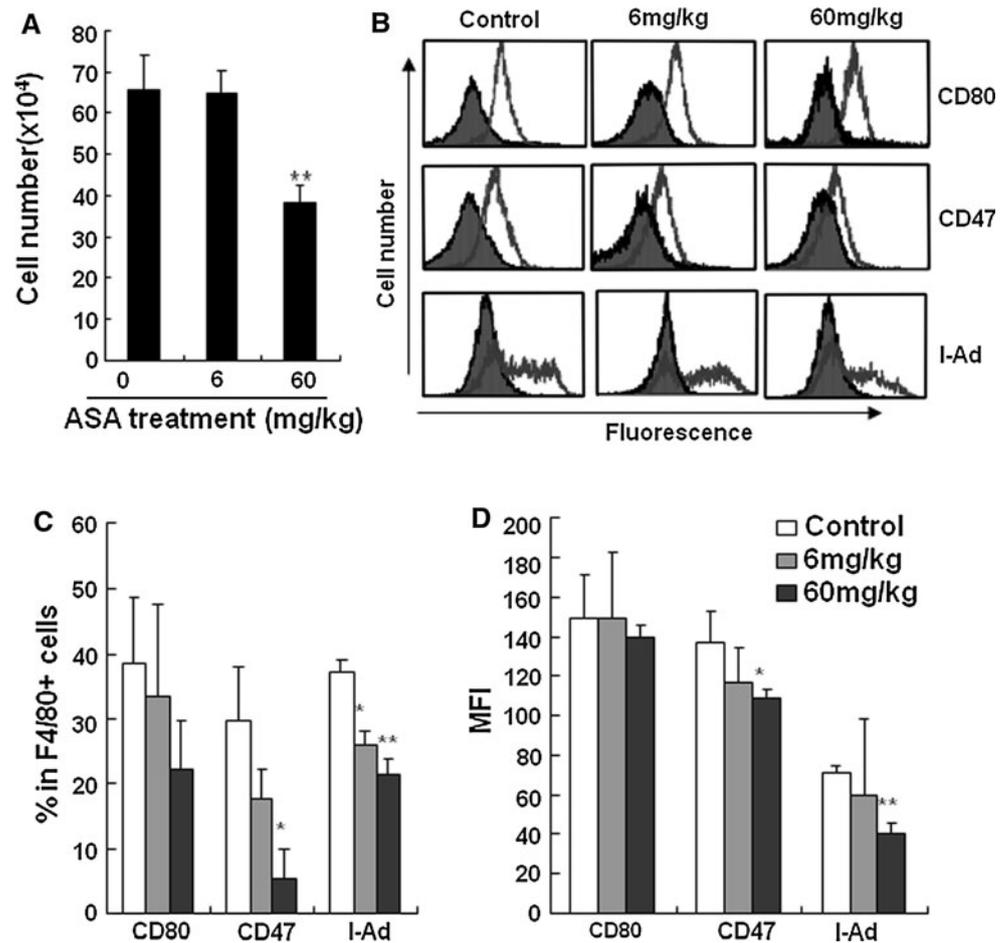
#### ASA significantly altered cell number and phenotype of mouse PEMs in vivo

The cell numbers and phenotype of macrophages were firstly evaluated 1 week after BALB/c mice were treated with ASA (6 or 60 mg/kg/day, respectively). As shown in Fig. 1a, a significant decrease in cell numbers of PEMs in mice treated with the high dose (60 mg/kg/day) of ASA for 1 week was observed compared with control-treated mice ( $P < 0.01$ ). The expression of co-stimulatory molecules on antigen presenting cells (APCs) is crucial in determining the nature and extent of the immune response. We therefore studied the expressions of major histocompatibility complex (MHC)-II molecules (I-A<sup>d</sup>), co-stimulatory molecules (CD80), and CD47 on murine F4/80<sup>+</sup> PEMs. When the phenotype of PEMs were detected using two-color FCM, significantly lower percentages of F4/80<sup>+</sup> PEMs expressed MHC II and CD47 molecules in ASA-treated mice than those in control mice (Fig. 1 b, c), while the expression of CD80 did not change significantly after ASA treatment. The expression levels of CD47 and I-A<sup>d</sup> molecules on F4/80<sup>+</sup> cells were significantly decreased after ASA treatment ( $P < 0.05$ ; Fig. 1d). Thus, alteration of cell numbers and phenotype of F4/80<sup>+</sup> PEMs occurred in the ASA-treated mice.

#### ASA significantly decreased the nonopsonic phagocytosis and immunogenicity of mouse PEMs in vivo

To determine whether or not ASA has the ability to alter the functions of PEMs in vivo, we performed nonopsonic phagocytosis and DTH assays. Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens. Mouse F4/80<sup>+</sup> PEMs separated from ASA-treated mice were co-cultured with CFSE-labeled cRBCs for 2–2.5 h and the phagocytic ability of macrophages was investigated using FCM. Mouse F4/80<sup>+</sup> PEMs in control mice have the ability to phagocytize cRBCs efficiently, as reported previously (Fig. 2). However, macrophages separated from mice treated with ASA showed significantly decreased nonopsonic phagocytosis compared with the control mice ( $P < 0.01$ ; Fig. 2b).

**Fig. 1** The cell numbers and phenotype of PEMs from mice treated with or without ASA for 1 week. **a** Cell numbers of PEMs from mice treated with or without ASA. **b** Phenotype characteristics of murine F4/80<sup>+</sup> PEMs. The murine PEMs were stained with PE-labeled anti-F4/80 mAb versus FITC-labeled I-Ad, CD80, CD86, or CD47 mAbs. Ten thousand F4/80<sup>+</sup> cells were analyzed by FCM. A representative of the phenotype of murine F4/80<sup>+</sup> PEMs was assessed by FCM. The *black filled* histograms represent the non-specific mAb staining and *open* histograms represent the indicated mAb staining. **c** The percentages of I-A<sup>d</sup>, CD80, and CD47-positive cells in murine F4/80<sup>+</sup> PEMs treated with or without ASA. **d** The levels of I-A<sup>d</sup>, CD80, or CD47 molecules in murine F4/80<sup>+</sup> PEMs treated with or without ASA as determined by two-color FCM. Data are shown as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with the corresponding groups. More than six mice in each group were examined



We then observed the immunogenicity of macrophages of ASA-treated BALB/c mice *in vivo* by using an allo-DTH assay. Sensitized C57 BL/6 CD4<sup>+</sup> T cells were co-injected intradermally with allogeneic PEMs separated from ASA-treated BALB/c mice into the pinna of naïve C57BL/6 mice. The changes in ear thickness were measured at 24 h after challenge. As shown in Fig. 2c, significant DTH responses were observed if sensitized C57BL/6 T cells were stimulated by allogeneic BALB/c PEMs. However, PEMs from BALB/c mice treated with 6 or 60 mg/kg/day ASA for 1 week induced significantly lower DTH responses of sensitized C57BL/6 T cells did than those of control mice ( $P < 0.05$ ,  $P < 0.01$ , respectively; Fig. 2c).

ASA inhibited the differentiation of bone marrow stem cells into macrophages *in vitro*

Mononuclear phagocytes develop in the bone marrow, circulate in the blood as monocytes, and are resident in all tissues of the body as macrophages. To explore whether the alteration of macrophages induced by ASA *in vivo* was a result of changes in macrophage differentiation, we

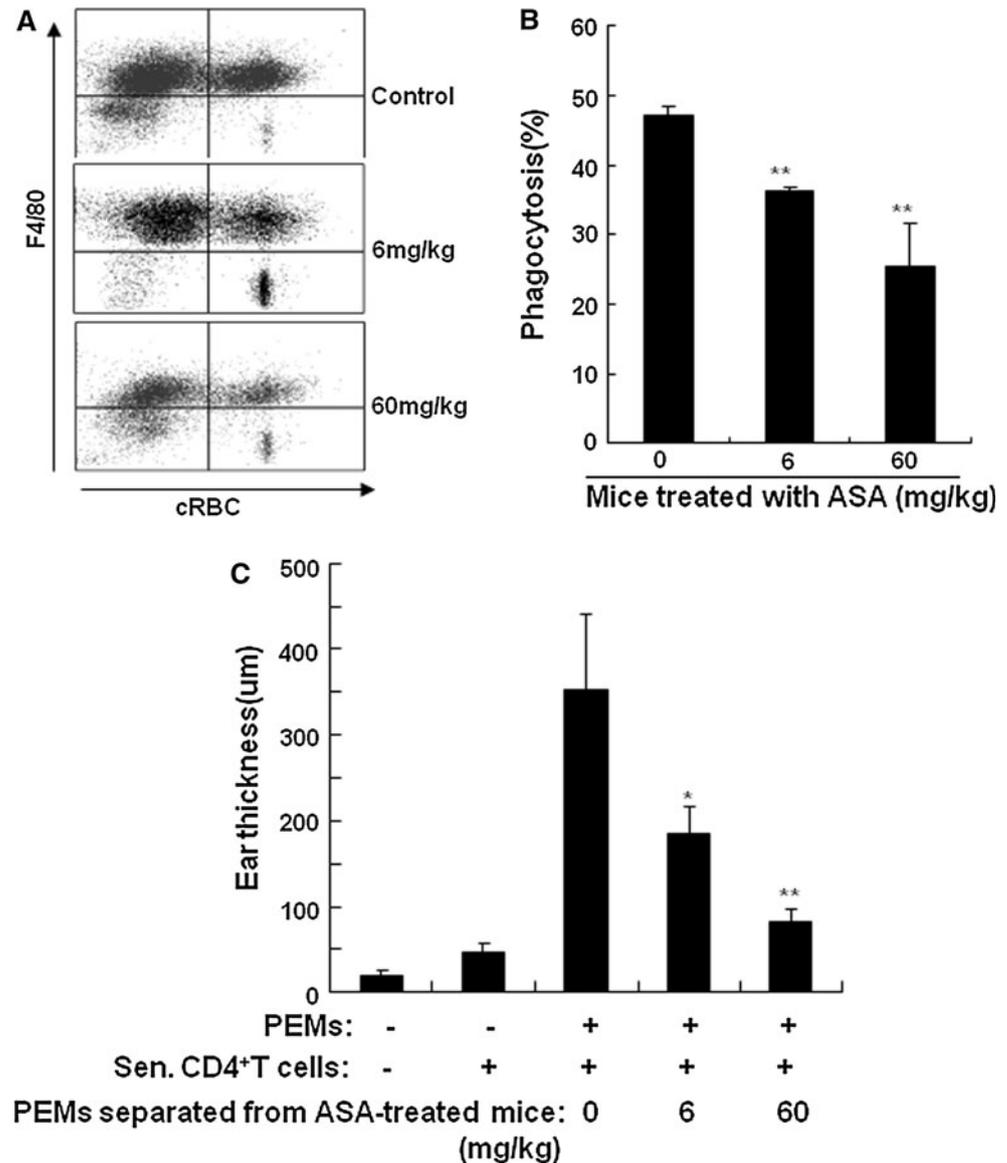
examined the effect of ASA on the differentiation of bone marrow stem cells into macrophages *in vitro*. As shown in Fig. 3, ASA markedly reduced the number of BMMs, indicating that ASA may block the differentiation of bone marrow stem cells into macrophages. In addition, the phenotypic analysis of BMMs by two-color FCM revealed that ASA-treated BMMs expressed lower levels of CD80, CD47, and I-A<sup>d</sup> as compared to control (Fig. 3b).

To evaluate the function of these BMMs, we performed nonopsonic phagocytosis by FCM. These BMMs treated with or without ASA *in vitro* were co-cultured with CFSE-labeled cRBCs for 2–2.5 h, and the phagocytic ability of macrophages was investigated using two-color FCM. As shown in Fig 3c, ASA-treated BMMs exhibited a significant lower phagocytic ability as compared to control BMMs ( $P < 0.01$ ).

Effect of ASA on the phenotype and nonopsonic phagocytosis of mouse PEMs *in vitro*

To investigate the potential direct effect of low (1 mM) and high (5 mM) doses of ASA on the phenotypic characteristics of F4/80<sup>+</sup> PEMs *in vitro*, we studied the expressions

**Fig. 2** Significantly decreased phagocytosis and immunogenicity of PEMs from mice treated with ASA for 1 week. **a** One representative showing BALB/c F4/80<sup>+</sup> PEM phagocytosis of xenogeneic cRBCs as detected by FCM. BALB/c PEMs were stained with PE-labeled anti-F4/80<sup>+</sup> mAb, whereas cRBCs were labeled with CFSE. BALB/c PEMs were co-cultured with cRBCs as described in the “Materials and methods” section. **b** The mean percentages of phagocytosis cells in F4/80<sup>+</sup> PEMs isolated from mice treated with or without ASA. **c** DTH responses induced by BALB/c PEMs from ASA treated or control mice were detected at 24 h after injection into the pinna of naïve C57BL/6 mice. Six mice in each group were assayed and three independent experiments were performed. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with the corresponding groups



of MHC-II molecules (I-A<sup>d</sup>), co-stimulatory molecules (CD80), and CD47 on murine F4/80<sup>+</sup> PEMs. In vitro treatment with ASA in both low and high doses for 24 h significantly down-regulated the expression of CD80, CD47 and I-A<sup>d</sup> on macrophages (Fig. 4).

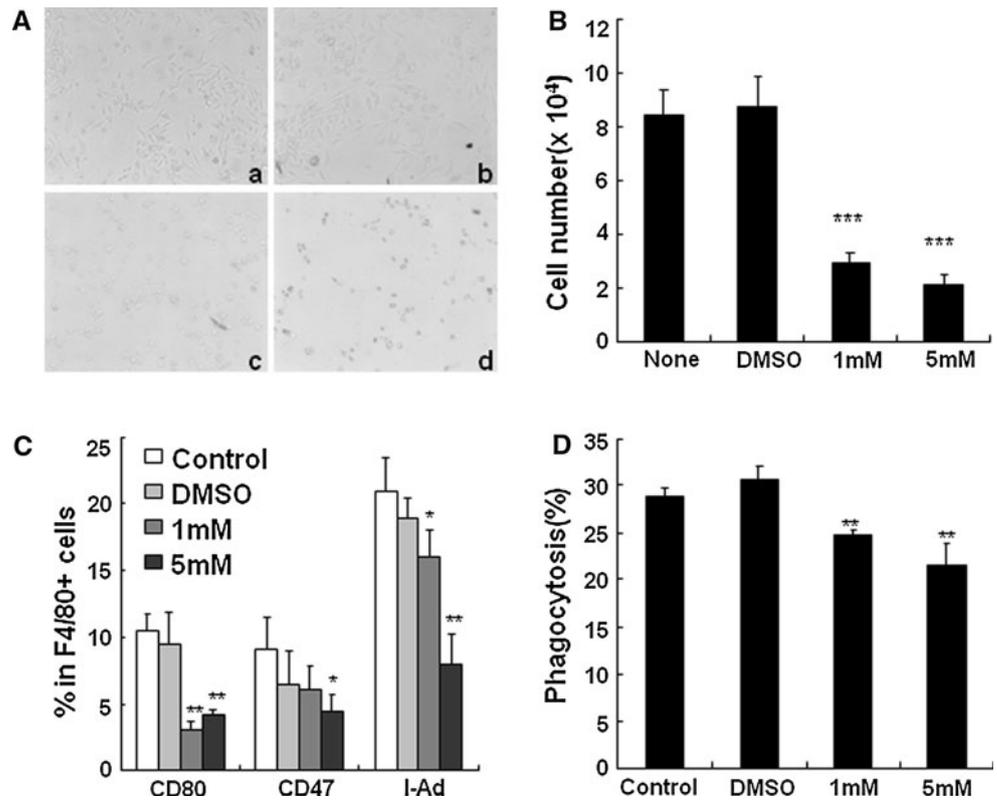
Murine PEMs treated by ASA in vitro were co-cultured with CFSE-labeled cRBCs for 2–2.5 h and the phagocytic ability of macrophages was investigated using FCM. As shown in Fig. 5, the incubation of murine F4/80<sup>+</sup> PEMs with ASA for 12 h in concentrations of 0.5, 1, and 2 mM did not exert significant effects on nonopsonic phagocytosis but exerted a significant inhibitory effect on nonopsonic phagocytosis at the concentration of 5 mM (Fig. 5b). However, incubation of murine F4/80<sup>+</sup> PEMs with ASA for 24 h at concentrations of 0.5, 1, 2, and 5 mM

exerted significant inhibitory effects on the nonopsonic phagocytic activity of murine PEMs (Fig. 5c).

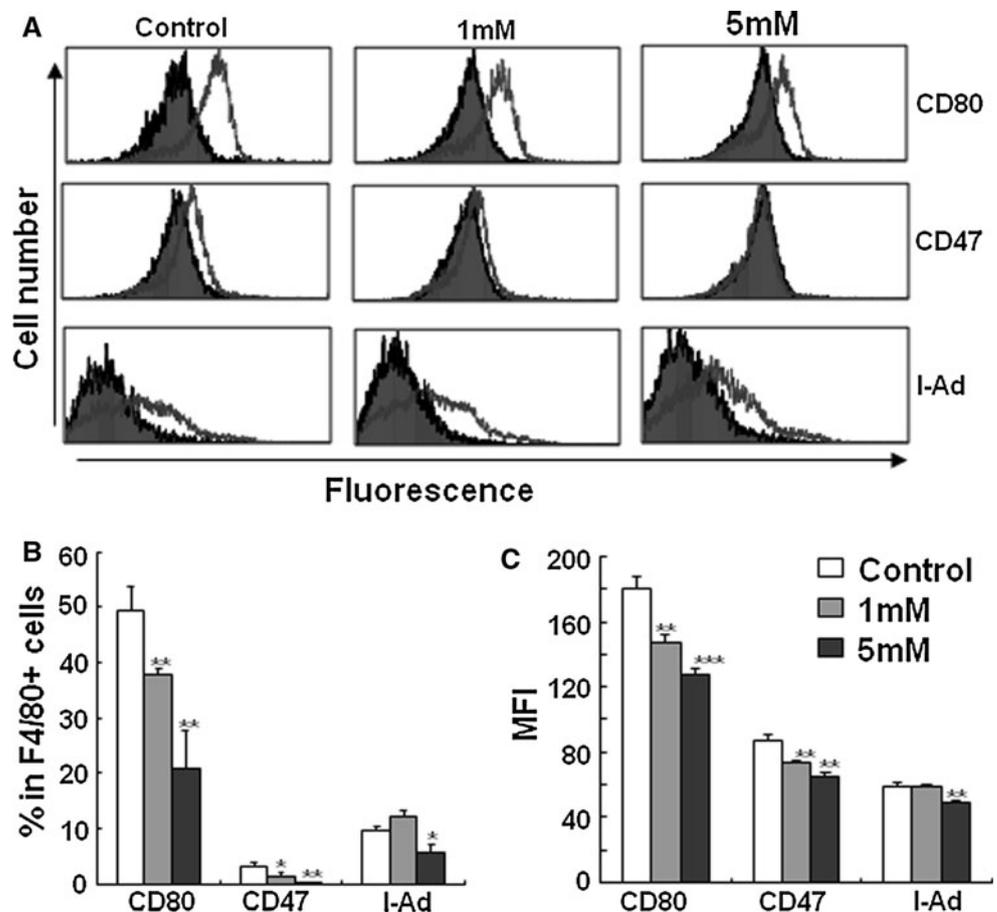
The direct effect of ASA on the immunogenicity of murine PEMs

The direct effect of ASA on the immunogenicity to allogeneic T cells of BALB/c PEMs was determined in BALB/c PEMs treated with low (1 mM) and high (5 mM) doses of ASA or without ASA in vitro. BALB/c PEMs treated with ASA showed significantly decreased immunogenicity to allogeneic T cells compared with control, as evaluated in a MLR assay (*P* < 0.001; Fig. 6a). To further determine the immunogenicity of ASA-treated murine PEMs by DTH assay, sensitized C57BL/6 CD4<sup>+</sup> T cells were co-injected

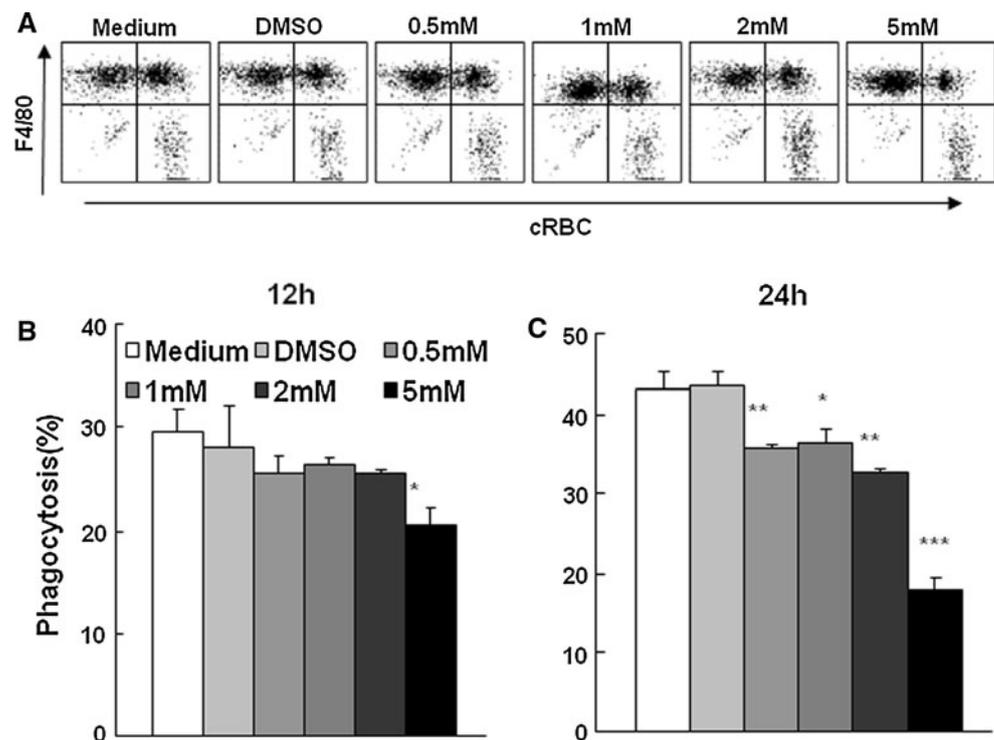
**Fig. 3** The direct effect of ASA on the differentiation of BMMs in vitro. **a** ASA blocked the differentiation of macrophages from bone marrow cells in the presence of M-CSF. *a* control; *b* DMSO control; *c* 1 mM ASA; *d* 5 mM ASA. **b** ASA decreased the cell numbers of macrophages derived from bone marrow cells in the presence of M-CSF. **c** ASA changed the phenotype of BMMs. BMMs were stained with PE-labeled anti-F4/80 mAb versus FITC-labeled I-A<sup>d</sup>, CD80, and CD47 mAb, respectively. Data are presented as mean ± SD. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with the corresponding groups



**Fig. 4** The direct effect of ASA on the phenotype of murine PEMs in vitro. Murine PEMs were cultured with or without ASA (1 and 5 mM) for 24 h in vitro, and then stained with PE-labeled anti-F4/80 mAb versus FITC-labeled I-A<sup>d</sup>, CD80, or CD47 mAb. Ten thousand F4/80<sup>+</sup> cells were analyzed by FCM. **a** One representative of the phenotype of murine F4/80<sup>+</sup> PEMs was assessed by FCM. The *black filled* histograms represent the non-specific mAb staining and *open* histograms represent the indicated mAb staining. **b** The percentages of I-A<sup>d</sup>, CD80, and CD47-positive cells in murine F4/80<sup>+</sup> PEMs treated with or without ASA. **c**. The levels of I-A<sup>d</sup>, CD80, or CD47 molecules in murine F4/80<sup>+</sup> PEMs treated with or without ASA as determined by two-color FCM. Data are presented as mean ± SD. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with the corresponding groups



**Fig. 5** The direct effect of ASA on the phagocytosis of PEMs treated with ASA. Mouse PEMs were cultured with different levels of ASA (0.5, 1, 2, or 5 mM) in vitro. The phagocytosis was detected by FCM. *Medium* and *DMSO* indicate control. **a** One representative of the phagocytosis of murine F4/80<sup>+</sup> PEMs (treated with ASA for 24 h) to cRBCs as determined by FCM. The mean percentage of phagocytosis cells in F4/80<sup>+</sup> PEMs treated with ASA for 12 h and 24 h is summarized in **(b)** and **(c)**, respectively. Data are one representative of three independent experiments. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with the corresponding groups



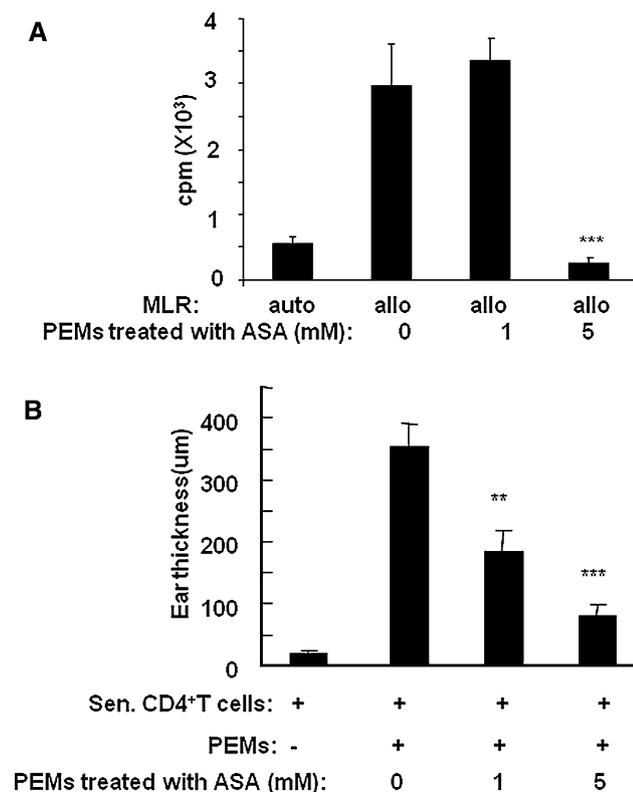
intradermally with allogeneic BALB/c PEMs (treated with or without ASA in vitro) into the pinna of naïve C57BL/6 mice. As shown in Fig. 6b, significant DTH responses were observed if sensitized C57BL/6 CD4<sup>+</sup> T cells were stimulated by allogeneic BALB/c PEMs. However, BALB/c PEMs treated with low and high doses of ASA in vitro induced significantly lower DTH responses of sensitized C57BL/6 T cells than did those of control mice ( $P < 0.01$ ; Fig. 6b).

## Discussion

The effect of ASA on macrophages in transplantation recipients is of interest since macrophages are key components in the immune response, performing different functions including phagocytosis and antigen presentation [8]. Resident macrophages are generally considered to be derived from circulating monocytes [26, 27]. In the present study, the effect of ASA on cell numbers, phenotype, and functions were detected in resident murine PEMs in both in vivo and in vitro assays. These observations indicated that ASA decreased cell numbers and functions and changed the phenotype expressions of PEMs. The decrease in cell numbers may be caused by the effect of ASA on macrophage viability and/or blocking the differentiation of macrophages from monocytes or bone marrow cells (BMCs). Our results showed that various concentrations of ASA used in the present study did not affect the viability of

macrophages, based on MTT assay. In addition, ASA-treated murine BMCs produced a significantly lower number of F4/80<sup>+</sup> macrophages than did ASA-untreated murine BMCs. These data support the possibility that the decrease in macrophage cell numbers in ASA-treated mice is due, at least in part, to the deficient differentiation of macrophages from BMCs, though other possibilities were not excluded.

Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes and tissue macrophages [28]. Phagocytosis by macrophages is an essential step and a part of innate immunity for protection against foreign pathogens, microorganisms, or dead cells [29]. ASA has previously been shown to enhance the phagocytosis of IgG-opsonized sheep RBCs by murine PEMs [30]. Nevertheless, other authors showed no significant effect of ASA on opsonic phagocytosis of *Candida albicans* by murine tumor-associated and peritoneal macrophages [31]. However, the effect of ASA on nonopsonic phagocytosis of xenogeneic cells (cRBCs) by macrophages is unknown. Therefore, our study focused particularly on the effect of ASA on the nonopsonic phagocytosis of xenogeneic cells by macrophages, which plays an important role in the rejection response to allografts [10]. Murine F4/80<sup>+</sup> PEMs treated with ASA both in vitro and in vivo have significantly lower phagocytosis capacity against cRBCs than those of control groups as assayed by TPM or FCM. Consistently with the ASA-treated murine F4/80<sup>+</sup>



**Fig. 6** The direct effect of ASA on the immunogenicity of murine PEMs in vitro. **a** The immune responses of C57BL/6 CD4<sup>+</sup> T cells stimulated by allogeneic BALB/c PEMs treated with or without ASA in vitro as determined by MLR. **b** DTH response induced by allogeneic BALB/c PEMs treated with or without ASA was detected in C57BL/6 mice. Data are presented as mean  $\pm$  SD. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with the corresponding groups

PEMs, ASA-treated BMMs showed significantly lower phagocytosis capacity against cRBCs than those of control groups. A significantly decreased phagocytosis capability of host macrophages exerted by ASA may be related to its immunosuppressive effect protecting allograft function and survival as well as reducing complications in transplant recipients, as previously reported [3–5, 32]. ASA might enhance the opsonic phagocytosis of macrophages [30], though some reports showed inconsistent data [31]. The reasons for the different effects of ASA on opsonic and nonopsonic phagocytosis are not clear at this point. It may be due to the different molecular networks involved in opsonic and nonopsonic phagocytosis, which need to be determined in the near future.

In order to gain more insight into the effect of ASA on the functional activity of macrophages, we also studied the effect of ASA on the immunogenicity of murine PEMs both in vitro and in vivo by MLR and DTH assays, respectively. Our results indicated that murine PEMs treated with a high dose of ASA (5 mM) showed significantly decreased immunogenicity to allogeneic T cells of

murine PEMs as compared to control. Significant DTH responses were consistently observed if sensitized C57BL/6 T cells were stimulated by allogeneic BALB/c PEMs. However, BALB/c PEMs treated with ASA both in vivo and in vitro induced significantly less DTH responses of sensitized T C57BL/6 cells than did those of control mice, which may be related to their low levels of MHC-II and co-stimulatory molecule expressions, because many different molecules and signaling networks cooperate to activate and regulate T cells when they encounter a display of immunogenic peptides by APCs. It should be noted that the ASA doses used for our present in vivo and in vitro studies are not comparable and that the doses of ASA used in vitro and in vivo are working via different mechanisms. Previous studies in vitro have indicated that ASA as low as 1 mM inhibited NF- $\kappa$ B activity [33] whereas only a dose of ASA 600 mg/kg/day could inhibit NF- $\kappa$ B in vivo [34]. The related molecular mechanisms should be clarified in the future.

In summary, ASA decreases cell numbers, phagocytosis and immunogenicity of macrophages as well as changing the phenotype expressions of macrophages. ASA has the ability to block the differentiation of macrophages. These data may offer important implications for the clinical application of ASA to prevent graft rejection and treat individuals with autoimmune diseases.

**Acknowledgments** The authors wish to thank Ms. Jianxia Peng for her expert technical assistance and Ms. Qinghuan Li for providing excellent laboratory management. This work was supported by grants from the National Natural Science Foundation for Key Programs (C30630060, Y.Z.), the National Natural Science Foundation for Distinguished Young Scholars (C03020504, Y.Z.), the Knowledge Innovation Program of Chinese Academy of Sciences (KSCX2-SW-333, Y.Z.), and the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry (2005-546, Y.Z.).

## References

- Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA*. 1993;90:11693–7.
- Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M, Wu KK. Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci USA*. 1999;96:5292–7.
- Grotz W, Siebig S, Olschewski M, Strey CW, Peter K. Low-dose aspirin therapy is associated with improved allograft function and prolonged allograft survival after kidney transplantation. *Transplantation*. 2004;77:1848–53.
- Jablonski P, Howden BO. Oral buprenorphine and aspirin analgesia in rats undergoing liver transplantation. *Lab Anim*. 2002;36:134–43.
- Stechman MJ, Charlwood N, Gray DW, Handa A. Administration of 75 mg of aspirin daily for 28 days is sufficient prophylaxis

- against renal transplant vein thrombosis. *Phlebology*. 2007;22:83–5.
6. Rees MA, Butler AJ, Brons IG, Negus MC, Skepper JN, Friend PJ. Evidence of macrophage receptors capable of direct recognition of xenogeneic epitopes without opsonization. *Xenotransplantation*. 2005;12:13–9.
  7. He C, Heeger PS. CD8 T cells can reject major histocompatibility complex class I-deficient skin allografts. *Am J Transplant*. 2004;4:698–704.
  8. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A. Alternatively activated macrophages during parasite infections. *Trends Parasitol*. 2004;20:126–33.
  9. Bondanza A, Zimmermann VS, Rovere-Querini P, Turnay J, Dumitriu IE, Stach CM, Voll RE, Gaipf US, Bertling W, Poschl E, Kalden JR, Manfredi AA, Herrmann M. Inhibition of phosphatidylserine recognition heightens the immunogenicity of irradiated lymphoma cells in vivo. *J Exp Med*. 2004;200:1157–65.
  10. Koyamada N, Sato A, Takayama J, Usuda M, Kawagishi N, Doi H, Fujimori K, Satomi S. Macrophage depletion prevents anti-graft antibody production and results in long-term survival in xenotransplantation. *Transplant Proc*. 2005;37:514–5.
  11. Aronoff DM, Canetti C, Peters-Golden M. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol*. 2004;173:559–65.
  12. Kepka-Lenhart D, Chen LC, Morris SM Jr. Novel actions of aspirin and sodium salicylate: discordant effects on nitric oxide synthesis and induction of nitric oxide synthase mRNA in a murine macrophage cell line. *J Leukoc Biol*. 1996;59:840–6.
  13. Joerink M, Ribeiro CM, Stet RJ, Hermsen T, Savelkoul HF, Wiegertjes GF. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *J Immunol*. 2006;177:61–9.
  14. Liu G, Ma H, Jiang L, Peng J, Zhao Y. The immunity of splenic and peritoneal F4/80(+) resident macrophages in mouse mixed allogeneic chimeras. *J Mol Med (Berlin, Germany)*. 2007;85:1125–35.
  15. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest*. 2007;117:175–84.
  16. Zhang X, Edwards JP, Mosser DM. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. *J Immunol*. 2006;177:1282–8.
  17. Liu G, Xia XP, Gong SL, Zhao Y. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. *J Cell Physiol*. 2006;209:341–52.
  18. Ing R, Segura M, Thawani N, Tam M, Stevenson MM. Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *J Immunol*. 2006;176:441–50.
  19. Clanchy FI, Holloway AC, Lari R, Cameron PU, Hamilton JA. Detection and properties of the human proliferative monocyte subpopulation. *J Leukoc Biol*. 2006;79:757–66.
  20. Mortara L, Ploquin MJ, Faye A, Scott-Algara D, Vaslin B, Butor C, Hosmalin A, Barre-Sinoussi F, Diop OM, Muller-Trutwin MC. Phenotype and function of myeloid dendritic cells derived from African green monkey blood monocytes. *J Immunol Methods*. 2006;308:138–55.
  21. Lee JK, Byun JA, Park SH, Choi HJ, Kim HS, Oh HY. Evaluation of the potential immunotoxicity of 3-monochloro-1,2-propanediol in Balb/c mice II. Effect on thymic subset, delayed-type hypersensitivity, mixed-lymphocyte reaction, and peritoneal macrophage activity. *Toxicology*. 2005;211:187–96.
  22. Zhao Y, Ohdan H, Manilay JO, Sykes M. NK cell tolerance in mixed allogeneic chimeras. *J Immunol*. 2003;170:5398–405.
  23. Sun Y, Ge BS, Kasai M, Diffendaffer C, Parks N, Li H, Peng J, Langnas AN, Zhao Y. Induction of regulatory T cells from mature T cells by allogeneic thymic epithelial cells in vitro. *Transpl Int*. 2006;19:404–14.
  24. Wang H, Zhao L, Sun Z, Sun L, Zhang B, Zhao Y. A potential side effect of cyclosporin A: inhibition of CD4(+) CD25(+) regulatory T cells in mice. *Transplantation*. 2006;82:1484–92.
  25. Zhao Y, Xiong W, Yang T, Prall A, Baxter BT, Langnas AN. Xenogeneic skin graft rejection in M-CSF/macrophage deficient osteopetrotic mice. *Xenotransplantation*. 2003;10:232–9.
  26. Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. *Trends Immunol*. 2002;23:201–8.
  27. Kaufmann SH, Schaible UE. Antigen presentation and recognition in bacterial infections. *Curr Opin Immunol*. 2005;17:79–87.
  28. Fujiwara N, Kobayashi K. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy*. 2005;4:281–6.
  29. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005;5:953–64.
  30. Yamada A, Suzuki T. Fc gamma 2b receptor-mediated phagocytosis by a murine macrophage-like cell line (P388D1) and peritoneal resident macrophages. Up-regulation by the inhibitors of phospholipase A2 and cyclooxygenase. *J Immunol*. 1989;142:2457–63.
  31. Valdez JC, Perdigon G. Piroxicam, indomethacin and aspirin action on a murine fibrosarcoma. Effects on tumour-associated and peritoneal macrophages. *Clin Exp Immunol*. 1991;86:315–21.
  32. Schill D. Better transplantation survival with aspirin? *Med Monatsschr Pharm*. 2005;28:248–9.
  33. Shackelford RE, Alford PB, Xue Y, Thai SF, Adams DO, Pizzo S. Aspirin inhibits tumor necrosis factor alpha gene expression in murine tissue macrophages. *Mol Pharmacol*. 1997;52:421–9.
  34. Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. *J Immunol*. 1996;156:3961–9.