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ORIGINAL ARTICLE

The immunity of splenic and peritoneal F4/80$^+$ resident macrophages in mouse mixed allogeneic chimeras

Guangwei Liu · Haixia Ma · Lingling Jiang · Jianxia Peng · Yong Zhao

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Abstract Mixed allogeneic chimeras are emerging as a prospective approach to induce immune tolerance in clinics. However, the immunological function of macrophages in mixed chimeras has not been evaluated. Using a B6→BALB/c mixed chimera model, we investigated the phenotype and function of F4/80$^+$ resident peritoneal exudate macrophage (PEMs) and splenic macrophages (SPMs) in vitro and in vivo. Recipient F4/80$^+$PEMs and SPMs in mixed chimeras expressed significantly lower levels of MHC-II, CD54, and CD23 than those in non-chimeric mice before lipopolysaccharide stimulation. Recipient F4/80$^+$PEMs and SPMs in mixed chimeras induced normal cell proliferation and delayed-type hypersensitivity of allo-T cells, but they induced more IFN-γ and IL-2 products and less IL-10 and TGF-β products of allo-T cells compared with those of non-chimeras. Furthermore, recipient F4/80$^+$PEMs and SPMs had significantly higher phagocytotic capacity against chicken red blood cells or allo-T cells than those of controls while they had normal phagocytosis to *Escherichia coli*. Although some slight but significant alterations of recipient macrophages have been detected, these results provide direct evidences for the efficient immunity of recipient macrophages in mixed allogeneic chimeras. The present study also, for the first time, offered basic information for macrophages maturing in heterogeneous environments.

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Abbreviations
APCs antigen-presenting cells

CFSE	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
cRBC	chicken red blood cells
DTH	delayed-type hypersensitivity
E. coli	*Escherichia coli*
FCM	flow cytometry
IFN-γ	interferon-γ
IL-10	interleukin-10
LPS	lipopolysaccharide
MFI	median fluorescence intensity
MLR	mixed leukocyte reactions
NO	nitric oxide
PEMs	peritoneal exudate macrophages
SPMs	splenic macrophages
TBI	total body irradiation
TPM	two photon microscope
TGF-β1	transforming growth factor-β1

Introduction

Macrophages, a highly heterogeneous population of antigen-presenting cells (APCs), are one of the important components for the first defense line (innate immunity) against invading microorganisms and transformed cells [1]. Importantly, by releasing cytokines, presenting antigens or phagocytosis effects, macrophages, also function in the secondary defense line, both humoral and cell-mediated immunity, so that they link the innate and adaptive immune systems [2]. A common progenitor gives rise to tissue macrophages, dendritic cells, and osteoclasts, which are distinct, irreversibly differentiated sublineages [3]. Once distributed through the blood stream, monocytes constitutively enter all tissue compartments of the body, including the peritoneal cavity, to differentiate into macrophages [1, 4]. Resident macrophage populations are the important part of the body to participate in innate immunity and immediate immune defense [5].

Mixed allogeneic chimerism, in which both donor and host stem cells contribute to hematopoiesis, can achieve robust donor-specific immune tolerance [6–8]. Mixed chimerism is associated with improved immunocompetence compared with full allogeneic chimerism [9]. Mixed allogeneic bone marrow chimeras have been believed as one of the most prospective approaches to induce transplant immune tolerance in clinical organ transplantation after its success in rodents and large animals [10]. It was been demonstrated that T, B, and NK cells had normal immune capacity and were specifically tolerant to donor antigens in mixed chimeric animal models by a large amount of studies [6, 11, 12]. Although some studies on the immune function of macrophages, which differentiate from myeloid precursors, in the early stage of mixed allogeneic chimeras has been previously studied [13–15], the immune state of recipient macrophages in mixed allogeneic chimeras has not been fully addressed so far. On the other hand, detailed studies on whether macrophages maturing in heterogeneous surroundings will show normal phenotype and function may help us better understand the basic biology of macrophages.

To evaluate immunological defensive capability of macrophages in mixed allogeneic chimeras, we detected the cell numbers, phenotypes, phagocytosis ability, cytokine release of recipient F4/80$^+$ resident peritoneal exudate macrophage (PEMs), and splenic macrophages (SPMs) in a B6→BALB/c mixed chimera model. Although some alterations were observed, recipient F4/80$^+$ macrophages in mixed chimeras have fairly normal immunity as determined in vitro and in vivo. The present results provide direct evidence for the normal immune responsive state of macrophages in mixed allogeneic chimeras, which support the application of mixed chimeras in clinics.

Materials and methods

Animals Five- to 7-week-old C57BL/6 (B6; H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) 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.

Preparation of mixed allogeneic chimeras B6→ BALB/c mouse allogeneic mixed chimeras were prepared as previously described [16, 17]. Briefly, BALB/c recipients received 8.0 Gy of total body irradiation (TBI) and an i.v. administration of ten million T cell-depleted B6 and BALB/c bone marrow cells (BMCs) on day 0. By 2 months, donor cells were 50±8% (N=32) in the peripheral blood and spleens as assessed by a flow cytometry (FCM; Becton Dickinson, CA). Some non-chimeric BALB/c control mice received TBI and syngeneic BMCs alone.

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), FITC-labeled hamster anti-mouse CD54 (ICAM-1) mAb (3E2), FITC-labeled hamster anti-mouse CD80 (B7-1) mAb (16-10A1), FITC-conjugated rat anti-mouse CD86 (B7-2) mAb (GL1), FITC-labeled mouse anti-mouse H-2D^b mAb (AF6-88.5), PE-labeled

anti-mouse H-2D^d (34-2-12), FITC-labeled mouse anti-mouse I-A^b mAb (AF6-120.1), phycoerythrin (PE)- or FITC-labeled rat anti-mouse CD4 mAb (RM4-5), PE- or 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 IFN-γ mAb (GIR-208), and anti-mouse IL-10 mAb (JES5-16E3). In addition, rat anti-mouse FcR mAb (2.4G2) was produced by 2.4G2 hybridoma (ATCC, Rockville, MD) in our laboratory.

Preparation of PEMs and SPMs Mouse PEMs and SPMs were prepared, and the purity was more than 90% of F4/80$^+$ macrophages as reported previously [18, 19]. PEMs and SPMs were labeled with biotinylated anti-mouse H-2D^d mAb (34-2-12) and BD IMag™ streptavidin particles-DM (BD Biosciences PharMingen). After labeling, the cells were separated using the BD IMaget™, and the negative (H-2D^{d-}) and positive (H-2D^{d+}) fractions were collected. The purity of recipient H-2D^{d+}F4/80$^+$PEMs or SPMs was more than 95% as determined by FCM.

Morphology observation BALB/c F4/80$^+$PEMs and SPMs were cultured in RPMI1640 medium 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 [18, 20].

Immunofluorescence staining and FCM BALB/c PEMs and SPMs (5×10^5) were washed once with FACS buffer [phosphate buffered saline (PBS), pH 7.2, containing 0.1% NaN_3 and 0.5% bovine serum albumin). For two-color staining, cells were stained with PE-labeled anti-mouse F4/80 mAb versus FITC-labeled anti-I-A^d, CD11c, CD80, CD86, CD40, CD54 or CD23, or the non-specific staining mAb, respectively. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least 10,000 cells were assayed using a FASCalibur flow cytometry (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). Non-viable cells were excluded using the vital nucleic acid stain propidium iodide. The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs [21]. The cell size of F4/80 positive cells was determined by the analysis of the forward scatter side with gating on F4/80$^+$ cells.

To determine intracellular cytokine levels of macrophages and CD4$^+$ T cells in mixed cell reaction (MLR), the IFN-γ and IL-10 production in CD4$^+$ T cells stimulated by allogeneic PEMs and SPMs were detected using BD cytofix/cytoperm plus (with GolgiPlug™) 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 six-well plates for 48 h. Cells were then pulsed with 1.0 μl/ml Brefeldin A (BD GolgiPlug; BD Biosciences PharMingen) for the last 8 h of culture. The non-adherent cells were collected and washed once with FACS buffer. After incubation with FcR blockade (2.4G2), cells were stained with FITC-conjugated anti-mouse CD4 mAb. These cells were then fixed and permeabilized with 500 μl of BD cytofix/cytoperm solution at room temperature in the dark for 20 min according to the manufacturer's instructions. They were stained with 0.25 μg of anti-mouse IFN-γ mAb and anti-mouse IL-10 mAb, respectively, for 30 min. Ten thousand CD4$^+$ cells were analyzed by FCM.

Allogeneic MLR CD4$^+$T cells were purified by negative selection of mouse splenocytes using mouse CD4$^+$T lymphocyte enrichment set-DM (BD Biosciences PharMingen). Triplicate wells containing 2×10^5 CD4$^+$T cell responders with 1×10^5 or with the indicated doses of macrophage stimulators (pre-treated 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 [22]. 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 Instruments, USA). 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 [21, 23].

The phagocytosis of chicken red blood cells (cRBCs), allogeneic T cells, and Escherichia coli AB1157 (E. coli) by macrophages in vitro and in vivo A single-cell suspension of cRBCs was 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). A suspension of *E. coli* containing a bacterial concentration corresponding to 10^8 colony-forming units (CFU) was used. After two washes with PBS, 1×10^7 cells/ml cRBC, CD3$^+$T, or 10^8 CFU *E. coli* were labeled with 5.0 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 15 min at 37°C. These cells were then washed thoroughly and resuspended at a concentration of 1×10^7cells/ml. Cell viability was determined by trypan blue exclusion. Cell viability was usually

more than 95%. F4/80$^+$PEMs or SPMs (1×10^6) were co-incubated with 1×10^6 CFSE-labeled cRBC, CFSE-labeled CD3$^+$T cells, or 10^8 CFU CFSE-labeled *E. coli* in six-well plates (Costar, Cambridge, MA) that had been preset with cover glass at 37°C and 5% CO_2 for 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 (BM8) mAb. Three-channel images were taken with a two-photon laser scanning microscope (TPM; 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. Meanwhile, the dynamic phagocytosis of CFSE-labeled cRBCs or CFSE-labeled *E. coli* by macrophages was observed with a two-photon laser scanning microscope (LSM510, Zeiss) [20].

In addition, a suspension of CFSE-labeled *E. coli* AB1157 containing a bacterial concentration corresponding to 10^8 CFU was injected into murine peritoneal cavity. At 15, 30, 60 and 120 min after injection, PEMs were collected as described above [18, 24]. Some cells were blocked with anti-mouse FcγR mAb (clone 2.4G2) and stained with Cy5-conjugated anti-F4/80 mAb (eBioscience, BM8; San Diego, CA) and PE-cojugated anti-H-2D^d mAb. After washing with cold PBS three times, the phagocytosis percentages of H-2D^{d+}F4/80$^+$ gate cells were determined using a FCM. Meanwhile, some PEMs were dropped on the cover glasses and cultured for 30 min. The adherent cells were stained by Giemsa and Wright staining solution (Sigma) after using methanol to fix, as reported previously [25].

The response to LPS of SPMs and PEMs Mouse PEMs or SPMs at $1X10^6$ 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 [18].

Detection of IL-2 and TGF-β1 levels in MLR by ELISA Mouse CD4$^+$T cells were cultured with allogeneic or syngeneic macrophages in 96-well plates for 3–4 days. The supernatants were harvested and analyzed for the levels of transforming growth factor-β1 (TGF-β1) and IL-2 using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D).

Delayed-type hypersensitivity About 2 weeks after C3H mice were immunized with BALB/c splenocytes, C3H CD4$^+$T cells were enriched using the negative selecting MACS kit for CD4$^+$T lymphocytes (BD Biosciences PharMingen). BALB/c F4/80$^+$PEMs or SPMs were used as stimulator cells. C3H effector CD4$^+$T cells and macrophage stimulators (5×10^5 cells/each) were injected intradermally into the pinnate of naïve C3H mice. The changes in ear thickness were measured using an engineer's micrometer at 24 or 48 h after challenge [26]. The ear thickness change was calculated by subtracting the thickness of the same ear before injection from that after injection [18].

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

Cell numbers and morphology of F4/80+ SPMs and PEMs in mixed chimeras We first detected the cell numbers of macrophages in spleen and peritoneal cavity in mixed chimeras and non-chimeric mice. The total cell numbers of F4/80$^+$PEMs, F4/80$^+$ SPMs, Mac-1$^+$ PEMs, and Mac-1$^+$ SPMs in mixed chimeras were significantly higher than those in control mice ($P<0.05$, Fig. 1a). Among them, the level of donor cells in mixed chimeras was about 50% as determined by a FCM. Recipient F4/80$^+$PEMs or SPMs of mixed chimeras showed typical globular characteristics in shape with irregular and intensive Wright- and Giemsa-stained nuclei as well as the high karyoplasmic ratio cytoplasm as in normal mice (Fig. 1b and data not shown) [18, 24]. However, the cell size of F4/80$^+$PEMs, but not F4/80$^+$SPMs, in mixed chimeras was significantly smaller than those of control ($P<0.05$, Fig. 1c). After stimulation with LPS, F4/80$^+$PEMs in all groups became significantly larger in size than the un-stimulated cells (Fig. 1b and c).

The phenotypes of recipient F4/80+PEMs and SPMs in mixed chimeras The expression of co-stimulatory molecules on APCs is crucial in determining the nature and extent of the immune response. The expressions of MHC-II and co-stimulatory molecules on recipient F4/80$^+$PEMs or SPMs in mixed chimeras were detected using a FCM. Without LPS stimulation, recipient F4/80$^+$PEMs and SPMs in both chimeric and non-chimeric mice express relatively low levels of MHC-II, CD80, CD86, CD40, CD11c, CD54, and CD23 molecules (Fig. 2a) [27]. However, significantly decreased percentages of recipient F4/80$^+$PEMs or SPMs expressing MHC-II, CD54, or CD23 molecules were detected in mixed chimeras compared with non-chimeric mice ($P<0.01$, Fig. 2a). After the treatment with LPS, recipient F4/80$^+$PEMs or SPMs in both groups expressed enhanced levels of MHC-II, CD80, CD86, CD40, CD11c, CD54, and CD23 molecules (Fig. 2a). After F4/80$^+$PEMs or SPMs were co-cultured with allogeneic T cells for 3 days, recipient F4/80$^+$PEMs or SPMs in mixed chimeras

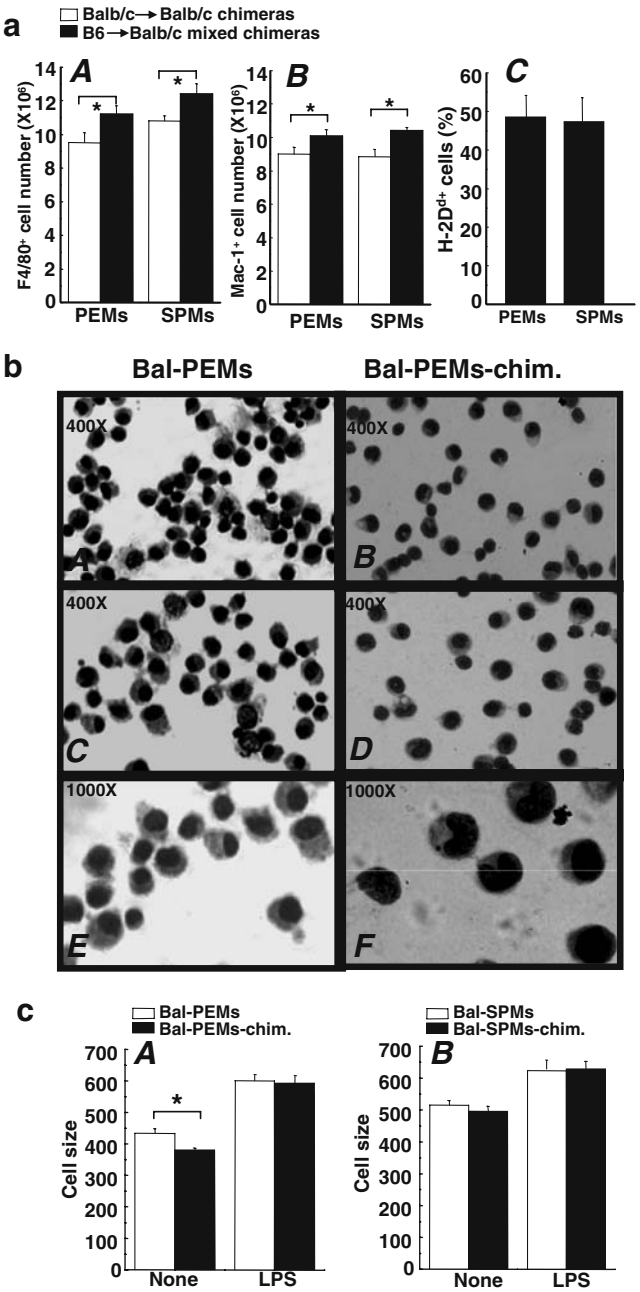


Fig. 1 Morphology of recipient F4/80$^+$ PEMs and SPMs in B6→BALB/c mixed chimeras assayed by Wright–Giemsa staining and FCM. **a** The total cell numbers of F4/80$^+$ PEMs (*A*), F4/80$^+$ SPMs (*A*), Mac-1$^+$ PEMs (*B*), and Mac-1$^+$ PEMs (*B*) as well as the percentages of recipient cells in PEMs and SPMs (*C*) in mixed chimeras. **b** Morphology of recipient PEMs in mixed chimeras with or without LPS stimulation. Freshly isolated BALB/c PEMs in non-chimeric mice (Bal–PEMs) (*A*) and recipient BALB/c PEMs in mixed chimeras (Bal-PEMs-chim) without (*A* and *B*) or with 0.5 μg/ml LPS stimulation for 24 h (*C*, *D* and *E*, *F*). **c** The cell size of Balb/PEMs (*A*) or SPMs (*B*) in mixed chimeras with or without LPS stimulation as detected by FCM. **P*<0.05 between the indicated groups. Results were shown as mean±SD (*N*=6). One representative of four independent experiments with identical results was shown

expressed significantly low levels of MHC-II and CD54 molecules compared with those of control mice ($P<0.05$; Fig. 2b). Thus, recipient F4/80$^+$PEMs and SPMs showed certain phenotype alteration in mixed chimeras.

Immunogenicity of recipient F4/80$^+$PEMs and SPMs in mixed chimeras to allogeneic CD4$^+$T cells The allogeneic immunogenicity of recipient F4/80$^+$PEMs and SPMs in mixed chimeras were determined in the present study. The in vitro results showed that both recipient BALB/c F4/80$^+$SPMs or PEMs in mixed chimeras could induce the proliferative reaction of allogeneic C3H CD4$^+$T cells in a dose-dependent manner in vitro as efficiently as those of control mice without any significant difference ($P>0.05$, Fig. 3a).

The immunogenicity of recipient F4/80$^+$PEMs and SPMs in mixed chimeras was further determined in vivo using an assay in which DTH reaction was induced by allogeneic F4/80$^+$PEMs or SPMs as described in "Materials and methods". Sensitized C3H CD4$^+$T lymphocytes were co-injected intradermally with allogeneic BALB/c F4/80$^+$PEMs or SPMs, respectively, into the pinnate of naïve C3H mice. The changes in ear thickness were measured at 24 or 48 h after challenge. As shown in Fig. 3b, significant DTH responses were observed if sensitized C3H T cells were stimulated by allogeneic BALB/c F4/80$^+$PEMs or SPMs, whereas there were no significant DTH responses if un-sensitized C3H T cells were stimulated by allogeneic BALB/c F4/80$^+$PEMs or SPMs, as reported [18]. Importantly, recipient BALB/c F4/80$^+$PEMs and SPMs in mixed chimeras induced DTH responses of sensitized allogeneic CD4$^+$T cells as efficiently as those of control group ($P>0.05$, Fig. 3b).

Cytokine secretion of allogeneic CD4$^+$T cells stimulated by F4/80$^+$SPMs and PEMs in mixed chimera in vitro IFN-γ, IL-2, TGF-β, and IL-10 are undoubtedly important in the regulation of adaptive immune responses and also in activating different subtype macrophages [28, 29]. After allogeneic C3H CD4$^+$T cells co-cultured with recipient F4/80$^+$ SPMs or PEMs, respectively, for 4 days, the levels of IFN-γ$^+$ cells in CD4$^+$T cells were determined by the intracellular staining method. As shown in Fig. 4, significantly increased levels of IFN-γ, IL-10, IL-2, and TGF-β produced by C3H CD4$^+$T cells were detected when they were cultured with allogeneic F4/80$^+$ SPMs or PEMs, regardless whether F4/80$^+$SPMs or PEMs were from mixed chimeras or not ($P<0.01$, Fig. 4). However, allogeneic F4/80$^+$SPMs or PEMs induced significantly higher percentages of IFN-γ$^+$ cells in C3H CD4$^+$T cells than those of non-chimeric mice ($P<0.05$, A in Fig. 4a). Recipient F4/80$^+$SPMs, but not PEMs, in mixed chimeras

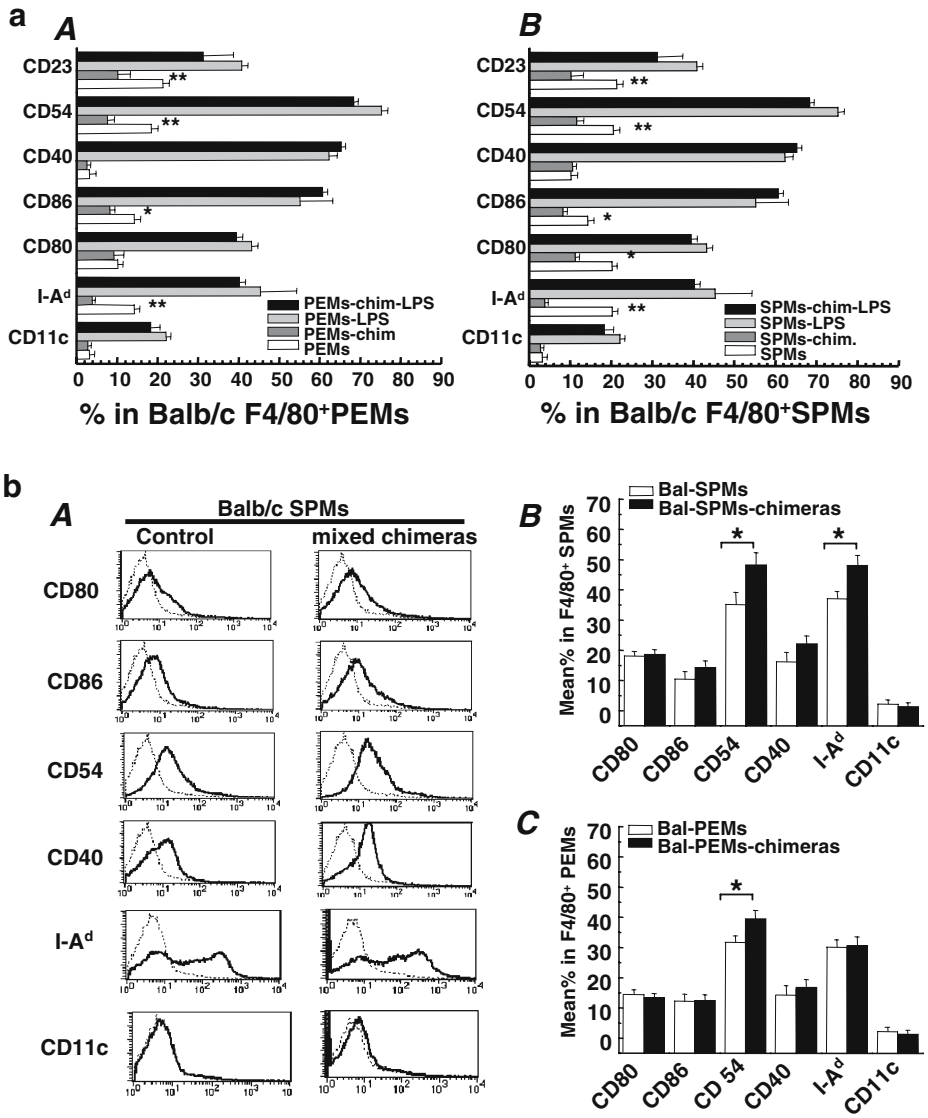


Fig. 2 Phenotype characteristics of recipient F4/80$^+$ PEMs and SPMs in B6→BALB/c mixed chimeras assayed by FCM. BALB/c recipient macrophages in mixed chimeras were cultured with or without the stimulation with 0.5 μg/ml LPS or with allogeneic C3H CD4$^+$T cells, and then stained with PE-labeled anti-F4/80 mAb versus FITC-labeled anti-CD11c, I-A^d, CD80, CD86, CD40, CD54, or CD23 mAb. *Dotted lines* represent the non-specific staining. **a** The percentages of CD11c, I-A^d, CD80, CD86, CD40, CD54, and CD23$^+$ cells in BALB/c F4/80$^+$ PEMs (*A*) or SPMs (*B*) in B6→BALB/c mixed chimeras or control mice stimulated with or without LPS. **b** The percentages of CD11c, I-A^d, CD80, CD86, CD40, and CD54$^+$ cells in BALB/c F4/80$^+$ PEMs or SPMs in B6→BALB/c mixed chimeras or control mice stimulated with or without allogeneic T cells. *A* One representative of the *staining* for BALB/c F4/80$^+$ SPMs in mixed chimeras or control mice assessed by FCM. The *dotted lines* represent the non-specific mAb staining, and *solid lines* were the indicated mAb staining. The percentages of CD11c, I-A^d, CD80, CD86, CD40, CD54, and CD23$^+$ cells in BALB/c F4/80$^+$ SPMs (*B*) or PEMs (*C*) in B6→BALB/c mixed chimeras or control mice stimulated with or without allogeneic C3H CD4$^+$ T cells. *P<0.05; **P<0.01 compared with the corresponding groups. Results were shown as mean±SD. More than eight mice in each group were examined

induced significantly higher percentages of IL-10$^+$ cells in C3H CD4$^+$T cells than those of non-chimeric mice (P< 0.05, B in Fig. 4a). Recipient F4/80$^+$PEMs in mixed chimeras induced significantly higher levels of IL-2 product by C3H CD4$^+$T cells than those of non-chimeric mice (P<0.05, A in Fig. 4b), while F4/80$^+$SPMs in mixed chimeras induced significantly lower levels of TGF-β1 product by C3H CD4$^+$T cells than those of non-chimeric mice (P<0.05, B in Fig. 4b).

The phagocytosis of allogeneic and xenogeneic cells by F4/80$^+$PEMs in mixed chimeras Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens [30, 31]. BALB/c F4/80$^+$PEMs were co-cultured with CFSE-labeled cRBCs for 4 h, and the phagocytic ability of macrophages was investigated using a TPM and FCM. The process for the phagocytosis of cRBCs by macrophages could be recorded (Fig. 5a) and observed by a TPM (Fig. 5b). When the phagocytosis of macro-

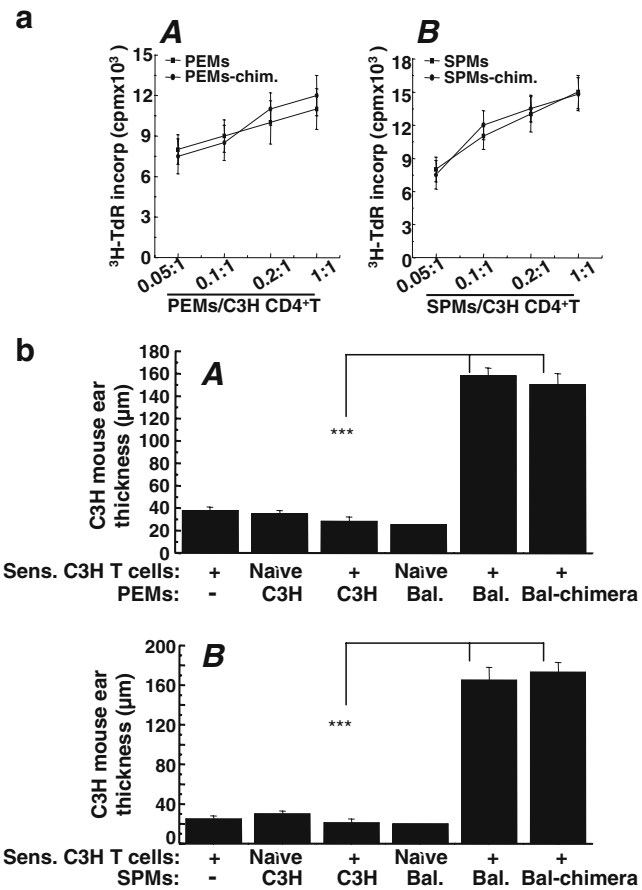


Fig. 3 The immunogenicity of recipient F4/80$^+$ PEMs and SPMs in B6→BALB/c mixed chimeras to allogeneic CD4$^+$ T cells in vitro and in vivo. **a** The proliferation of allogeneic C3H CD4$^+$ T cells induced by BALB/c F4/80$^+$ PEMs (*A*) or SPMs (*B*) in mixed chimeras or non-chimeric mice in vitro. Data were presented as mean±SD of triplicate wells. One representative of four independent experiments with similar data was shown. **b** DTH responses of allogeneic C3H CD4$^+$ T cells induced by BALB/c F4/80$^+$ PEMs (*A*) or SPMs (*B*) in mixed chimeras or control mice, respectively. Results were shown as mean±SD. Nine mice in each group were assayed

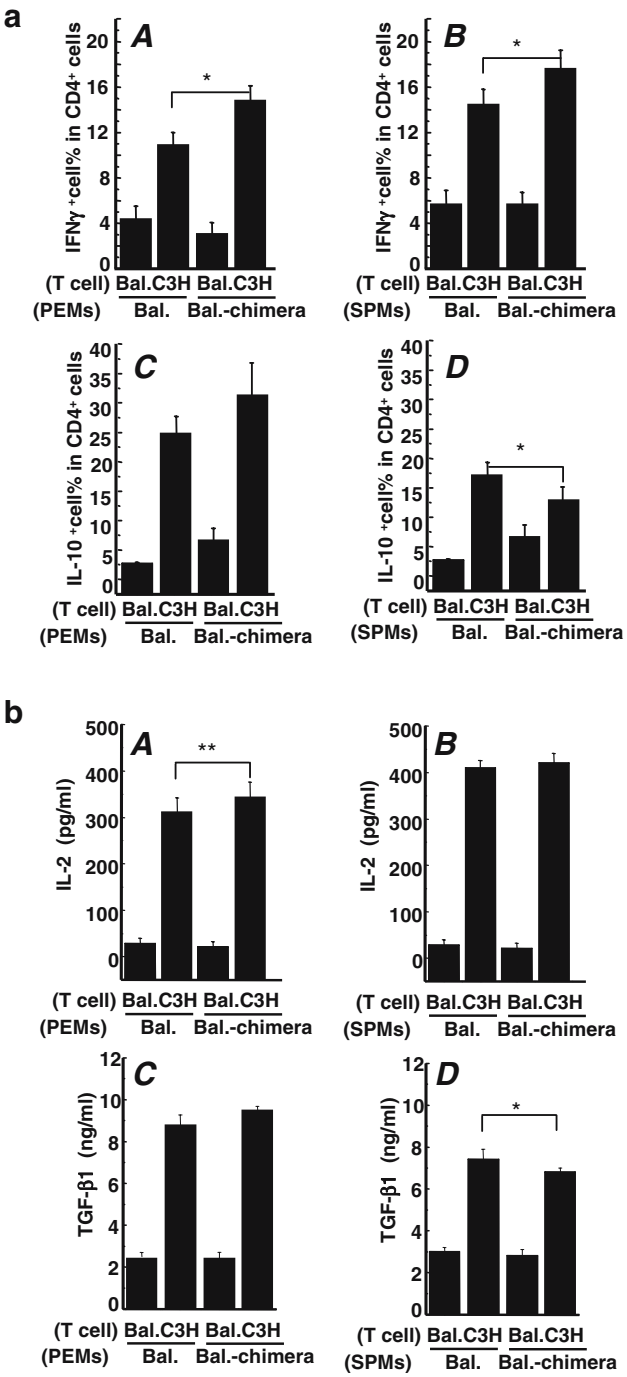
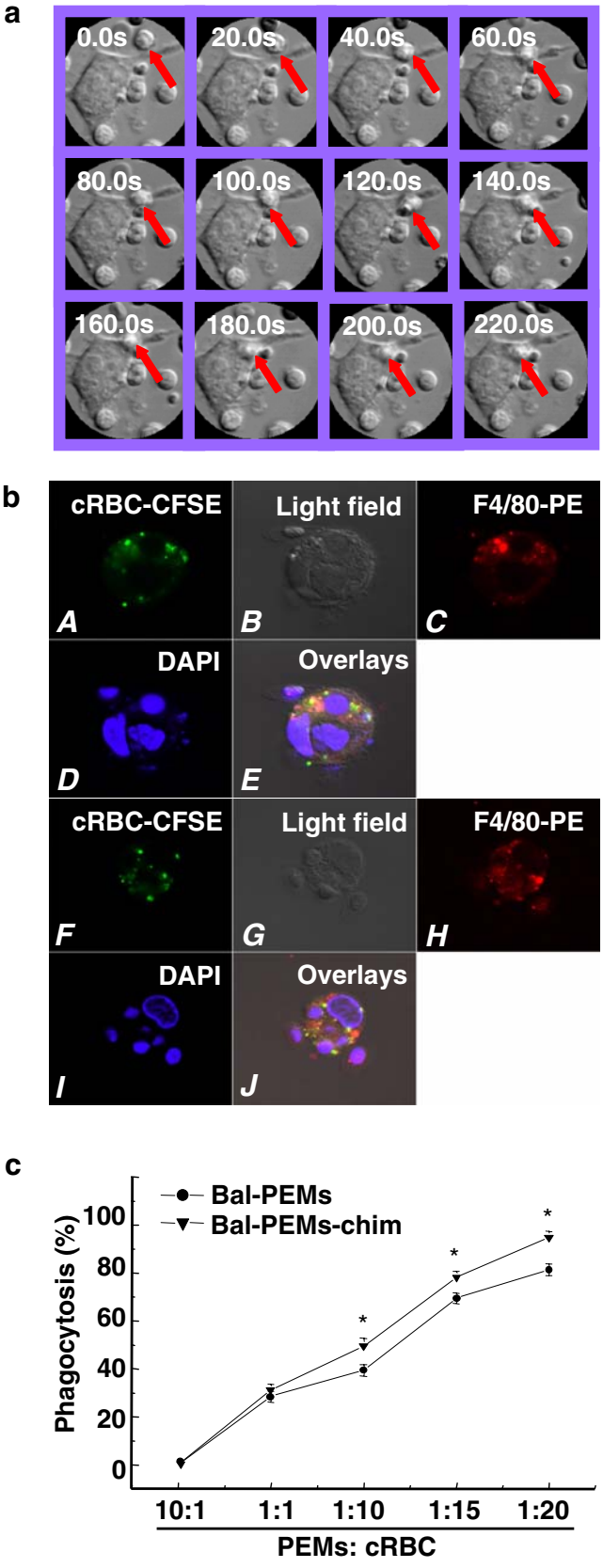


Fig. 4 Cytokine products of allogeneic CD4$^+$T cells stimulated by recipient F4/80$^+$ PEMs and SPMs in B6→BALB/c mixed chimeras. **a** IFN-γ (*A* and *B*) and IL-10 (*C* and *D*) productions by C3H CD4$^+$ T cells stimulated by recipient F4/80$^+$ PEMs (*A* and *C*) or SPMs (*B* and *D*) in mixed chimeras or control mice were determined by two-color intracellular staining FCM. **b** IL-2 and TGF-β1 products by C3H CD4$^+$ T cells stimulated by recipient F4/80$^+$ PEMs (*A* and *B*) and SPMs (*C* and *D*) in mixed chimeras or control mice was determined by ELISA. Data were presented as mean±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<0.05, **P<0.01 compared with the indicated groups

phages was qualified by a FCM, recipient BALB/c F4/80$^+$PEMs in mixed chimeras could phagocytize cRBCs cells in a higher efficiency compared with those in non-chimeric mice (P<0.05, Fig. 5c).

In addition, the phagocytosis of allogeneic T cells by macrophages was investigated using a TPM and FCM. As shown in Fig. 6, the phagocytosis of CFSE-labeled allogeneic T cells by macrophages in mixed chimeras or not could be observed by a TPM (Fig. 6a). Interestingly, recipient F4/80$^+$PEMs in mixed chimeras could phagocytize allogeneic T cells in a higher efficiency compared with those in non-chimeric mice as qualified by a FCM (P <0.05, Fig. 6b).



◀ **Fig. 5** Phagocytosis of cRBCs by BALB/c recipient F4/80$^+$PEMs in mixed chimeras as detected by TPM and FCM. **a** Continuous dynamic observation on the phagocytosis course of recipient F4/80$^+$PEMs in mixed chimeras on cRBCs in vitro as detected by TPM. **b** The phagocytosis of F4/80$^+$PEMs to cRBCs observed using a TPM. *A–E* BALB/c F4/80$^+$ PEMs in non-chimeric mice(×630); *F-J* BALB/c F4/80$^+$PEMs in mixed chimeras(×630). One representative of five independent experiments with similar results was shown. **c** The phagocytosis percentages of cRBCs by BALB/c F4/80$^+$ PEMs in mixed chimeras and non-chimeric mice. *P<0.05 vs corresponding PEMs. Six mice in each group were assayed and three independent experiments were performed

The phagocytosis of E. coli by F4/80$^+$PEMs in mixed chimeras Anti-microbe infection ability is an important index of macrophage immune function [32]. The phagocytosis of *E. coli* by PEMs in mixed chimeras was performed using a TPM and FCM. As shown in Fig. 7a, dynamic course shows that recipient F4/80$^+$PEMs in mixed chimeras have the ability to engulf *E. coli* in vitro. Furthermore, at 15, 30, 60, and 120 min after injection of CFSE-labeled *E. coli*, PEMs were collected, and phagocytosis percentage of recipient H-2D^{d+}F4/80$^+$PEMs were detected by a FCM and Wright–Giemsa staining as described in "Materials and methods". The phagocytic rate to *E. coli* by recipient F4/80$^+$PEMs in mixed chimeras did not show significant difference compared with those in non-chimeric mice (P> 0.05, Fig. 7b and c), indicating that recipient F4/80$^+$PEMs in mixed chimeras have normal phagocytosis ability against microorganisms in vivo.

Discussion

Macrophages play critical roles in initial defense against pathogens and that macrophage immunity significantly influences subsequent acquired immune responses [33, 34]. Our present study showed that recipient F4/80$^+$ resident macrophages in mixed chimera had relatively normal immunogenicity to allo-T cells, non-opsonic phagocytosis of allogeneic, xenogeneic, and pathogenic target cells in vitro and in vivo. These data provide the first evidence that macrophages maturing in heterogeneous surroundings have immunocompetence. It is of significance for the clinical application of mixed allogeneic chimeras to induce transplant tolerance, as the ensured healthy immunity of the reconstitute immune system in this approach is critical, otherwise, the advantage of the induced transplant tolerance over immunosuppression will disappear.

Recipient F4/80$^+$PEMs and SPMs in mixed chimeras with typical sphere characteristics including large karyoplasmic ratio and deeply stained nuclei with Wright–Giemsa staining are significantly smaller than those of control mice. However, after the treatment with LPS,

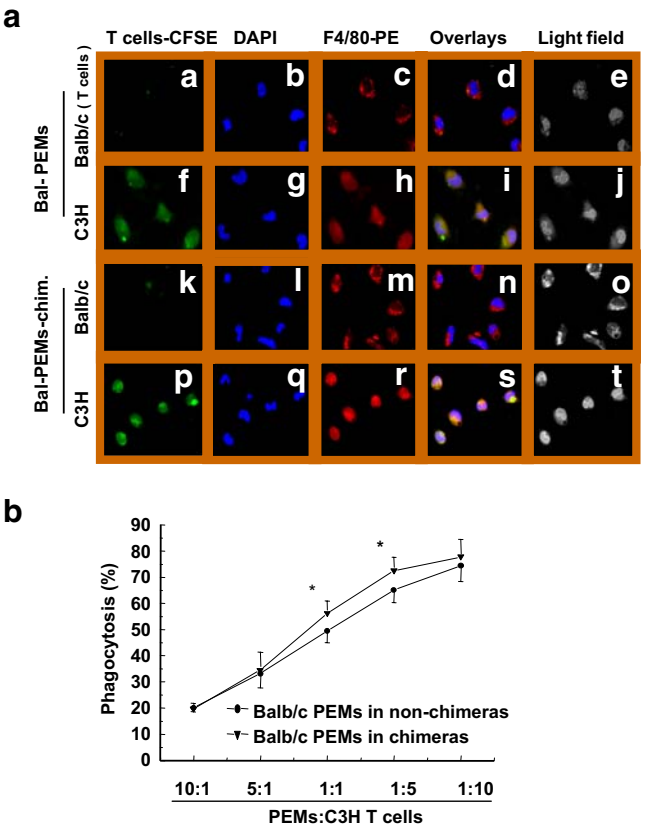


Fig. 6 Phagocytosis of allogeneic T cells by recipient F4/80$^+$ PEMs in mixed chimeras. **a** Continuous dynamic observation on the phagocytosis course of recipient PEMs in mixed chimeras on allogeneic C3H T cells in vitro. **b** The in vitro phagocytosis effects of BALB/c recipient F4/80$^+$ PEMs in mixed chimeras and control mice against C3H T lymphocytes detected with a TPM (magnification, ×630). One representative of the four independent experiments is shown. **c** Recipient F4/80$^+$PEMs phagocytized allogeneic C3H CD3$^+$ T cells as determined by FCM. BALB/c PEMs were stained with PE-labeled anti-F4/80 mAb, whereas C3H T cells were labeled with CFSE. BALB/c PEMs were co-cultured with BALB/c, C57BL/6, and C3H T cells. *P<0.05 vs the corresponding PEMs in control mice. Six mice in each group were used

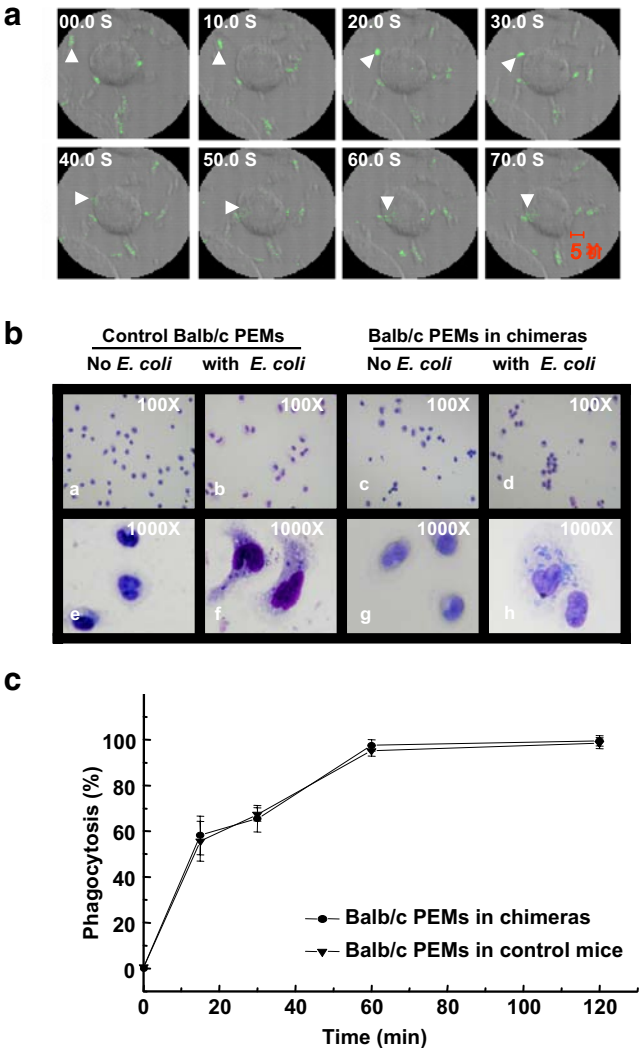


Fig. 7 Phagocytosis against *E. coli* by recipient F4/80$^+$ PEMs in mixed chimeras as detected by TPM and FCM. **a** Continuous dynamic observation on phagocytosis of recipient F4/80$^+$ PEMs in mixed chimeras on *E. coli* target cells in vitro. *E. coli* was pre-labeled with CFSE. **b** Morphology of recipient PEMs in mixed chimeras or control mice after culture without or with *E. coli* **c** The phagocytosis of *E. coli* by recipient F4/80$^+$ PEMs in mixed chimeras or control BALB/c mice in vivo. *E. coli* cells were injected into peritoneal cavity as described in "Materials and methods". Six mice in each group were assayed and two independent experiments were performed. Data are shown as mean±SD

recipient F4/80$^+$ macrophages in mixed chimeras acquired remarkably larger size, and their nuclei appeared much more irregular, which showed no detectable difference with control group stimulated with LPS. These data indicate that F4/80$^+$ macrophages in mixed chimeras might have the same potential ability for the enlarging cell size after stimulation [1, 18, 35]. Different subpopulations of macrophages were identified according to their cell size. It is true that cells with different sizes may have a different function no matter whether they are in different developmental/activation phases or specialized subsets [35]. The reasons for the smaller cell size of recipient macrophages in mixed chimeras are not clear at this moment.

Significantly lower percentages of un-stimulated recipient F4/80$^+$PEMs or SPMs expressing MHC-II, CD54 or CD23 molecules were detected in mixed chimeras compared with those in non-chimeric mice. These data, together with the small cell size of macrophages in mixed chimeras as mentioned above, indicate that more immature macrophages seem to present in mixed chimeras. However, after the treatment with LPS, recipient F4/80$^+$PEMs and SPMs in mixed chimeras expressed similar levels of MHC-II and co-stimulatory molecules as those cells in non-chimeric mice.

Furthermore, after the co-culture with allogeneic T cells, significantly higher percentages of recipient F4/80$^+$PEMs or SPMs in mixed chimeras expressed MHC-II and CD54

molecules compared with those cells in non-chimeric mice. Consistent with the phenotype alteration, recipient F4/80$^+$PEMs or SPMs in mixed chimeras induced more IFN-γ and IL-2 productions as well as less IL-10 or TGF-β productions of allogeneic CD4$^+$T cells than those control macrophages, respectively, although they showed similar immunogenicity as determined by cell proliferation and DTH reaction of allogeneic T cells. These results suggest that macrophages in mixed chimeras might be in favor to induce Th1 immune response of allogeneic T cells.

The phagocytosis of cRBCs and allogeneic T cells by recipient F4/80$^+$PEMs and SPMs in mixed chimeras is slightly, but significantly, higher than those cells in non-chimeric mice. This is reversely related to the levels of MHC-II and co-stimulatory molecules expressed on macrophages. It is known that macrophage phagocytosis ability changes might be related to the developmental stages of macrophages [35, 36]. So, these data indicate that although macrophages in mixed chimeras have normal immune response ability, these cells maybe have different maturation subtypes from normal macrophages, which needs to be confirmed.

Anti-microbe infection ability is one of the important functions of macrophages [37]. Recipient F4/80$^+$PEMs in mixed chimeras have the same phagocytosis ability of *E. coli* as those of control mice in an in vivo experimental model. This suggests that recipient macrophages in mixed chimeras might have normal immune defensive ability to pathogen infection. It is an important hint for the immunocompetence of macrophages in mixed allogeneic chimeras.

In summary, recipient F4/80$^+$ macrophages in mixed allogeneic chimeras were fully immunofunctional as determined by their phenotypes including co-stimulatory and MHC II molecules, immunogenicity, as well as phagocytosis to allo- and xeno- or bacterial targets, although some slight but significant alterations in these cells were observed. These results provide evidences for the immunobiological capacity of recipient F4/80$^+$ resident macrophages in allogeneic mixed chimeras. The immunocompetence of recipient macrophages in mixed chimeras indicates that the mixed surroundings with both donor- and recipient-derived cells may not alter the immunity of macrophages during the later cell development. Due to the importance of macrophages in defending against infection and tumor cells, the present study offered support for the potential clinical application of mixed allogeneic chimeras to induce transplant tolerance. In addition, these data indirectly support the speculation that macrophages in bone-marrow-transplanted patients may have efficient immunity.

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