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Characterization of the liver-macrophages isolated from a mixed primary culture of neonatal swine hepatocytes☆

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ABSTRACT

We recently developed a novel procedure to obtain liver-macrophages in sufficient number and purity using a mixed primary culture of rat and bovine hepatocytes. In this study, we aim to apply this method to the neonatal swine liver. Swine parenchymal hepatocytes were isolated by a two-step collagenase perfusion method and cultured in T75 culture flasks. Similar to the rat and bovine cells, the swine hepatocytes retained an epithelial cell morphology for only a few days and progressively changed into fibroblastic cells. After 5–13 days of culture, macrophage-like cells actively proliferated on the mixed fibroblastic cell sheet. Gentle shaking of the culture flask followed by the transfer and brief incubation of the culture supernatant resulted in a quick and selective adhesion of macrophage-like cells to a plastic dish surface. After rinsing dishes with saline, the attached macrophage-like cells were collected at a yield of 10^6 cells per T75 culture flask at 2–3 day intervals for more than 3 weeks. The isolated cells displayed a typical macrophage morphology and were strongly positive for macrophage markers, such as CD172a, Iba-1 and KT022, but negative for cytokeratin, desmin and α-smooth muscle actin, indicating a highly purified macrophage population. The isolated cells exhibited phagocytosis of polystyrene microbeads and a release of inflammatory cytokines upon lipopolysaccharide stimulation. This shaking and attachment method is applicable to the swine liver and provides a sufficient number of macrophages without any need of complex laboratory equipments.

1. Introduction

Liver-macrophages, or Kupffer cells, are the resident macrophages in the liver [1]. These cells comprise approximately 10–15% of all liver cells [2]. These cells are localized along the sinusoidal space or its immediate vicinity in the liver, and are involved in the regulation of physiological homeostasis and immunosurveillance in this organ [3]. The liver-macrophages also play a protective role against liver damage and promote the regeneration and fibrosis in cholestatic liver injury [1]. However, in pathological circumstances, activated liver-macrophages may aggravate liver damage, leading to cirrhosis and eventually failure of the organ. Therefore, liver-macrophages are considered to be an important therapeutic target for pharmacological intervention [4,5]. Thus, various methods of isolating the liver-macrophages for *in vitro* studies have been elaborated and refined in a number of mammalian species.

The conventional method of liver-macrophages starts with dissociation by collagenase perfusion, pronase treatment to remove parenchymal hepatocytes and finally counterflow centrifugal elutriation to separate the liver-macrophages from other nonparenchymal cells [6,7]. In addition, various modifications of this method for specific animal species have been reported, including human [8,9] and bovine [10,11] applications. However, all of these methods still require complex equipment and technical skill.

To circumvent these technical difficulties, we recently developed a novel procedure for obtaining liver macrophages in sufficient number and purity, using a mixed primary culture of liver cells from the adult rat [12,13] and bovine [14]. In this study, we applied this simple and efficient method to the neonatal swine liver and succeeded repeatedly isolating sufficient numbers of swine liver-macrophages. These cells are useful tools for the functional analyses of the innate immune response in this important livestock species.

Abbreviations: CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial to mesenchymal transition; FACS, fluorescent activated cell sorter; SMA, α-smooth muscle actin; DES, desmin; M-CSF, macrophage colony-stimulating factor; LPS, lipopolysaccharide.

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2. Materials and methods

2.1. Liver cell dissociation and primary culture

Swine neonates at 1–7 days of age were obtained from the animal facility in the National Institute of Animal Health, according to the institutional guidelines for animal experiments (Approval no. 12-085). After deep anesthesia by intravenous injection of sodium pentobarbital (20 mg/kg) and exsanguination, the lobes of the swine liver were dissected out and parenchymal hepatocytes were isolated by the perfusion of saline followed by collagenase into the portal vein [10,15,16]. Parenchymal hepatocytes were suspended in growth medium composed of DMEM (D6429, high-glucose type, Sigma-Aldrich, St. Louis, MO) containing 10% heat inactivated FCS (Sanko Junyaku Co. Ltd., Tokyo, Japan), supplemented with 100 μM β-mercaptoethanol (M3148, Sigma-Aldrich), 10 μg/ml insulin (I5500, Sigma-Aldrich), 100 μg/ml streptomycin and 100 U/ml penicillin (15140-122, Life Technologies, Carlsbad, CA), and seeded into tissue culture flasks (surface area: 75 cm^2, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of 6.7×10^4 cells/cm^2, as described [12–14]. The culture flasks were coated with type I collagen (Cellmatrix Type I–C, Nitta Gelatin Inc., Osaka, Japan). Culture medium was replaced every 2–3 days (intervals).

2.2. Isolation of macrophage-like cells by shaking and attachment method

After 5–13 days of culture, when most of the hepatocytes had transformed into fibroblastic cells, round macrophage-like cells started to proliferate vigorously on the cell sheet. These macrophage-like cells were suspended by reciprocal shaking of the culture flasks at 120 strokes/min for 10–15 min at 37 °C. The fibroblastic cell sheet remained intact, but occasionally a few fibroblastic cells were suspended into the culture medium. The culture medium was transferred into 90 mm non-tissue culture grade plastic dishes (MS-1390R, Sumitomo Bakelite Co., Ltd.). After incubation for 6 h at 37 °C, followed by rinsing with PBS, the macrophage-like cells attached onto the dish surface were harvested by treatment with TrypLE Express (Life Technologies), as described elsewhere [12–14]. Contaminating fibroblastic cells did not attach onto non-tissue culture grade plastic dishes and were removed during rinse with PBS.

2.3. Immunocytochemistry

The isolated macrophage-like cells were seeded in eight-well chamber glass slides (354118, BD Biosciences) at the density of 10^5 cells/well with the growth medium. The next day, the cells were washed with PBS, fixed with 95% ethanol and 1% acetic acid and processed for immunocytochemistry, as described [17]. The primary antibodies were as follows: mouse monoclonal anti-CD172a (VMRD, Inc., Pullman, WA); rabbit polyclonal anti-Iba 1 (Wako Purechemical Industries, Ltd, Osaka, Japan); mouse monoclonal anti-macrophage scavenger receptor MSR-A: CD204 (KT022; TransGenic, Inc., Kumamoto, Japan); mouse monoclonal anti-cytokeratin 18 (CK18; Millipore Co., Billerica, MA); mouse monoclonal anti-α smooth muscle actin (SMA; Progen); mouse monoclonal anti-desmin (DES; Thermo Scientific, Rockford, IL). After rinsing the slides with PBS containing 0.05% Tween 20, an EnVision system (DAKO, Japan) was used to visualize the antibody–antigen reaction according to the manufacturer's protocol. The immunostained slides were observed and photographed with a Leica DM5000B microscope equipped with a digital camera system.

2.4. Phagocytic assay

Fluorescence-labeled polystyrene microbeads (1.0 μm diameter, #17154, Polysciences, Inc., Warrington, PA) were diluted at 1:800 in the growth medium, and added to the isolated macrophage-like cells seeded in eight-well chamber glass slides (10^5 cells/well) or 60 mm non-tissue culture grade plastic dishes (10^6 cells/plate). After incubation for 0.5, 1 and 2 h at 37 °C, the cells in the chamber glass slides were rinsed with PBS three times to remove any non-phagocytosed beads [18,19], and fixed with a mixture of 95% ethanol and 1% acetic acid. The slides were incubated with a mouse monoclonal anti-CD172a antibody, followed by goat anti-mouse IgG labeled with Alexa Fluor 594 (Life Technologies), covered with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and photographed with a Leica DM5000B fluorescent microscope system equipped with a digital camera. For analysis using a fluorescence-activated cell sorter (FACS), the cells in the plastic dishes were harvested with TrypLE Express at the time points indicated, rinsed with PBS three times to remove nonphagocytosed beads and fixed with 3.7% formalin in PBS at room temperature for 15 min. After washing with PBS, cells were suspended in 0.5 ml of Iso Flow (Beckman Coulter, Fullerton, CA) and analyzed with a flow cytometer (Epics XL-MCL, Beckman Coulter) for the phagocytosis of the fluorescence-labeled microbeads.

2.5. Cytokine production

The isolated macrophage-like cells were seeded in 60 mm non-tissue culture grade plastic dishes (MS-1160R, Sumitomo Bakelite Co., Ltd.) at a density of 10^6 cells/plate. The next day, the medium was replaced by growth medium containing lipopolysaccharide (L3129, Sigma-Aldrich) at 0.1–1.0 μg/ml. After incubation for 24 h at 37 °C, the culture supernatant was collected, filtered with a membrane filter (0.45 μm pore size, Millipore Millex) and stored at −80 °C until use. Aliquots of the samples were assayed using swine cytokine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The experiments were independently performed at least three times, and the cytokine concentrations in the culture supernatant are expressed as the mean value ± SEM.

2.6. Culture and expansion of macrophages from adult pig blood

For comparison, macrophages from adult pig blood were selectively expanded and cultured on STO mouse fibroblasts (RCB0536, RIKEN, Cell Bank, Tsukuba, Japan) according to the method described [20]. The isolated macrophages were seeded in eight-well chamber glass slides (10^5 cells/well) and processed for immunocytochemistry, as described above.

3. Results

3.1. Proliferation of macrophage-like cells in the mixed primary culture of swine hepatocytes

Swine parenchymal hepatocytes readily became attached to the surface of a collagen-coated tissue culture flask, as reported previously [16]. They spread to form a polygonal cobblestone-like monolayer after 2 days of incubation (Fig. 1). Immunocytochemistry showed that almost all the cells at this stage were positive for CK18 (Fig. 2A), which is a marker for parenchymal hepatocytes. On the other hand, small numbers of contaminating cells, such as hepatic stellate cells (positive for SMA), were detected in the cell culture (Fig. 2A). In addition, small numbers of CD172a-positive cells were observed among the hepatocytes (Fig. 2A, arrowheads). These cells displayed slender cell processes, which are typically observed

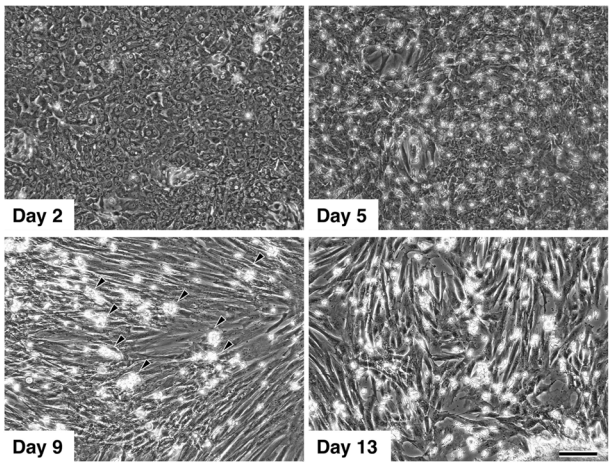

Fig. 1. Primary culture of swine hepatocytes and proliferation of macrophage-like cells. After 2 days of culture, parenchymal hepatocytes spread on to the surface of the culture flasks and displayed a typical polygonal cobblestone-like morphology. Parenchymal hepatocytes lost their epithelial cell morphology after 5 days in culture changed into more flattened, fibroblastic cells. Around day 9, phase contrast-bright, round macrophage-like cells started to proliferate on the fibroblastic cell sheet (arrowheads). The proliferation of the macrophage-like cells continued and reached a maximum on day 13 and thereafter. Scale bar = 100 μm.

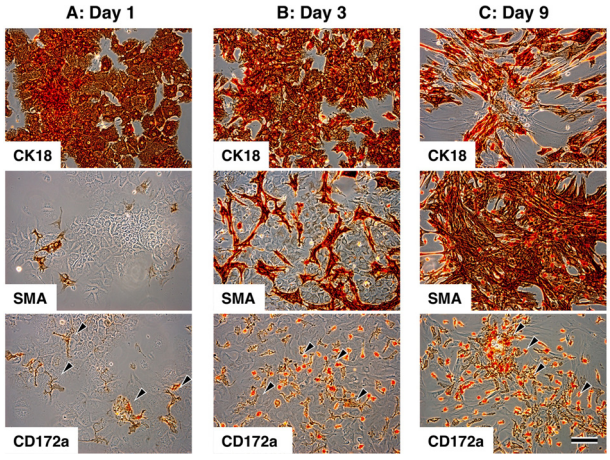

Fig. 2. Immunocytochemical staining of swine hepatocytes in primary culture. Cells that were mainly composed of parenchymal hepatocytes were obtained and seeded in eight-well chamber glass slides. After culture for the indicated periods, the cells were fixed and immunocytochemically stained with the primary antibodies indicated. As shown in Panel A (Day 1), the culture is mostly dominated by CK18-positive parenchymal hepatocytes, whereas the numbers of SMA-positive mesenchymal cells were relatively small. There were a certain number of slender cells which were positive for the swine macrophage marker CD172a (arrowheads), suggesting the starting cell population contained a small numbers of macrophages. In Panel B (Day 3), most of the fibroblastic cells were positive for CK 18, whereas SMA-positive cells started to increase their number. Many CD172a-positive cells were observed on the flat cell sheet, indicating that macrophage-like cells were steadily increasing their numbers in this specific culture environment. In Panel C (Day 9), the culture was dominated by SMA-positive cells. Also, large numbers of CD172a-positive cells were proliferating on the mixed fibroblastic cell layer. Scale bar = 100 μm.

in resting macrophages in culture. This suggests that the parenchymal hepatocyte-rich cell fraction contained a small but nevertheless substantial number of macrophage-related cells.

As the culture proceeded from day 5–13, the parenchymal hepatocytes lost the epithelial cell morphology and morphologically turned into more flattened, fibroblast-like cells (Fig. 1). Some of these transformed hepatocytes were positive for SMA (Fig. 2B) around day 3, although most of the other cells still continued to express CK18 (Fig. 2B). The number of SMA-positive cells increased as the culture proceeded further after 9 days of culture (Fig. 2C). These results suggest that

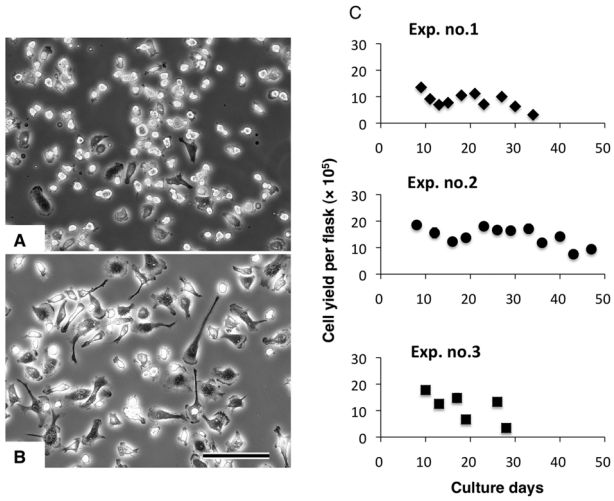

Fig. 3. Selective isolation of swine macrophage-like cells by shaking and attachment method. Cells were suspended into the culture medium by gently shaking the flasks, subsequently transferred into a non-tissue culture grade plastic dish and incubated at 37 °C. Macrophage-like cells promptly attached to the dish surface, while other, contaminating fibroblastic cells remained suspended. After a rinse with PBS, a highly purified macrophage population was obtained and they exhibited mainly a round shape after 6 h of incubation (A). These cells showed a typical macrophage morphology after 1 day of culture (B). Subsequent changes in the cell numbers harvested from the T75 culture flasks at different culture periods are shown in C as the average ± SEM from five flasks. Experiments were repeated three times and the data are indicated by different symbols. The error bars for SEM are smaller than the size of the symbols. Scale bar = 100 μm.

the epithelial to mesenchymal transition (EMT) was progressively induced in swine hepatocytes under these culture conditions, and the SMA-positive hepatic stellate or smooth muscle cells became dominant. Concomitantly, phase contrast-bright, round macrophage-like cells started to proliferate on the fibroblastic cell sheet around day 9 (Figs. 1 and 2B, arrowheads). The growth of the macrophage-like cells continued and reached a plateau around day 13 and thereafter (Fig. 1). In accordance with the phase contrast microscopy, immunocytochemistry demonstrated many CD172a-positive cells on the fibroblastic cell sheet (Fig. 2C). Therefore, as observed in the rat [12] and bovine [14] livers, macrophage-like cells actively proliferated on a cell sheet having a mixed primary culture of swine hepatocytes.

3.2. *Isolation and purification of macrophage-like cells from mixed culture flasks*

Macrophage-like cells were suspended in the culture medium by shaking the culture flasks, followed by transfer into plastic dishes. After incubation for 6 h at 37 °C, macrophage-like cells readily became attached to the dish surface (Fig. 3A), whereas fibroblastic cells or other cells remained suspended in the medium. These contaminating cells were removed by washing with PBS. After 24 h culture, these cells exhibited a typical macrophage-like morphology, extending filopodia and lamellipodia (Fig. 3B).

The average yields of the macrophage-like cells, which were repeatedly harvested from the flasks at different culture periods, are shown in Fig. 3C. The macrophage-like cells were harvested as early as day 9; thereafter, more than 10^6 cells were harvested from each T75 flask repeatedly at 2–3 day intervals for at least 3 weeks or more. These results demonstrate that the shaking and attachment method is applicable to primary cultures of swine liver cells, with a total macrophage-like cell yield per flask of more than 10^7.

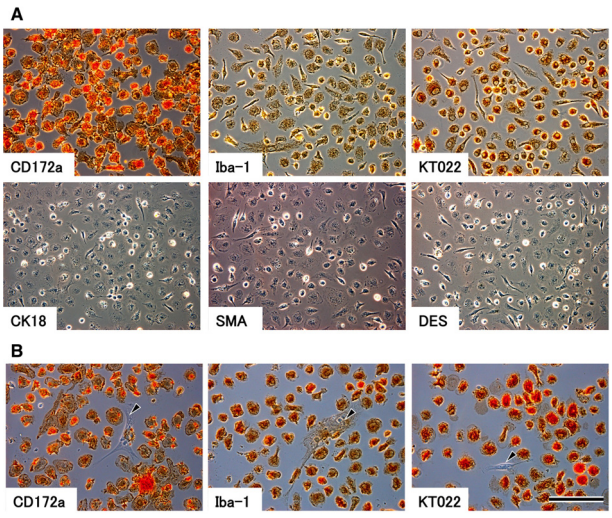

Fig. 4. Immunocytochemical staining of swine macrophage-like cells selectively isolated by shaking and an attachment method from the mixed primary culture of swine hepatocytes (A). The cells were plated in eight-well chamber glass slides in DMEM-based medium containing 10% FCS. The next day, the cells were fixed and stained with specific antibodies against CD172a, Iba-1, KT022, CK18, SMA and DES, as described in Materials and Methods. For comparison, macrophages from adult pig blood were selectively expanded and cultured on STO mouse fibroblasts. Harvested macrophages were seeded in eight-well chamber glass slides, fixed and stained accordingly (B). A contaminating STO fibroblast (arrowheads) was negative for macrophage markers. Scale bar = 100 μm.

3.3. Immunocytochemical characterization

Immunocytochemsitry with cell type-specific antibodies demonstrated that almost all the isolated cells were strongly positive for macrophage markers, such as CD172a, Iba-1 and KT022, but negative for CK18, SMA and DES (Fig. 4A). The proportions of contaminating epithelial and mesenchymal cells, which are positive for CK18, or SMA and DES, respectively, comprised less than 1% by immunocytochemistry (data not shown), indicating the purity of the cell population of swine macrophage to be more than 99%. In addition, most of the isolated cells showed round or flat ameboid morphology with filopodia and lamellipodia, suggesting that they are activated in this culture condition. Taken together, the isolated cells are almost purely composed of macrophages, with little contamination by other cell types.

For comparison, macrophages from adult pig blood which were selectively expanded and cultured on STO mouse fibroblasts showed a similar morphology. Almost all of macrophages were strongly positive for CD172a, Iba-1 and KT022, whereas a few contaminating STO fibroblasts were negative for these macrophage markers (Fig. 4B). Therefore, CD172a, Iba-1 and KT022 would be useful markers for swine macrophages in our culture condition. These cells showed morphological resemblance to the liver macrophages obtained from the mixed primary culture of neonatal swine hepatocytes (Fig. 4A). So, swine macrophages originated from different tissues might exhibit a similar morphology under the present culture condition. In addition, macrophages derived from adult pig blood showed strong phagocytic activity against polystyrene microbeads (data not shown), similar to the liver macrophages described below.

3.4. Phagocytic activity

The isolated CD172a-positive macrophage-like cells phagocytosed polystyrene microbeads as early as at 0.5 h, and continued to do so until almost all the cells incorporated the beads after 2 h of administration (Fig. 5). The phagocytic activities of the cells were quantitatively demonstrated by FACS (Fig. 5), indicating the proportions of the fluorescence-positive cells increased as follows; 77.2% at 0.5 h, 83.6% at 1 h and 92.6% at 2 h. These results demonstrate the strong phagocytic activity of the isolated cells, which is a distinctive characteristic of macrophages in the liver [16,18,9].

3.5. ELISA analysis of cytokine release

We assayed the capabilities of the isolated cells to produce inflammatory and anti-inflammatory cytokines in response to lipopolysaccharide. These cells secreted substantial amounts of both inflammatory (TNFα, IL-1β, IL-6 and IL-12) and anti-inflammatory (IL-10) cytokines after stimulation of lipopolysaccharide for 24 h (Fig. 6). In untreated negative controls, concentrations of all the cytokines, except for IL-10, were under the detection limit of the ELISA kits. These results show that the isolated cells produce and release specific cytokines in response to bacterial endotoxin.

Notably, the swine liver-macrophages secreted very high levels of IL-1β (6000 pg/ml) after stimulation with lipopolysaccharide alone. This is in contrast to the macrophages of human or murine origin, which usually require a second stimulus, such as ATP, for the maturation and release of this cytokine [21,22]. In our previous investigation of rat liver-macrophages that were obtained in an identical manner and stimulated with lipopolysaccharide alone, these cells hardly released any IL-1β after stimulation, only at the levels of 10–15 pg/ml (unpublished observation). These results may suggest that the swine-liver macrophages are in activated condition at the time they are harvested from T-75 flasks.

4. Discussion

Here, we applied the shaking and attachment method to the mixed primary cultures of neonatal swine hepatocytes, and repeatedly obtained liver macrophages in similar number and purity, as we previously reported in the rat [12] and bovine [14] livers. The applicability of this method to the swine liver provides a simple and efficient protocol to obtain large numbers of swine macrophages, which could be used for functional analyses of the innate immune response in this important livestock species.

Starting with a primary culture of swine parenchymal hepatocytes, followed by proliferation of the macrophages on a mixed cell sheet, liver macrophages were isolated and purified based on their biological characteristics. These cells most probably originated from Kupffer cells, which mainly reside in the sinusoidal space in the liver and function as the resident macrophages in this organ [1,2]. As Kupffer cells reportedly exhibit a high turnover rate in mouse models of bone marrow transplantation [23], these cells might be continuously supplied from bone marrow as precursor cells. Thus, it is speculated that Kupffer cells or their precursor cells that are intermingled with the parenchymal fraction (Fig. 2A) responded to the specific environment provided by the mixed liver cell cultures and contributed to the continuous expansion of macrophages (Fig. 2C). In relation to this, Talbot and Paape reported that normal pig tissue-derived macrophages can be continuously grown in simple explant cultures on STO feeder cells [24]. They reported that fetal and newborn pig liver and testicle tissues give rise to large numbers of macrophage in cultures, which is quite consistent with our mixed primary culture of swine hepatocytes. The precise origin of the macrophages that proliferated in the mixed primary cultures of swine hepatocytes requires further investigation.

As observed in the rat [12] and the bovine [14] applications, swine parenchymal hepatocytes quickly underwent phenotypic conversion, i.e. an epithelial to mesenchymal transition (EMT). During EMT, morphological and functional changes in epithelial cells are associated with the expression of specific cytoskeletal proteins in mesenchymal cells, such as α-smooth muscle actin and vimentin, and a concomitant decrease in the level of epithelial cytokeratins, as well as the

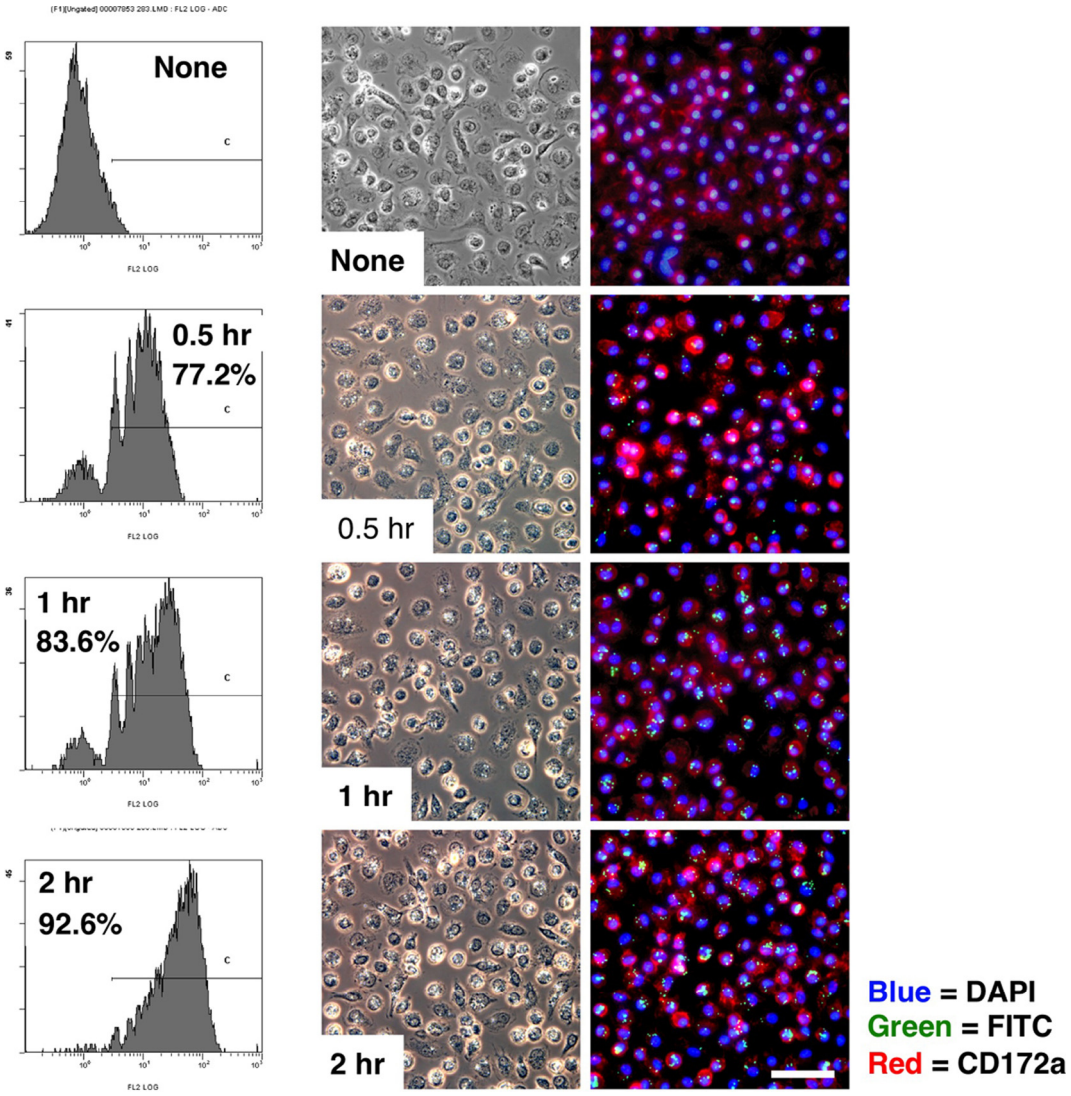

Fig. 5. Phagocytosis of fluorescence-labeled microbeads by swine macrophage-like cells. After incubation for the indicated periods at 37 °C, cells were fixed, immunostained with an anti-CD172a antibody and observed under phasecontrast/fluorescence microscopy, or analyzed by FACS, as described in Section 2. Scale bar = 50 μm.

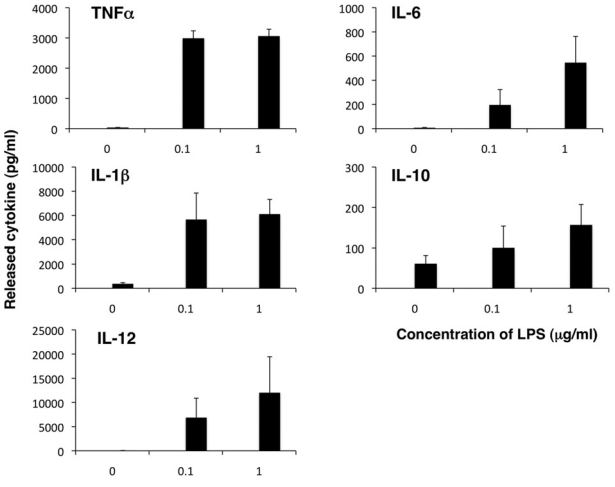

Fig. 6. Secretion of inflammatory and anti-inflammatory cytokines from isolated macrophage-like cells after stimulation with lipopolysaccharide for 24 h. The experiments were independently performed three times. The cytokine concentrations in the culture supernatant were quantified using specific swine ELISA kits and expressed as the mean value ± SEM.

deposition of extracellular matrix [25,26]. In fact, immunocytochemical analyses indicated these phenotypic changes and the transition of cell population from epithelial (CK18-positive) to mesenchymal (SMA-positive) were observed in the mixed primary cultures of swine liver cells (Fig. 2A–C). Therefore, it may be that Kupffer cells or their precursors responded to the specific culture environment caused by EMT in the mixed culture and started to proliferate. In addition, a specific type of cells in the liver, such as intrahepatic bile duct cells [27] may have expanded in the mixed culture and contributed the proliferation of Kupffer cells. Further studies are required to determine whether any specific cytokines, such as M-CSF [28], are secreted from the mixed epithelial and mesenchymal cell sheet to stimulate the macrophage proliferation. Alternatively, deposition of extracellular matrix proteins such as collagen and fibronectin during EMT process may be necessary for the macrophage proliferation. It would be interesting to know whether the mixed primary culture of swine hepatocytes also supports the proliferation of macrophages from other tissues.

The isolated liver macrophages secrete substantial amounts of inflammatory (TNFα, IL-1β, IL-6 and IL-12) cytokines after stimulation of lipopolysaccharide. These results suggest that these cells primarily have M1 phenotype [29]. In addition, these cells secrete anti-inflammatory (IL-10) cytokines in un-stimulated control cultures, and the levels of IL-10 increased after stimulation of lipopolysaccharide. These results may suggest that some of the isolated liver macrophages display anti-inflammatory M2 properties [29,30] in the present culture condition. Expression of scavenger receptor MSR-A:CD204 (revealed by KT022 monoclonal antibody) in these cells may support M2 phenotype. Further studies are necessary to evaluate the activation state of the liver macrophages.

The release of IL-1β from macrophages is tightly regulated because this cytokine induces a very powerful, and in some cases, detrimental inflammatory response [21]. IL-1β is first synthesized as a biologically inactive procytokine (pro- IL-1β) that accumulates in the cytoplasm. Then, pro-IL-1β is cleaved into the mature form by a specific protease, caspase-1, and released from the cell. Although, lipopolysaccharide primes macrophages to increase IL-1β gene expression and the synthesis of pro-IL-1β, the stimulation by lipopolysaccharide alone is insufficient and a secondary endogenous signal, such as ATP, is required for the maturation and release of this cytokine [21,22]. However, the swine macrophages released a significant amount of IL-1β after stimulation by lipopolysaccharide alone (Fig. 6). This suggests there may be a different regulatory mechanism for the synthesis, maturation and release of IL-1β in the swine macrophage-lineage cells. Ferrari et al. [31] reported that microglial cells release ATP when stimulated with LPS and such an LPS-dependent release of ATP is also observed in human macrophages. Thus, LPS treatment alone might be sufficient to activate an autocrine/paracrine loop of ATP stimulation [31] in swine liver-macrophage. If so, this mechanism may explain why these cells release high levels of IL-1β upon LPS stimulation alone.

Cells in the macrophage-lineage express a specific plasma membrane receptor for extracellular ATP [32]. The P2X7 receptor, one of the purinergic receptor families, responds to relatively higher concentrations of ATP ($>$ 1 mM) and facilitates the processing and release of IL-1 β in LPS-primed microglia [22, 33]. Activation of the P2X7 receptor and its downstream signaling trigger a key step of the inflammatory response of IL-1β, the induction of NLRP3/caspase-1 inflammasome complexes that promote the proteolytic maturation and secretion of IL-1β [34]. Little is known about the P2X7 receptor regulation of the NLRP3 inflammasome in swine macrophages and the processing and secretion of IL-1β. Further studies are needed to address the mechanism of the release of IL-1β from swine liver-macrophages and its biological significance in the inflammatory response of the swine.

In our method, only a relatively small number of liver cells (5×10^6 cells per T75 flask) are required, and active proliferation of macrophages in the mixed liver cell culture, followed by selective harvest of these cells, yields a large number of pure macrophages (c.a. 10^7 in total) from a single T75 culture flask during a comparatively long culture period (i.e. more than 3 weeks). The average number of macrophages harvested from a single T75 flask is relatively low (c.a. 10^6 cells) compared to large-scale harvest by lung or peritoneal lavage in pig. However, the main advantage of our method is to provide a certain number of pure macrophages originated from the same animal repeatedly at a few days interval for more than 3 weeks. By increasing the number of T75 flasks appropriately, the harvest scale of macrophage would be adjusted to meet a specific experimental design. As the Swine Genome Project provides comprehensive genetic marker information for the selective breeding of this important livestock species [35], appropriate swine cellular models need to be developed to verify the linkage between genetic polymorphisms and cellular phenotypes. In this context, our method can provide swine liver-macrophages in reasonable quantity and purity from a swine individual of a specific genetic background and thus advance the application of functional genomics on the innate immune responses to the specific infectious diseases of swine [36,37]. In addition, permanent cell lines will come to be established, such as by the transfection of SV40T oncogene [38], or the utilization of specific growth factors to support the continuous proliferation of swine macrophages [39]. These permanent swine macrophage cell lines, once established, will constitute valuable tools for the *in vitro* study of the swine innate immune system.

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