Phenotypic characterization of swine peripheral blood monocyte-derived macrophages and ex vivo infection with *Salmonella enterica* serovar Typhimurium

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Abstract

Macrophages are critical mediators of the inflammatory process, playing a relevant role in the pathogenesis of *Salmonella* Typhimurium. The protocols for isolation, culture, and differentiation of monocytes into macrophages and their interaction with *Salmonella* are well established in humans and murine models, but little information is available in swine. The aims of this study were to establish an efficient protocol for macrophage culture and to evaluate the interaction of the *invA* mutant strain and the wild type (WT) *Salmonella* Typhimurium with porcine macrophages. Peripheral blood monocyte-derived macrophages from pigs were obtained, separated by density-gradient centrifugation, and cultured in Teflon vials for 10 days. After the differentiation period, cultures consisted of 92.4% CD14⁺ cells. In addition, these cells showed phagocytic ability, demonstrated by the presence of the same amount of WT and *invA* mutant *Salmonella* Typhimurium 1 h after interaction with macrophages. The early cytotoxic effect was *Salmonella* pathogenicity island (SPI)-[1]dependent, in which log-phase WT strains were more efficient ($p < 0.01$) than the *invA* mutant strain at inducing the death of macrophages.

Keywords *Salmonella* Typhimurium · Pigs · Macrophage · Cytotoxicity

Introduction

Macrophages play important roles in the innate and adaptive immune responses against invading pathogens, contributing to the elimination of microorganisms through bactericidal activity while also being able to present antigens to T cells. Conversely, macrophages may be targeted by intracellular bacteria that remain viable and multiply within the intracellu-

lar environment, leading to persistence and chronic infections [1, 2].

A standardized protocol for primary culture of peripheral blood monocyte-derived swine macrophages is not available, although such a protocol would be highly desirable for advancing our knowledge on swine macrophage function. However, the low concentration of monocytes in the peripheral blood of pigs may pose a challenge for the isolation and primary culture of these cells [3, 4].

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular bacterium capable of infecting several animal species, including pigs, causing considerable economic losses and relevant public health problems [5]. Pigs infected with *S.* Typhimurium become a source of infection for other animals, the environment, and carcasses at the slaughterhouse [6]. The carrier status of infected animals may be due to the ability of this pathogen to survive within the macrophage [7]. After contact with enterocytes and macrophages, *S.* Typhimurium has the ability to be internalized, to survive and, in some cases, to multiply in the intracellular

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environment [8, 9]. Invasion of intestinal epithelial cells is mediated by the *Salmonella* pathogenicity island 1 (SPI-1), which encodes a type III secretion system (T3SS-1) [10]. Once *S. Typhimurium* adheres to epithelial cells, a process that is mediated by fimbrial adhesin, the T3SS-1 translocates effector proteins, including SopE, SopE2, SopB, SipC, and SipA, into the cytosol of host cells, leading to cytoskeletal changes that result in a process similar to endocytosis and consequent internalization of the bacteria [10–12]. The *inv* operon, which encodes T3SS-1 inner membrane proteins, is also essential for invasion of epithelial host cells. Indeed, *invA* mutant strains lose their ability to actively invade host cells; therefore, these strains can only be internalized by eukaryotic cell-dependent mechanisms such as phagocytosis [13, 14]. Within immortalized murine macrophages, these mutant strains can survive and multiply at wild-type levels, causing late cytotoxicity, in contrast to the wild type strain, which is able to induce very early cytotoxicity [15–17]. Most of these studies have been performed using murine models. Importantly, the course of *S. Typhimurium* infection in vivo may differ between mice and pigs [18]. Considering that research on the interaction between *Salmonella* and porcine macrophages is scarce, it is important to evaluate the species-specific interaction mechanism between the pathogen and this host.

The aims of this study were to use an efficient protocol for macrophage culture and to phenotypically characterize peripheral blood monocyte-derived swine macrophages and to evaluate their interaction with *S. Typhimurium* (wild type or an *invA* mutant), and to evaluate possible effects of the age of pigs on macrophage function under experimental conditions in vitro.

Material and methods

Blood samples and ethics statement

Peripheral blood was drawn from four young female pigs (60 days of age, nursery pigs) and four adult female pigs (> 1 year of age, sows) by puncture of the jugular vein and collected in tubes with anticoagulant (EDTA and heparin).

This experimental protocol has been reviewed and approved by the Ethics in Animal Use Committee at the Universidade Federal de Minas Gerais (CEUA/UFMG protocol number 249/2015).

Salmonella Typhimurium strains

Wild-type (WT) *Salmonella Typhimurium* (strain IR715; ATCC14028) [19] and an isogenic *invA* mutant strain [20] were used in this study. Bacteria were grown in Luria-Bertani broth (LB) under agitation at 37 °C overnight to reach

the stationary phase. A similar procedure was carried out for 5 h to obtain cultures in the logarithmic phase.

Leukogram

Whole blood samples collected in EDTA were used for global counts and differential leukocyte analysis in an automated Vet ABC Hematology Analyzer (Heska Corporation, Waukesha, WI, USA).

Peripheral blood mononuclear cells—isolation, culture, and in vitro macrophage differentiation

Peripheral blood mononuclear cells (PBMC) were isolated by differential centrifugation of whole blood samples on a Histopaque cushion. Briefly, 60 mL of heparinized whole blood was collected from each animal and diluted (1:1 proportion) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, USA). Diluted blood samples were carefully layered onto Histopaque solution (Sigma-Aldrich, St. Louis, MO, USA) at a proportion of 1:2 v/v Histopaque: blood in 50-mL polypropylene conical tubes (BD Bioscience, San Jose, CA, USA), followed by centrifugation at 1065xg for 40 min at 18 °C. Mononuclear cells were collected from the interface between the plasma and Histopaque with a Pasteur pipette and transferred to another tube, where they were resuspended in erythrocyte lysis buffer for 5 min at room temperature, and washed twice with DMEM by centrifugation at 1065xg for 15 min at 4 °C. Subsequently, the cells were transferred to Teflon flasks (Nalge Nunc, Rochester, USA), and cultured for 10 days at 37 °C with 5% CO₂ in 10 mL of RPMI-1640 supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 7.5% w/v sodium bicarbonate, 10 mM non-essential amino acids, 10 mM sodium pyruvate, 50 IU/mL penicillin, and 50 µL/100 mL streptomycin. Following 24 h of incubation, the culture medium was replaced to remove non-adherent cells and antibiotics. The cultures remained under the same conditions for 10 days, and the medium was replaced every 3 days. After this time, the monocyte-derived macrophages were recovered for flow cytometric phenotypic characterization and transferred to 96-well plates to assess in vitro *S. Typhimurium* survival in monocyte-derived macrophages and the cytotoxic effect of *S. Typhimurium* to monocyte-derived macrophages.

Flow cytometric analysis of porcine circulating leukocytes, peripheral blood mononuclear cells, and monocyte-derived macrophages

Aliquots of heparinized whole blood (50 µL aliquots), mononuclear cells (approximately 2.5×10^5 cells in 50 µL), and monocyte-derived macrophages (1.5×10^5 cells in 50 µL) were incubated with 1 µL of anti-CD14 monoclonal antibody

(TüK4 clone) labeled with Alexa Fluor 647 (Serotec Inc., Oxford, UK) for 30 min at room temperature in the dark. After incubation, red blood cells were lysed (in whole blood samples) and cells prefixed by the addition of 3 mL commercial lysing solution (FACS Lysing Solution, BD Biosciences, San Jose, CA, USA) with vortexing, followed by incubation for 10 min at room temperature. The cells were washed once with phosphate-buffered saline (PBS) by centrifugation at 400 $\times$ g for 7 min at room temperature. Cells were then fixed in 300 μ L of Macs FACS Fix (10.2 g/L sodium cacodylate, 10.0 g/L paraformaldehyde, 6.63 g/L sodium citrate, pH 7.2) and maintained at 4 °C for up to 24 h prior to analysis with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The software CellQuest™ was used for data acquisition and storage. The software FlowJo version 7.6.5 (TreeStar, San Diego, CA, USA) was used for morphometric and immunophenotypic analysis.

***Salmonella* Typhimurium infection and intracellular survival in porcine monocyte-derived macrophages**

Monocyte-derived macrophages were seeded in 96-well plates (50,000 cells/well) for evaluation of *S. Typhimurium* (WT and *invA*) internalization, survival, and multiplication at 1, 6, and 24 h post infection (hpi) at a multiplicity of infection (MOI) of 50, in triplicate. Bacteria were inoculated, and the plates were centrifuged at 1,000 $\times$ g for 5 min to synchronize infection. The plates were then incubated for 30 min at 37 °C with 5% CO₂. Subsequently, 50 μ g/mL of gentamicin (Sigma-Aldrich) was added to remove extracellular bacteria, and the cells were then incubated under the same conditions for another hour. After this last procedure (T1), the wells were washed with PBS, and the cells were lysed with 0.1% Triton X-100 (Roche, Germany). The samples were diluted, and 100 μ L of each dilution was plated on LB agar for CFU counting. The procedure was repeated at 6 (T6) and 24 (T24) hpi. Additional macrophages were cultured without bacteria (negative control), and the culture medium with antibiotic was incubated with the bacteria (control for antibiotic efficacy).

Evaluation of the cytotoxic effect of *S. Typhimurium* on monocyte-derived macrophages

Macrophages were seeded in 96-well plates (50,000 cells/well) to evaluate the cytotoxic effect of *S. Typhimurium* (log-phase WT and *invA* mutant strains) with an MOI of 50 at 1 and 6 hpi (T1 and T6, respectively), in triplicate. The supernatant from the wells was frozen at –80 °C. Lactate dehydrogenase (LDH) was measured by using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, USA) to evaluate the cytotoxic effect according to the manufacturer's

instructions, and optical density (OD) values were measured with an ELISA reader.

Statistical analysis

Data were subjected to ANOVA followed by Student's *t* test. The software Prism GraphPad version 5.0 was used for statistical analysis to compare means. Differences were considered significant if $p < 0.05$. GraphPad Prism 5.0 and Microsoft Excel were used for graphic arts.

Results

Morphometric and immunophenotypic features of porcine whole blood leukocytes, peripheral blood mononuclear cells, and monocyte-derived macrophages

Flow cytometric analysis was performed to evaluate the morphometric features (size and granularity) of peripheral blood leukocytes, isolated peripheral blood mononuclear cells, and monocyte-derived macrophages (Fig. 1a–c). Additionally, specific staining with anti-CD14 monoclonal antibody was used to characterize the distinct cell populations by flow cytometry (Fig. 1d–f). The differential stainability of porcine peripheral blood leukocytes with anti-CD14 monoclonal antibody (TüK4 clone) allowed the discrimination of distinct clusters of cells, including lymphocytes, monocytes, neutrophils, and eosinophils. Monocytes had the highest and most heterogeneous fluorescence intensity (Fig. 1b). Similar profiles were observed for nursery pig and sow samples (data not shown).

The morphometric profile clearly indicated the purity of the mononuclear cell suspension obtained after Histopaque separation (Fig. 1c). The immunophenotypic profile of peripheral blood mononuclear cells also revealed that the anti-CD14 monoclonal antibody (TüK4 clone) allowed the discrimination of distinct clusters of cells, including lymphocytes and monocytes. In this case, monocytes exhibited a homogeneous staining profile (Fig. 1d).

The morphometric and immunophenotypic pattern of monocyte-derived macrophages demonstrated a homogeneous staining profile with anti-CD14 antibody (Fig. 1f), but revealed great variation in granularity among the monocyte-derived macrophages (Fig. 1e). This feature may indicate the existence of distinct cell subsets or distinct statuses of cell activation among these cells.

Overall recovery of swine peripheral blood monocyte-derived macrophages

The results of monocyte quantification in whole blood and PBMC, as well as the overall recovery of monocyte-

Flow Cytometry Morphometric and Immunophenotypic Features of Porcine Whole Blood Leukocytes, Peripheral Blood Mononuclear Cells and Monocyte-derived Macrophages

Fig. 1 Flow cytometric analysis of morphometric and immunophenotypic features of porcine whole blood leukocytes, peripheral blood mononuclear cells, and monocyte-derived macrophages. Morphometric features (size × granularity) of **a** whole blood leukocytes [LYM, MON, NEU, and EOS], **c** peripheral blood mononuclear cells

[LYM and MON], and **e** monocyte-derived macrophages [debris and Mφ]. Immunophenotypic features (CD14⁺ × granularity of **b** whole blood leukocytes [LYM, MON, NEU, and EOS], **d** peripheral blood mononuclear cells [LYM and MON], and **f** monocyte-derived macrophages [debris and Mφ])

derived macrophages, are summarized in Fig. 2. Macrophage differentiation after in vitro culture of PBMC in Teflon flasks yielded a high purity profile of CD14⁺ cells, demonstrating the efficiency of this approach for selecting porcine monocytes based on their plastic-adherent properties. This procedure yielded 97.5% and 89.3% CD14⁺ cells in nursery pigs and sows, respectively (Fig. 2c). During the PBMC separation, we recovered 36.5 ± 4.5% and 44 ± 6.5% from the monocytes in nursery pig and sow whole blood, respectively (Fig. 2g). The percentage of monocyte-derived macrophages recovered from the monocytes in PBMC was 4.9 ± 0.7% and 6.2 ± 2.9% in nursery pigs and sows, respectively (Fig. 2g). The overall recovery of cell subsets is shown in Fig. 2g, demonstrating that, from the total absolute counts of monocytes in whole blood, only 1.8 ± 0.3 and 4.2 ± 1.8 macrophages were recovered from nursery pigs and sows, respectively.

Nursery pigs had higher concentrations of mononuclear cells in whole blood ($p < 0.05$) (Fig. 2d), but the age of the animals did not significantly influence the rate of recovery of monocyte-derived macrophages after in vitro differentiation.

Intracellular survival and cytotoxic effect of *Salmonella Typhimurium*

Intracellular survival of *S. Typhimurium* in porcine macrophages is detailed in Fig. 3. There was no significant difference between the strains (WT and *invA*), indicating that the bacteria were internalized by phagocytosis without variation in resistance or susceptibility according to age. Intracellular multiplication of both *S. Typhimurium* strains was observed at 6 and 24 hpi (Fig. 3a).

The cytotoxic effect of the WT strain (100% cell death) was significantly higher ($p < 0.05$) than that induced by the *invA*

Fig. 2 Overall recovery of monocyte-derived macrophages from nursery pig and sow whole blood monocytes. **a** Percentage of monocytes in whole blood; **b** percentage of CD14⁺ cells (monocytes) among PBMC; **c** percentage of CD14⁺ after 10 days of cultivation (macrophages); **d** absolute counts of monocytes in whole blood; **e** absolute counts of CD14⁺ cells (monocytes) among PBMC; **f** absolute counts of CD14⁺ after 10 days of cultivation (macrophages); **g** recover of monocytes/macrophages. * $p < 0.05$

mutant strain (24.75% cell death at 6 hpi) (Fig. 3b). Macrophages infected with both strains exhibited no cytotoxicity at 1 h after infection (data not shown).

Discussion

The efficacy of the protocol employed in this study for monocyte purification from pigs was demonstrated by phenotypic characterization of cultured macrophages. Immediately after PBMC separation, the percentage of CD14⁺ cells was 9.1%, whereas at the end of the differentiation process, 93.2% of

cultured cells were CD14⁺. These results clearly demonstrate the effectiveness of PBMC selection, since CD14[−] cells do not adhere to Teflon vials. These results are similar to those observed in other animal species [21, 22]. Morphologically, an increase in macrophage size and granularity was observed, and these features are compatible with changes that take place during the differentiation process [22].

The percentage of monocytes in the peripheral blood of pigs varies widely [3, 4], reaching extremely low values [23]. As a result, highly efficacious techniques are needed to isolate and culture this cell type. Interestingly, in the present study, the quantity of ex vivo monocytes was significantly

Impact of *Salmonella enterica* serovar Typhimurium Infection on Nursery pig and Sow Monocyte-derived Macrophages *in vitro*

Fig. 3 Impact of *Salmonella enterica* serovar Typhimurium infection on nursery pig and sow monocyte-derived macrophages *in vitro*. **a** Time course of *Salmonella* intracellular survival in sow macrophages (filled square: *Salmonella* WT; empty square: *Salmonella invA* mutant) and nursery pig macrophages (filled circle: *Salmonella* WT; empty circle:

Salmonella invA mutant), at 1, 6, and 24 h post infection. **b** Comparison of cell death between nursery pig and sow macrophages 6 h post infection with *Salmonella* Typhimurium (wild type and *invA* mutant in the logarithmic phase)

higher in nursery pigs ($p < 0.05$), a result that has not been found in other studies evaluating this variable [3, 24]. However, the higher quantity of monocytes in peripheral blood of nursery pigs did not result in a greater quantity of macrophages after 10 days of differentiation.

An important virulence factor of *S. Typhimurium* is related to its ability to survive and proliferate within macrophages. In this study, we used an *invA* mutant strain to evaluate the role of that gene, which is required for *Salmonella* invasion of epithelial cells. Considering that primary macrophages should uptake *Salmonella* by phagocytosis, an invasion-deficient strain, namely *invA* mutant, should have similar intracellular numbers when compared to the wild type strain. Indeed, the *invA* mutant was internalized in similar quantities when compared to the WT strain, which evidences the phagocytic capacity of the cultured cells. In addition, the ability of these two strains to multiply intracellularly was assessed. Our results agree with those previously reported [25, 26] demonstrating the ability of *Salmonella* to subvert lysosomal digestion by porcine macrophages. Mechanisms required for intracellular survival are related to SPI-2, which encodes T3SS-2, responsible for secreting effector proteins (i.e., SopD2, SifA, SopB) that alter trafficking of the *Salmonella*-containing vacuole, preventing endosome maturation and fusion with lysosomes, thus establishing a *Salmonella* replication niche within the macrophages [27–30].

In addition to its capacity to survive and multiply within macrophages, *S. Typhimurium* can induce the death of these cells through a specific process called pyroptosis [31]. The evaluation of the cytotoxic capacity of the two strains during the log phase of culture, demonstrated that the *invA* mutant strain had a lower and later cytotoxic effect when compared to the WT strain. During the log phase of growth, *Salmonella* has an increased expression of SPI-1, which is involved in inducing rapid cell death [32]. Impaired cytotoxicity caused by strains with lower SPI-1 expression occurs because these strains do not translocate flagellin into the host cell cytosol, an event that is responsible for activating the mechanism of pyroptosis [16, 17, 33].

Conclusion

The protocol described in this study was efficient for obtaining differentiated monocyte-derived swine CD14⁺ macrophages. The interaction between *Salmonella* and porcine macrophages demonstrated the phagocytic capacity of these cells. In addition, the early cytotoxic effects of SPI-1 have been demonstrated in swine macrophages. Together, our results clearly characterized peripheral blood monocyte-derived swine macrophages both phenotypically and functionally, thus supporting further studies on macrophage function and pathogen interactions.

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Compliance with ethical standards

This experimental protocol has been reviewed and approved by the Ethics in Animal Use Committee at the Universidade Federal de Minas Gerais (CEUA/UFMG protocol number 249/2015).

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