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Expression profile of porcine scavenger receptor A and its role in bacterial phagocytosis by macrophages

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Abstract

Expression of scavenger receptor A (SRA) in macrophages plays key role in macrophage mediated uptake of microbes. However, little is known about the role of porcine scavenger receptor A (pSRA) in phagocytic function of macrophages in swine species. In this study, polyclonal antibody against pSRA was generated by using recombinant proteins to study expression and function of pSRA. We report broad expression of pSRA in different tissues. In the lungs, pSRA is mainly expressed by alveolar macrophages. Blockade of class A scavenger receptor by fucoidan treatment demonstrates that pSRA has role in bacterial phagocytosis by macrophages. Furthermore, importance of SRA-mediated bacterial phagocytosis has been shown using CHO cell line expressing pSRA. In summary, these findings reveal that pSRA, which is predominantly expressed in alveolar macrophages is likely to be an important receptor mediating recognition and uptake of bacteria in pig lungs.

Keywords

Scavenger receptor A; Phagocytosis; Macrophages; Bacterial infections; Lung

1. Introduction

Scavenger receptor A (SRA), one of class A scavenger receptors, also named the macrophage scavenger receptor and cluster of differentiation 204 (CD204) was the first and most well-known scavenger receptor to be identified so far (Kodama et al., 1988, 1990). There are three different isoforms (SRA I/II/III) which are differentially derived from the differently spliced variants from the same genes. In mammals, SRA I/II are the major isoforms which are often referred to in the literature as SRA. In contrast, SRA III is

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preferentially expressed but cannot functionally mediate lipoprotein binding and uptake (Gough et al., 1998).

SRA is found to be predominately expressed by macrophages although some other cells like human lung epithelial cells are also responsible for its expression (Limmon et al., 2008). In macrophages, SRA has been shown to mediate uptake of a variety of microbes (bacteria (Hampton et al., 1991; Dunne et al., 1994), fungi (Means et al., 2009), and viruses (Yamayoshi et al., 2009; Cheng et al., 2016; Stichling et al., 2018)), indicating that it is important for host defenses to microbial infections (bacteria, fungi, and viruses) in human, mouse and so on. While porcine scavenger receptor A (pSRA) gene and protein has been described the function of this receptor remains unknown.

We were particularly interested in possibility of SRA's function as the pathogen recognition/uptake receptor at the mucosal surface such as lungs (Qiu et al., 2013). In pig industry the infections at the mucosal sites (gastrointestinal and respiratory) are the most important causes of economic losses, however, little is known about class A scavenger receptors playing crucial role in mucosal defenses in pigs and what is their relative importance in the immune system. The unanswered questions we sought to address about the pSRA were including: 1) whether it is abundantly expressed by macrophages or other cells in the lungs? 2) whether it resembles structurally and functionally its homologues in other domestic animal species and humans? 3) Whether it is relevant for uptake of pathogens that are specifically important causes of swine diseases. In this study, for the first time we determined expression profiles of SRA in different tissues of pigs and clarified its effect on bacterial phagocytosis by pig macrophages.

2. Materials and methods

2.1. Generation of polyclonal anti-pSRA antibody

Generation of antibody was performed as previously described (Qiu et al., 2008). In brief, polyclonal anti-pSRA antibody was generated from rabbits immunized with purified recombinant protein (aa 205–404 fused with N-terminal His-Tag), which was expressed in *Escherichia coli* strain BL21 and purified using a His Band kit (Novagen, Madison, WI, USA). The titer of the polyclonal antibody was determined by direct ELSIA against the purified recombinant protein. Furthermore, the specificity of the polyclonal antibody was determined by Western blot using the protein samples of vector-transfected 293T cells and Flag-pSRA-transfected 293T cells.

2.2. Expression vector for pSRA, transient transfection

To express pSRA I as a C-terminal FLAG-tag protein, the open reading frame (ORF) encoding full-length pSRA (NM_001243874.1) was cloned from a pig lung and inserted into *Hind* III-*Bam*HI sites of p3 × Flag-CMV-14 vector (Sigma, St. Louis, MO, USA), named pFlag-pSRA. Furthermore, an amplified fragment encoding full-length pSRA I was subcloned into *Eco*R V-*Xho*I cloning sites of pcDNA3.0 vector (Invitrogen, Carlsbad, USA), named pcDNA-pSRA. To determine specificity of anti-pSRA antibody, pFlag-pSRA or pFlag-vector were transfected into HEK293T cells by Lipofectamine™ 2000 following

the manufacture's instruction. Furthermore, to determine the role of pSRA in bacterial phagocytosis, pcDNA-pSRA or vector were transfected into CHO cells shown as described above.

2.3. Isolation of porcine alveolar macrophages (PAM)

PAM isolation was performed as previously described (Qi et al., 2017). Briefly, lungs of euthanized pigs were washed by a pipette gun with 300–400 ml PBS containing 1 mM EDTA. The recovered fluid was spun at 500×g for 10 min and cell pellets were resuspended by complete medium (RPMI 1640, 10% FBS, 1% penicillin and streptomycin, 1% GlutaMAX). The cells were enumerated on a hemocytometer (Hausser Scientific, Horsham, PA, USA) before plating. All of experiments performed were in accordance with rules approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute (IACUC No: Shvri-po-201606 0501), Chinese Academy of Agriculture Science.

2.4. Western blot

Protein samples were prepared from the tissue homogenate or cell pellets as previously described (Qiu et al., 2008). By using the samples, transferred membranes were prepared and blocked with 5% skim milk (BD Difco™, Detroit, USA) 1 h at room temperature. Primary antibodies were incubated with membrane overnight at 4 °C [anti-Flag (1:1000, clone M2, Sigma), anti-pSRA (1:1000), anti-actin (1: 10,000, clone C4, Sigma)]. Secondary antibodies were incubated for 1 h at room temperature [goat anti-mouse HRP (1:5,000, Abcam, Cambridge, MA, USA), goat anti-rabbit (1:10,000, Abcam)].

2.5. Bacterial phagocytosis

PAM were harvested as described above, and cultured overnight at 37 °C and 5% CO₂ in 24-well tissue culture-treated plates (Corning Inc., Corning, NY, USA) with 10⁶ cells/well. The cells were treated with compounds (Cytochalasin D, fucoidan or DMSO) of interest and incubated for 30 min at 37 °C. Heat-inactivated bacteria were surfaced labeled with FITC as described in a previous report (Aronoff et al., 2008). FITC-labeled bacteria were incubated at a multiplicity of infection (MOI) of 300 bacteria:1 cell for 2 h at 37 °C. After 2 h at 37 °C, trypan blue was added to quench extracellular fluorescence. Cells were harvested and analyzed using a Cytomics FC500 (Beckman Coulter, Inc., Brea, CA, USA). Data were analyzed by FlowJo software (Tree Star Inc.). A minimum of 10,000 PAM events were collected per sample.

CHO cells (which do not express class A SR) were transfected with pcDNA-pSRA or vector as described above. At 24 h post-transfection, FITC-labeled bacteria were incubated as described above. After 2 h at 37 °C, trypan blue was added to quench extracellular fluorescence. The fluorescence was determined using a microplate fluorometer (485ex/535em, M3; Molecular Devices, Sunnyvale, CA, USA) and the phagocytic index (PI) was calculated as previously described (Thelen et al., 2010).

2.6. Statistical analysis

All data were analyzed with GraphPad Prism software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). An unpaired Student's t-test was used to determine significant

differences. Values were considered statistically significant when $p < 0.05$. Data were given as mean (SD) as indicated; 'n' refers to the sample size.

3. Results

3.1. Sequence analysis and generation of polyclonal antibody against porcine SRA

Based on porcine SRA sequence (GenBank accession number [NM_001243874.1](#)), a porcine SRA gene was amplified by RT-PCR from total RNA extracted from a pig lung. Sequence analysis through BLAST reveals that our cloned gene sequence is identical to porcine SRA sequence (GenBank accession number [NM_001243874.1](#)). Full-length cDNA of porcine SRA contains 1338 bps (GenBank accession number [MN227184](#)) which encodes a protein of 446 amino acid residues including cytoplasmic domain, extracellular domain, and transmembrane domain between them (Fig. 1). Furthermore, we performed a multiple sequence alignment among pig, human, mouse, bovine and rabbit by using full-length amino acid sequence. Results reveal that pSRA is more than 78% identical to that of human, bovine or rabbit despite of being 67% identical to mouse SRA.

Having determined the high level of conservation of SRA, which indicated its universal importance between human and animal we sought to study its expression in different tissues and cell types. Thus, we developed anti-pSRA antibody that would allow for detection of pSRA. We immunized rabbits with purified recombinant pSRA-c (aa 205–404, shown in Fig. 1) expressed in *E. coli*. The antibody titer in serum was measured using purified recombinant pSRA-c through ELISA. When the antibody ELISA titer was more than 10^6 , we examined its reaction with pSRA expressed in HEK293T cells by Western blot. Consistent with the results of anti-Flag antibody, the polyclonal antibody specifically detected expression of pSRA expression in HEK293T cells (Fig. 2A), indicating successful generation of a polyclonal antibody for detection of pSRA expression.

3.2. Expression profile of porcine SRA in different tissues

Subsequently the polyclonal antibody was used to determine expression profile of porcine SRA in different tissues using Western blot. The results show that pSRA is differentially expressed in all examined tissues, including liver, spleen, lungs, kidneys, lymph nodes and intestines (Fig. 2B). Furthermore, the relative expression of pSRA in different tissues by normalization to β -actin, showed that the most abundant expression of pSRA among these tissues was observed in inguinal lymph nodes (Fig. 2B). Moreover, except inguinal lymph nodes, the other two kinds of lymph nodes also had high abundance of SRA in comparison to lungs or kidneys. Apart from the lymphoid organs, SRA was also extensively expressed in small intestine (Fig. 2B). Collectively, these data indicate that global expression of SRA is well-represented in lymphoid tissues, organs with large mucosal surfaces (intestine and lung) and liver.

3.3. Alveolar macrophages mainly express SRA in pig lungs

Alveolar macrophages (AM), as the first line of defense, perform surveillance function to detect infectious microbes in the alveolar space (Broug-Holub et al., 1997) and are equipped by variety of receptors for pathogen recognition and uptake (Nakayama, 2018). SRA, has

been found to be abundantly expressed by macrophages in the lungs of mouse and human (Beamer and Holian, 2005; Tomokiyo et al., 2002), in which its role in host defense against microbial infections has been demonstrated (Arredouani et al., 2006). Our results showed a moderate expression of SRA in pig lungs relative to the lymphoid tissues (Fig. 2B), however how PAM contribute to global expression of SRA in the lungs remains to be determined. Next, the global lung SRA expression and its expression in purified PAM has been compared using Western blot. Of note, the abundance of SRA expression by PAM was much greater than the global lung SRA expression, indicating that alveolar macrophages are the major SRA positive cell subsets responsible for expression of SRA in pig lungs (Fig. 3A). Moreover, among all of tested the porcine cells, PAM also showed the highest protein level (Fig. 3B). Thus, our data reveal that porcine alveolar macrophages are the major SRA positive cells in the uninfected lungs.

3.4. SRA mediate bacterial phagocytosis in pig alveolar macrophages

SRA has been shown to play important role in bacterial phagocytosis (Thelen et al., 2010; Peiser et al., 2002). We next sought to determine the role of SRA in phagocytosis of unopsonized bacteria by PAM. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are universal opportunistic pathogens causing infections in many species, including pigs. Given that SRA can mediate the phagocytosis of *S. aureus* and *E. coli* by human and mouse alveolar macrophages (Peiser et al., 2000; Thomas et al., 2000), we choose porcine isolates of *S. aureus* and *E. coli* to determine the role of pSRA in their phagocytosis. Additionally, we chose two common respiratory swine pathogens *Haemophilus parasuis* (*H. parasuis*) (Macedo et al., 2015) and *Bordetella bronchiseptica* (*B. bronchiseptica*) (Opriessnig et al., 2011) for this assay. Cytochalasin D, as an inhibitor of actin polymerization was chosen as a negative control. Flow cytometry analysis showed that fucoidan (the pan class A SR inhibitor) significantly decreased phagocytosis for all of the tested bacteria in comparison to vehicle-treated cells, and to the degree similar to Cyto D (Fig. 4), strongly suggesting that class A SR was crucial for bacterial phagocytosis by PAM. To further verify effects of SRA on phagocytosis, we used SRA-expressing CHO cells and negative-control CHO cells to measure bacterial uptake. Our data showed that SRA expression promote uptake of all of those tested bacteria in comparison to control cells (Fig. 5). Collectively, our data reveals that SRA could mediate bacterial phagocytosis in pig alveolar macrophages.

4. Discussion

This study provides novel information regarding expression profile of pSRA and its role in bacterial phagocytosis. Rabbit polyclonal anti-pSRA antibody was generated to characterize its expression profile in different tissues and cells. Our data illustrate that porcine alveolar macrophages are the major SRA positive cells in the uninfected lungs. Furthermore, our data demonstrates that expression of pSRA in pig macrophages contributes to bacterial phagocytosis evidenced by fucoidan treatment in macrophages and SRA-transfected CHO cells. Collectively, these data provide novel demonstration that pig macrophages can recognize bacterial infections and mediate bacterial phagocytosis through SRA expression.

SRA characterized as cell surface membrane glycoprotein, has an increased molecular mass by N-linked glycosylation in comparison to the predicted protein (Hampton et al., 1991). Firstly, as shown in Fig. 2A, overexpressed SRA-I presents three distinct bands: 1) one at 55 kDa, consistent with the predicted mass; 2) two big ones, consistent with glycosylated form. Furthermore, by analysis of endogenous pSRA in tissues and cells, we only observed predominant bands around 70 kDa, consistent with glycosylated modification. Although it is not sure that the polyclonal anti-pSRA can detect three isoforms of SRA, our data suggest that the increased molecular mass of pSRA is attributed to protein modification.

SRA, also named as macrophage scavenger receptor, has been shown to be predominantly expressed by macrophages (Tomokiyo et al., 2002). Indeed, pig alveolar macrophages show robust expression of SRA in comparison to its expression in pig lungs (Fig. 3A). Furthermore, we demonstrated that pig alveolar macrophages show greater protein level than that in the other porcine cells. Interestingly, 3D4, a pulmonary macrophage cell line cannot robust express SRA like primary macrophages. Thus, this suggests that it may lose some unknown regulatory function on SRA expression during development of the immortal cell line.

It is not unexpected that pig SRA play role in mediating bacterial phagocytosis, which has been shown in mouse and human (Thelen et al., 2010; Peiser et al., 2002). By fucoidan treatment and over-expression of SRA, our data demonstrated that pig SRA can efficiently recognize and uptake *H. parasuis* and *B. bronchiseptica* besides *S. aureus* and *E. coli*. To our best knowledge, it is the first time to know that SRA can recognize and uptake *H. parasuis* and *B. bronchiseptica*. At present, how SRA affects those bacterial infections *in vivo* is not clear. Thus, future studies are needed for addressing this point. In summary, for the first time we provide the evidence that pig SRA contributes to bacterial phagocytosis. These finding could pave the way to gain insights to host-pathogen interaction in pigs.

Acknowledgments

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Fig. 1. Comparative sequence analysis shows high level of homology in the porcine macrophage scavenger receptor A (SRA/CD204) with other mammalian SRAs. Sequences of multiple species pig (NP_001230803.1), human (NP_619729.1), mouse (NP_001106797.1), bovine (NP_001106711.1) and rabbit (NP_001075717.1) per GenBank depository were aligned to determine level of homology. The black and gray shading highlight identity in all 5 species and similarity, respectively. The cytoplasmic and extracellular domain have been labeled in reference to the human SRA protein. Note blue line designating the recombinant pSRA peptide (truncated protein), which we generated for the purpose of anti-pSRA antibody development.

Fig. 2.

(A) HEK293T cells were transiently transfected with pSRA expression vector. Cell lysates were analyzed by Western blot with anti-pSRA or anti-Flag antibodies. (B) Expression of pSRA in different tissues of pig was measured by Western blot. The bar graph represents relative expression of pSRA by calculating the pSRA/actin ratio. The data shown are mean (SD) (n = 3) from three independent experiments.

Fig. 3.

(A) Western blotting showed expression of pSRA in pig lungs and alveolar macrophages, respectively. The bar graph represents relative expression of pSRA by calculating the pSRA/actin ratio. The data shown are mean (SD) ($n = 3$) pooled from three independent experiments, $**p < 0.01$. (B) Western blot analysis of expression of pSRA in porcine cell lines (PIEC, ST, PK15, 3D4) and primary macrophages (PAM). The bar graph represents relative expression of pSRA by calculating the pSRA/actin ratio.

Fig. 4.

Flow cytometry showed phagocytosis of FITC-*S. aureus*, FITC-*E. coli*, FITC-*H. parasuis*, and FITC-*B. bronchiseptica* in PAM pretreated with cyto D, Fucoidan and DMSO. PAM with FITC-labeled bacteria are shown as red solid line (DMSO-treated group), gray spot line (Fucoidan-treated group), and black dash line (cyto D-treated group) and negative control PAM as shaded histogram. The bar graph presents mean frequencies of FITC-positive cells derived from these histograms. The data shown are mean (SD) pooled from two independent experiments; n = 6; *p < 0.05, **p < 0.01.

Fig. 5. Phagocytosis of FITC-*S. aureus*, FITC-*E. coli*, FITC-*H. parasuis*, and FITC-*B. bronchiseptica* by control CHO cells (pcDNA3) and SRA-transfected CHO cells (pcDNA-pSRA) was measured. Results are presented relative to the average of control CHO values set to 100%; The data shown are mean (SD) pooled from three independent experiments; n = 9. *p < 0.05; **p < 0.01.