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TLR2, Siglec-3 and CD163 expressions on porcine peripheral blood monocytes are increased during sepsis caused by *Haemophilus parasuis*

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ABSTRACT

TLRs, Siglecs and CD163 are cell surface receptors that play an important role in immune response and sepsis. The objective of this study was to assess changes in the expression levels of several of these receptors (TLR2, TLR4, CD163, Siglec-1, Siglec-3, Siglec-5 and Siglec-10) on the surface of peripheral blood mononuclear cells from pigs with sepsis caused by *Haemophilus parasuis*. Flow cytometry was employed to analyze samples from an experimental infection and from cell cultures. A significant increase in CD163, TLR2 and Siglec-3 expression during infection was seen. However, *in vitro* exposure of peripheral blood monocytes to bacteria or sera from infected pigs did not increase the expression of these receptors. These changes may be due to recruitment of monocytes into the blood compartment in response to *H. parasuis*-induced sepsis.

1. Introduction

CD163 is a scavenger receptor expressed by monocytes and macrophages that recognizes complexes formed between haptoglobin and damage-associated molecular patterns (DAMPs), as free hemoglobin and the high mobility group box 1 (HMGB1). These interactions induce production of anti-inflammatory cytokines [1,2]. However, CD163 can also act as a surface receptor for recognizing intact bacteria by macrophages triggering pro-inflammatory cytokine production [3].

Toll like receptors (TLRs) are a kind of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) from different microorganisms. Interaction of TLRs with their ligands promotes the transcription of genes encoding inflammatory cytokines. Because an exacerbated innate response initiated by PRRs could be dangerous to the host, inhibitory mechanisms are necessary in order to prevent it [4]. Sialic acid-binding immunoglobulin-like lectins (Siglecs) are receptors constituting one of these mechanisms [5]. Most Siglecs have an immunoreceptor tyrosine-based inhibition motif (ITIM) and signal negatively when they bind their ligands (sialic acid attached to glycolipids, glycoproteins or proteoglycans) from the host, and therefore this mechanism enables the immune system to discriminate between self and non-self ligands because sialic acid should cover only the host's molecules [6]. However, several bacterial pathogens have developed strategies to coat their surface with sialic acid in order to avoid the immune response [7].

Haemophilus parasuis is a Gram-negative rod belonging to the family *Pasteurellaceae* that is part of the commensal microbiota in the upper respiratory tract of pigs. However, under certain conditions, such as stress during postweaning or pigs without previous contact to this agent, virulent strains can gain access to the blood stream and result in a sepsis with polyserositis, known as Glässer´s disease [8]. The sialization of the lipooligosaccharide of the virulent Nagasaki strain (reference strain of serovar 5) and the presence of enzymes implicated in metabolism of sialic acid in several strains have been previously reported [9].

Since TLRs, Siglecs and CD163 may play an important role in sepsis, the expression levels of TLR2, TLR4, Siglec-1, Siglec-3 (CD33), Siglec-5, Siglec-10 and CD163 in peripheral blood mononuclear cells (PBMCs) from pigs with sepsis after 24 h of *H. parasuis* infection were investigated in this study.

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2. Materials and methods

2.1. Bacterial strain, growth conditions and infective dose preparation

The *H. parasuis* Nagasaki strain was grown overnight at 37 °C in pleuropneumonia-like organism (PPLO) broth (Conda, Spain) supplemented with nicotinamide adenine dinucleotide (0.0004%) and glucose (0.0025%). Bacterial cells were recovered after centrifugation at 300 ×*g*, and the pellet was suspended in RPMI 1640 (Sigma-Aldrich). The infective dose was prepared from aliquots of 2 ml containing 10^8 colony forming units (CFUs) suspended in RPMI 1640 and was maintained at 4 °C until the challenge.

2.2. Experimental infection

A group of 10 colostrum-deprived Large White × Pietrain piglets coming from a farm with an excellent sanitary status and no previous clinical history of *H. parasuis* were housed in isolation rooms, hand reared and fed as previously described [8,10]. At 67 days of age, the 10 piglets were infected by intratracheal injection of 10^8 CFUs of the *H. parasuis* Nagasaki strain. Piglets with severe signs of distress were euthanized with T61® (Intervet, Spain). The experiment was conducted according to the guidelines of the University of León Ethical Committee (approval ULE 005/2015) and the Spanish Government.

2.3. Peripheral blood mononuclear cells (PBMCs) isolation and sera collection

Blood samples were collected immediately before infection and 24 h after it by aseptic venipuncture of the jugular vein using commercial tubes (Vacutainer, Becton Dickinson, Spain) containing EDTA for PBMCs isolation or without EDTA for sera collection. PBMCs were isolated by means of a density gradient using Histopaque 1077® (Sigma-Aldrich, Spain), according to the instructions of the manufacturers. Isolated PBMCs were suspended in Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich Spain) containing 2% fetal bovine serum (Sigma-Aldrich, Spain) and 10% of DMSO (Sigma-Aldrich, Spain); then, PBMCs were preserved in liquid nitrogen until use. Sera were collected and stored at −80 °C until use.

2.4. Clinical examination and bacterial isolation

Pigs were monitored for rectal temperature and other clinical signs every 12 h after infection. The dead animals were subjected to necropsy and bacterial isolation was attempted by collecting swabs from different tissues: meninges (cranial subarachnoid space), lungs, spleen, liver, pericardial cavity, and the carpal and tarsal joints. Samples were inoculated on chocolate agar plates and cultured for 24 h at 37 °C and 5% CO_2. The colonies whose morphology was compatible with *H. parasuis* were subcultured and confirmed by specific 16S *H. parasuis* PCR [11].

2.5. Flow cytometry

Expression changes in several receptors (TLR2, TLR4, Siglec-1, Siglec-3, Siglec-5 and CD163) on PBMCs after *H. parasuis* infection were analyzed by two-color flow cytometry with monoclonal antibodies (mAbs) against CD163, CD172a or granulocytes (Table 1). Samples from four animals were employed to perform preliminary assays. Double stains with a CD172a-specific antibody were performed in three animals as a preliminary approximation in order to assess expression changes in TLRs, Siglecs (1, 3 and 5) and CD163 on monocytes (defined as $CD172a^{+high}$ events in a FSC/SSC gate characteristic of monocytes) and Siglec-10 on lymphocytes (defined as $CD172a^{-}$ events in a FSC/SSC gate characteristic of lymphocytes). A specific granulocyte antibody 6D10 was used to perform double stains in three animals for the purpose of removing the influence of granulocytic contamination in PBMC samples during the post-infection time. In this double staining, expression changes for TLRs and Siglecs (3 and 5) were analyzed for monocytes defined as $6D10^{-}$ events in a FSC/SSC gate characteristic of this cell type. Based on the results from CD172a double stains, other double stains with CD163-specific antibodies were performed in six animals in order to study *H. parasuis*-induced changes in TLRs and Siglecs expressions on $CD163^{+}$ and $CD163^{-}$ subsets of monocytes (defined by the FSC/SSC gate). In addition, TLR2/CD163 double staining was employed to study CD163 expression changes on monocytes (defined as $TLR2^{+}$ events in a FSC/SSC gate characteristic of monocytes). mAbs and animals used in the double stains are shown in Table 1.

For two-color flow cytometric analyses, PBMCs were distributed in V-shaped 96-well microplates (0.4–1 × 10^6 cells/well) and 50 µl of each hybridoma supernatants were added and incubated for 30 min at 4 °C. After washing twice in FACS buffer (phosphate buffer saline [PBS] containing 0.1% bovine serum albumin [BSA] and 0.01% sodium azide), PBMCs were incubated for 30 min at 4 °C with each secondary labeled immunoglobulin according to the instructions of the manufacturers. Then, the PBMCs were washed twice with FACS buffer, collected, and fixed with FACS buffer containing 0.1% formaldehyde. Labeled cells were analyzed using a FACSCanto II flow cytometer (double staining with CD172a) or MACSQuant® Analyzer 10 flow cytometer (double staining with CD163 and 6D10), and data analysis was performed using FlowJo Software. Irrelevant isotype-matched mAbs were used as negative controls. With regard to the receptors showing a unimodal peak of fluorescence (TLR2, TLR4, Siglec-3 and Siglec-5), normalized mean fluorescence intensity (NMFI) was calculated by dividing mean fluorescence intensity (MFI) from the positive peak of the sample by MFI from the peak of the negative control.

With all of these double stains, the expression level of the different receptors of PBMCs was measured in colostrum-deprived piglets, and the changes induced by infection with *H. parasuis* were studied by comparing the respective MFI or NMFI values before and after experimental infection.

2.6. Culture of PBMCs

Fresh isolated PBMCs from healthy pigs were resuspended in complete medium with 20% serum from an infected pig or serum from the same pig before infection and cultured for 24 h at 37 °C with 5% CO_2 using 24-well tissue culture plates (0.5–1 × 10^6 cells/ml each well). This experiment was replicated four times. Each replica was carried out with sera from a different pig. As source of PBMCs, four healthy pigs were employed (one pig per replica).

The same methodology was employed to perform the exposure to bacteria: fresh isolated PBMCs were resuspended in complete medium with 10^7, 10^6 or 0 (control) CFUs of the heat-inactivated *H. parasuis* Nagasaki strain (45 min at 60 °C) [12] and cultured for 24 h. The experiment was replicated four times. Each replica was carried out with fresh PBMCs coming from a different healthy pig. Single color stains (Table 1) were performed to assess the influence of *in vitro* exposure to bacteria or sera from infected pigs in the expression of these receptors on monocytes whose expression was found to be modified during experimental infection.

2.7. Statistical analysis

Statistical analysis was performed using SPSS software (IBM, Spain). Data were tested for normality by the Shapiro-Wilk test, followed by analysis with paired-samples t-test. A p value < 0.05 was considered significant.

Table 1
List of mAbs and their combinations used in this study.

Porcine target studied	Clones of specific primary murine moAbs	Specifity and fluorchrome of secondary labeled moAbs, showing source in brackets	Number of pigs
CD172a / TLR2	74-22-15a / 1H11	IgG2b-APC (SB, 1090-11) / IgG1-FITC (BDP, A85-1)	3
CD172a / TLR4	74-22-15a / 3H3	IgG2b-APC (SB, 1090-11) / IgM-FITC (SB, 1020-02)	3
CD172a / Siglec-1	74-22-15a / 1F1/CR4	IgG2b-APC (SB, 1090-11) / IgG2a-FITC (SB, 1080-02)	3
CD172a / Siglec-3	74-22-15a / 5D5	IgG2b-APC (SB, 1090-11) / IgG1-FITC (BDP, A85-1)	3
CD172a / Siglec-5	74-22-15a / 4F7	IgG2b-APC (SB, 1090-11) / IgG1-FITC (BDP, A85-1)	3
CD172a / Siglec-10	74-22-15a / 2E9	IgG2b-APC (SB, 1090-11) / IgG1-FITC (BDP, A85-1)	3
CD172a / CD163	74-22-15a / 1C6-BM	IgG2b-APC (SB, 1090-11) / IgG2a-FITC (SB, 1080-02)	3
Granulocytes / TLR2	6D10 / 1H11	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	3
Granulocytes / TLR4	6D10 / 3H3	IgG2a-APC (SB, (1080-11) / IgM-FITC (SB, 1020-02)	3
Granulocytes / Siglec-3	6D10/ 5D5	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	3
Granulocytes / Siglec-5	6D10 / 4F7	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	3
CD163 / TLR2	1C6-BM / 1H11	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	6
CD163 / TLR4	1C6-BM / 3H3	IgG2a-APC (SB, (1080-11) / IgM-FITC (SB, 1020-02)	6
CD163 / Siglec-1	2A10/11 / 1F1/CR4	IgG1-FITC (BDP, A85-1) / IgG2a-APC (SB, (1080-11)	5
CD163 / Siglec-3	1C6-BM / 5D5	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	6
CD163 / Siglec-5	1C6-BM / 4F7	IgG2a-APC (SB, (1080-11) / IgG1-FITC (BDP, A85-1)	6
CD163	2A10/11	IgG1-FITC (BDP, A85-1)	–
TLR2	1H11	IgG1-FITC (BDP, A85-1)	–
Siglec-3	5D5	IgG1-FITC (BDP, A85-1)	–

Secondary moAbs are commercial antibodies obtained from Southern Biotech (SB) or BD-Pharmigen (BDP) and they are conjugated to fluorescein isothiocyanate (FITC) or allophycocyanine (APC). Primary moAbs are supernatant from hybridomas from the Porcine Immunology Laboratory of INIA (Spain).

3. Results

3.1. Clinical examination and bacterial isolation

Within the first 24 h post-inoculation, the pigs began to develop clinical signs compatible with the infection caused by *H. parasuis*. When post-infection blood samples were collected, all pigs showed the following severe signs: hyperthermia above 41 °C, dyspnea, abdominal respiration and prostration. Within the next 24 h, their symptomatology became so severe that the animals had to be euthanized. Necropsy studies revealed different degrees of fibrinous polyserositis (Table 2). All animals tested positive for *H. parasuis* isolation (confirmed by PCR) from more than one of the sampled tissues other than the lung (Table 3), thus providing evidence that *H. parasuis* had resulted in a systemic infection.

3.2. Expression changes on monocytes

As a preliminary approximation, we compared the proportion of positive cells and the levels of expression for TLRs, Siglecs (−1, −3 and −5) and CD163 on monocytes (defined as CD172a^{+high} events in a FSC/SSC gate characteristic of these cells) collected from three animals prior to and 24 h after infection (when sepsis induced by *H. parasuis* infection was already established) (Fig. 1E). Practically all monocytes were positive for TLR2, TLR4, Siglec-3 and Siglec-5, while two positive and negative subsets of monocytes were observed for CD163 and Siglec-1 expression. All three animals showed a decrease in the CD163^{+} and Siglec-1^{+} percentage after infection. The reduction in the percentage of positive cells for these two markers was not enough to achieve a significant difference ($p = 0.174$ for CD163 and $p = 0.104$ for Siglec-1). With regards to expression levels, an increase in NMFIs of TLR2 and Siglec-3 was observed 24 h after infection in the three animals (Fig. 1A and B), and significant differences were found when comparing with the time before infection ($p = 0.016$ for TLR2 and $p = 0.0326$ for Siglec-3). The influence of granulocytic contamination on the observed increase of TLR2 and Siglec-3 expression was discounted after studying the expression of these receptors on 6D10^{-} cells in three animals, finding that the expression of both receptors was again significantly higher ($p = 0.033$ and $p = 0.039$). For TLR4, Siglec-1, and Siglec-5, no significant differences in expression were observed ($p = 0.427$, $p = 0.260$ and $p = 0.447$, respectively). Concerning TLR4, a decrease in the NMFI was observed in two pigs (Fig. 1D), while NFMI was not modified in the third animal. The expression of CD163 increased in the three pigs analyzed but the change did not reach statistical significance ($p = 0.0723$) (Fig. 1C).

Table 2
Presence of exudate or fibrin in sampled joints or body cavities in the infected pigs with *H. parasuis* Nagasaki strain.

		Pig nº									
		1	2	3	4	5	6	7	8	9	10
Pericardial cavity	Exudate	+	–	+	+	–	–	–	–	+	–
	Fibrin	–	–	–	+	–	–	–	+	+	+
Pleural cavity	Exudate	+	–	+	+	–	–	+	+	+	+
	Fibrin	+	–	–	+	–	+	–	+	+	+
Abdominal cavity	Exudate	+	–	+	+	+	–	+	–	+	+
	Fibrin	+	+	+	+	+	+	+	+	+	+
Carpal joint	Exudate	+	+	+	–	+	+	+	+	+	+
	Fibrin	+	–	+	–	–	+	+	+	–	–
Tarsal joint	Exudate	+	+	+	+	+	+	+	+	+	+
	Fibrin	+	+	+	+	+	+	+	–	–	–

Table 3
Isolation of *H. parasuis* from different tissues of the pigs infected with *H. parasuis* Nagasaki strain.

	Pig nº										
	1	2	3	4	5	6	7	8	9	10	Positive/total
Meninges	+	+	+	+	+	+	–	–	+	–	7/10
Pericardium	+	+	+	+	+	–	+	–	+	+	8/10
Lungs	–	+	–	–	+	–	+	+	+	+	6/10
Liver	+	+	+	+	+	+	+	+	+	–	9/10
Spleen	–	+	–	–	–	–	–	–	+	–	2/10
Carpus	+	+	+	–	+	+	+	+	+	+	9/10
Tarsus	+	+	+	–	+	+	–	+	–	+	7/10

3.3. Siglec-10 expression on lymphocytes

No significant changes were observed after infection either in the proportion of Siglec10^{+} lymphocytes ($p = 0.952$) or in the level of expression (MFI) of this receptor ($p = 0.156$).

3.4. Expression changes on CD163^{+} and CD163^{-} subsets of monocytes

All CD163^{+} monocytes were positive for TLRs, Siglec-3, and Siglec-5, while the CD163/Siglec-1 double staining showed both CD163^{+}/

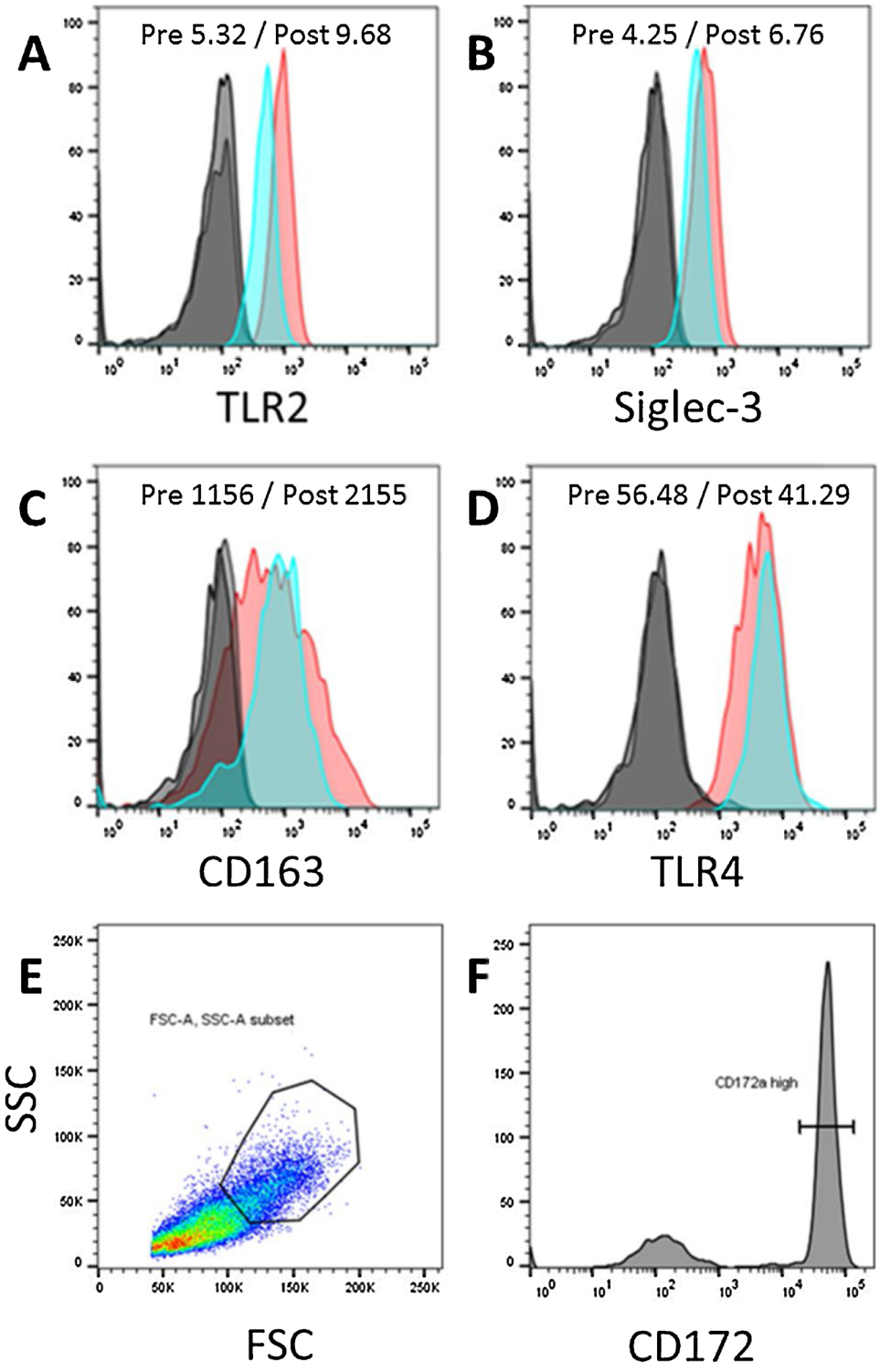

Fig. 1. Changes in expression of TLR2 (A), Siglec-3 (B), CD163 (C) and TLR4 (D) on monocytes after infection (sepsis time) defined by FSC/SSC gate (E) and CD172a^{+hi} events (F). Blue histograms correspond to fluorescence intensity (FI) at the preinfection time and red histograms at the post-infection time. Gray peaks correspond to negative controls using an irrelevant isotype antibody (light gray for pre-infection and dark gray for post-infection time). Panels A and B are representative of three independent experiments using cells from three different animals. Panel C show data from the animal in which the increase of CD163 MFI was more moderate. Figure D (TLR4) is representative of two animals in which a decrease of FI was observed. NMFI (A, B and D) or MFI (C) at preinfection (Pre) and post-infection (Post) are indicated.

Siglec-1$^+$ and CD163$^+$/Siglec-1$^-$ monocytes (Fig. 2). No significant differences were seen before and after infection either for Siglec-1 MFI ($p = 0.076$) or for the Siglec-1$^+$ percentage ($p = 0.871$). However, the subpopulation CD163$^-$/Siglec-1$^+$ that was present at the preinfection time tended to disappear after infection.

All six animals exhibited an increase in CD163 MFI on their monocytes after infection (Fig. 3), and this increase was significant ($p = 0.024$). The presence of statistical significance in this double staining and its absence in the previous double staining CD163/CD172a, where there was also increased CD163 MFI in all animals after infection, could be explained by the use of a small sample size in the first double staining ($n = 3$). The percentage of CD163$^+$ monocytes increased in three pigs, while it decreased in another three; therefore, no significant differences were observed between before and after infection ($p = 0.583$).

In all six animals, TLR2 NMFI increased after infection both on CD163$^+$ and CD163$^-$ monocytes (Fig. 4A and C). When the values of TLR2 NMFI were compared between the pre and post-infection times, a significantly higher value was observed after infection for both CD163$^+$ ($p < 0.001$) and CD163$^-$ monocytes ($p = 0.003$). In addition,

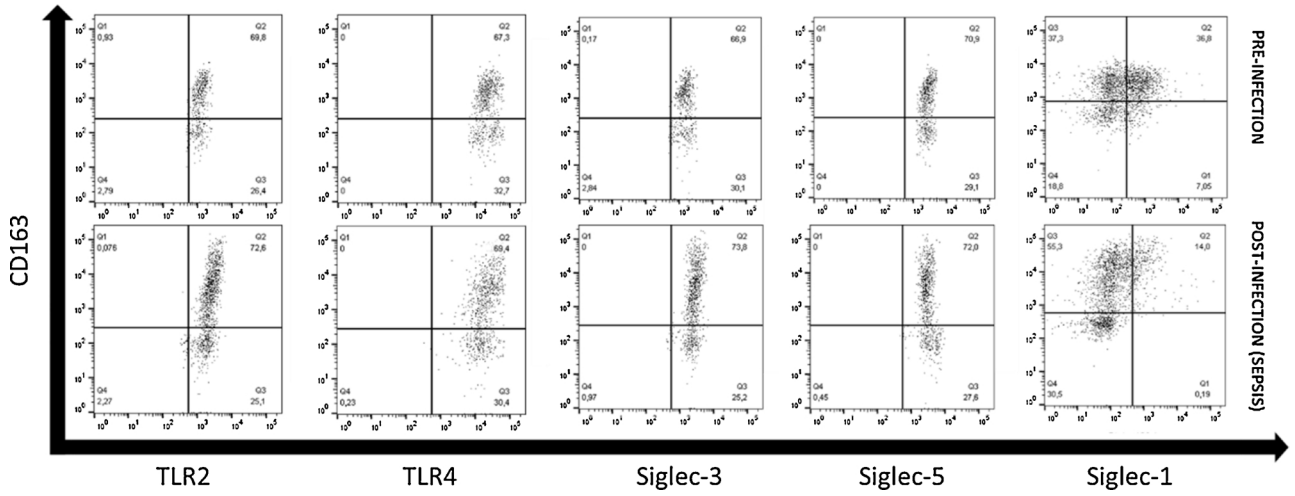

Fig. 2. Dot plots of double stains with CD163 in the preinfection time (upper panel) and the post-infection time (lower panel). The Y axis represents CD163 expression by monocytes, and the X axis represents TLRs and Siglecs expression on monocytes. Dot plots are representative of six independent experiments using cells from six different animals except CD163/Siglec-1, which is representative of five independents experiments using cells from five animals.

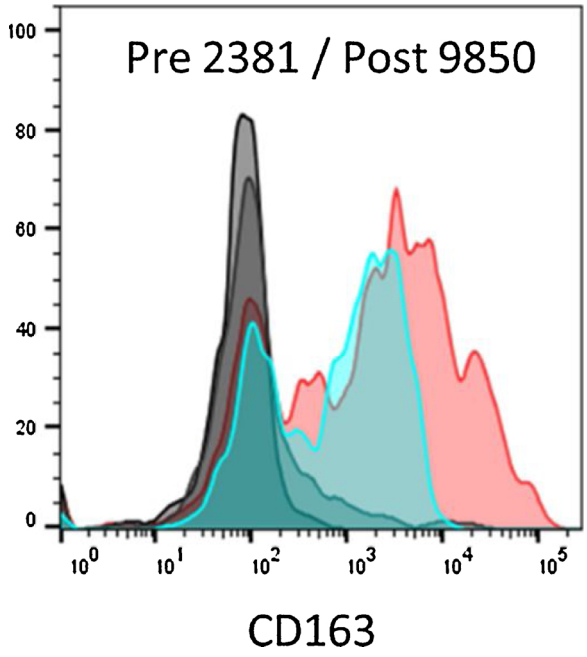

Fig. 3. Changes in CD163 expression on TLR2$^+$ cells (monocytes) after infection (sepsis time). Blue histograms correspond to the fluorescence intensity (IF) of CD163 at the preinfection time and red histograms at the post-infection time. Gray peaks correspond to negative controls using an irrelevant isotype antibody (light gray for preinfection and dark gray for sepsis time). This figure is representative of six independent experiments using cells from six different animals. MFI at preinfection (Pre) and post-infection (Post) are indicated.

significantly higher values were found for TLR2 NMFI on CD163$^+$ than on CD163$^-$ monocytes after infection (p = 0.002) (Fig. 4D), while no differences were observed prior to infection between these two monocyte subpopulations (p = 0.114) (Fig. 4B). A significant increase in Siglec-3 NMFI on CD163$^+$ (p = 0.001) and CD163$^-$ (p = 0.013) cells was observed after infection. All six animals showed an increase in the Siglec-3 NMFI on CD163$^+$ monocytes (Fig. 5A) after infection and five animals also showed this increase on CD163$^-$ (Fig. 5C), while Siglec-3 NMFI did not change in the remaining one. Similar to what was observed for TLR2, the Siglec-3 NMFI was significantly higher on CD163$^+$ than on CD163- monocytes after infection (p = 0.001) (Fig. 5D), while no significant differences were observed (p = 0.481) before it (Fig. 5B).

A positive correlation between CD163 and TLR2 (Fig. 4D) and CD163 and Siglec-3 (Fig. 5D) expression was seen after infection, while it was not observed before it (Figs. 4B and 5 B).

A slight decrease in TLR4 expression on the two monocyte subsets (CD163$^+$ and CD163$^-$) was observed after infection in five animals, while in the sixth animal, no changes were seen. No significant differences were observed on either CD163$^+$ or CD163$^-$ subpopulations (p = 0.122 and p = 0.255, respectively) when NMFI values from the pre- and post-infection times were compared. Siglec-5 expression showed a random behavior in the CD163$^+$ and CD163$^-$ subsets because animals with increasing and decreasing expression levels of this receptor were found after inoculation. Obviously, no significant differences for Siglec-5 NMFI by CD163$^+$ (p = 0304) or CD163$^-$ (p = 0075) cells were observed pre- and post-infection times.

3.5. Influence of in vitro bacteria exposure on TLR2, Siglec-3 and CD163 expression

There was a significant decrease in the expression of TLR2 (NMFI) with respect to the control when PBMCs were exposed to 10^6 CFUs of *H. parasuis* (p = 0.003) (Fig. 6A), while no significant differences were observed between the control and the PBMCs exposed to 10^7 CFUs (p = 0.231). CD163 expression was significantly lower after exposure to 10^7 CFUs than the control (p = 0.033) (Fig. 6B), while there were no significant differences between exposure to 10^6 CFUs and the control (p = 0.861). No significant differences were observed for Siglec-3 NMFI between the control and bacterial exposure of 10^6 (p = 0.187) or 10^7 CFUs (p = 0.917).

3.6. Influence of in vitro exposure to sera from infected pigs on TLR2, Siglec-3 and CD163 expression

No significant differences were observed in the expression of CD163 between monocytes exposed to sera from infected pigs and those incubated with sera from healthy animals (p = 0.221). With regards to TLR2 (Fig. 7A) and Siglec-3 (Fig. 7B), a slight increase in their NMFI was seen in all cases, but it did not result in a significant difference (p = 0.067 and 0.102, respectively).

4. Discussion

Severe symptomatology, a fast death, recovery of bacteria and pathological lesions indicate that at the time of blood sampling after

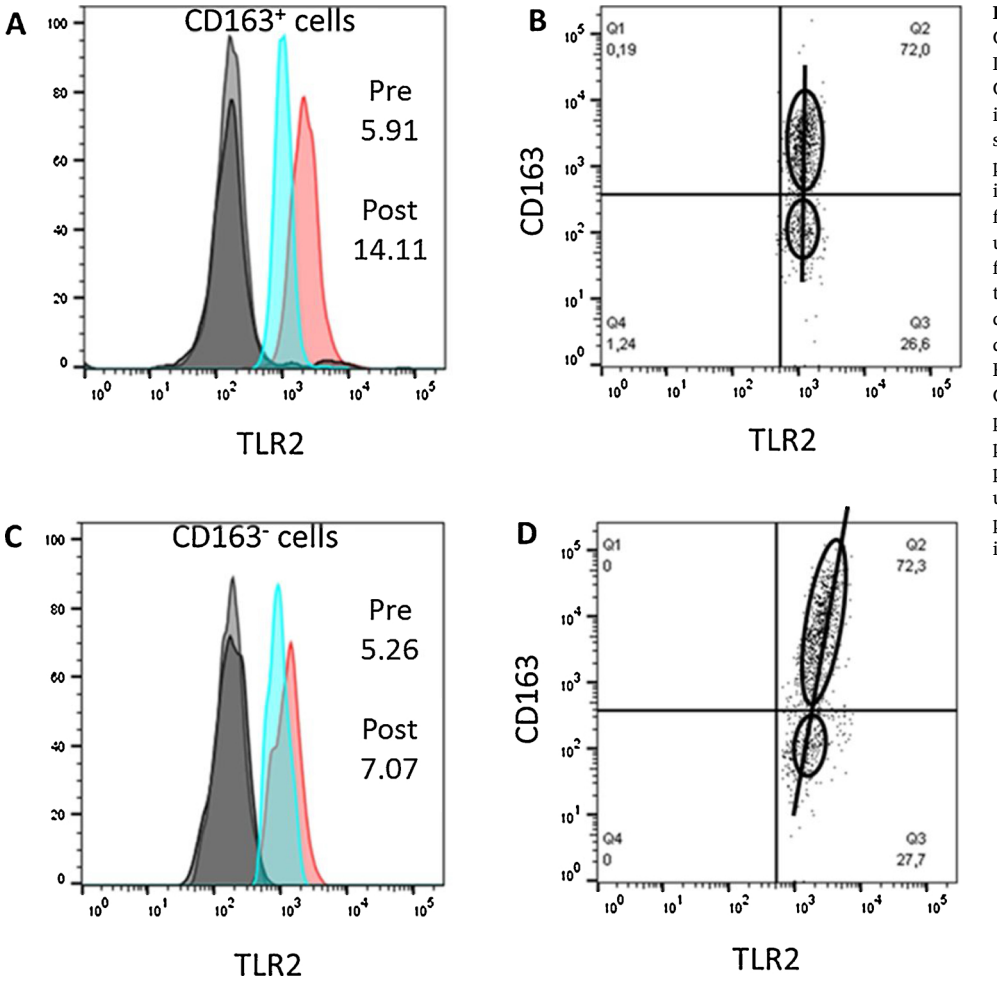

Fig. 4. Changes in expression of TLR2 on $CD163^+$ and $CD163^-$ monocytes.
Left: Changes in expression of TLR2 on $CD163^+$ (A) and $CD163^-$ (C) monocytes after infection (sepsis time). Blue histograms correspond to fluorescence intensity (FI) of TLR2 at preinfection time and red histograms at post-infection time. Gray peaks correspond to fluorescence intensity in negative controls using an irrelevant isotype antibody (light gray for preinfection and dark gray for post-infection time). Figures are representative of six independent experiments using cells from six different animals.
Right: Dot plots where the expression levels of CD163 (Y axis) and TLR2 (X axis) are represented on monocytes at preinfection (B) and post-infection (D) times. Dot plots are representative of six independent experiments using cells from six different animals. NMFI preinfection (Pre) and post-infection (Post) are indicated.

infection, all of the animals used in this study suffered from sepsis triggered by the experimental infection with *H. parasuis*. This disease progression is what is expected working with the selected infection model [10,13,14]. The aim of this study was to evaluate how the establishment of the septic process caused by *H. parasuis* could affect the expression of several immunological receptors related to sepsis (TLR2, TLR4, Siglec-1, Siglec-3, Siglec-5 and CD163) by comparing their expression on monocytes from the same pig before and after infection, when sepsis was already established.

Results from double stains showed that TLR2, Siglec-3 and CD163 expression were significantly increased on monocytes after the establishment of sepsis. These expression changes may be due to a recruitment of monocytes with different expression profile into the bloodstream, but it may also have resulted from a variation in the phenotype of the blood monocytes in response to interactions with bacteria and/or changes in the blood environment during sepsis. In addition, a combination of both factors, the recruitment of new monocytes into the bloodstream and the modification in the expression profile of the blood monocytes, may also have occurred.

To clarify this, PBMCs from healthy pigs were cultured for 24 h in the presence of inactivated bacteria or sera from pigs used in the experimental infection (before and after infection). The results of *in vitro* cultures showed different trends to those observed with the samples from infected animals. No significant differences were observed in the expression of these receptors between PBMCs exposed to pre-infection sera and the same cells exposed to post-infection sera. On the other hand, *in vitro* exposure to bacteria resulted in a decrease in the surface expression of TLR2 and CD163, while Siglec-3 did not seem to be influenced by exposure to the bacteria.

The differences between *in vivo* and *in vitro* results may be explained by the release of new monocytes with high levels of these receptors into the bloodstream, which overshadows the expression profiles of the old cells, including the bacteria-induced decrease of TLR-2 and CD163. Therefore, the observed changes during sepsis may be mostly due to a release of new monocytes into the bloodstream rather than the changes in the expression profiles of the blood monocytes in response to blood environment.

Another possibility is that the selected culture conditions failed to reproduce the host bloodstream environment and therefore, the interaction of peripheral monocytes with the blood environment during infection should not be completely ruled out to explain the increase in these receptors. However, our *in vitro* results to Siglec-3 and CD163 are similar to those observed by previous *in vitro* studies. With regard to Siglec-3, Álvarez et al. [15] observed that this receptor was not modified when porcine monocytes were exposed to several cytokines or ligands of TLRs, this result being similar to that obtained by us. Concerning the bacteria-induced decrease of CD163, Costa-Hurtado et al. [16] also observed an opposite trend of this receptor between *in vitro* and *in vivo* studies on porcine alveolar macrophages. These authors observed that infection of pigs with *H. parasuis* resulted in an increase of CD163 expression on porcine alveolar macrophages after 48 h of infection. However, they also observed that CD163 expression decreased on porcine alveolar macrophages when these cells were *in vitro* cocultured with *H. parasuis* for 20 h. Hintz et al. [17] obtained similar results using LPS-exposed human monocytes: the *in vitro* exposure caused the decrease of surface CD163 while the intravenous injection of LPS resulted in an increase of CD163 on circulating monocytes. TLR activation of monocytes induces the shedding of cell surface CD163

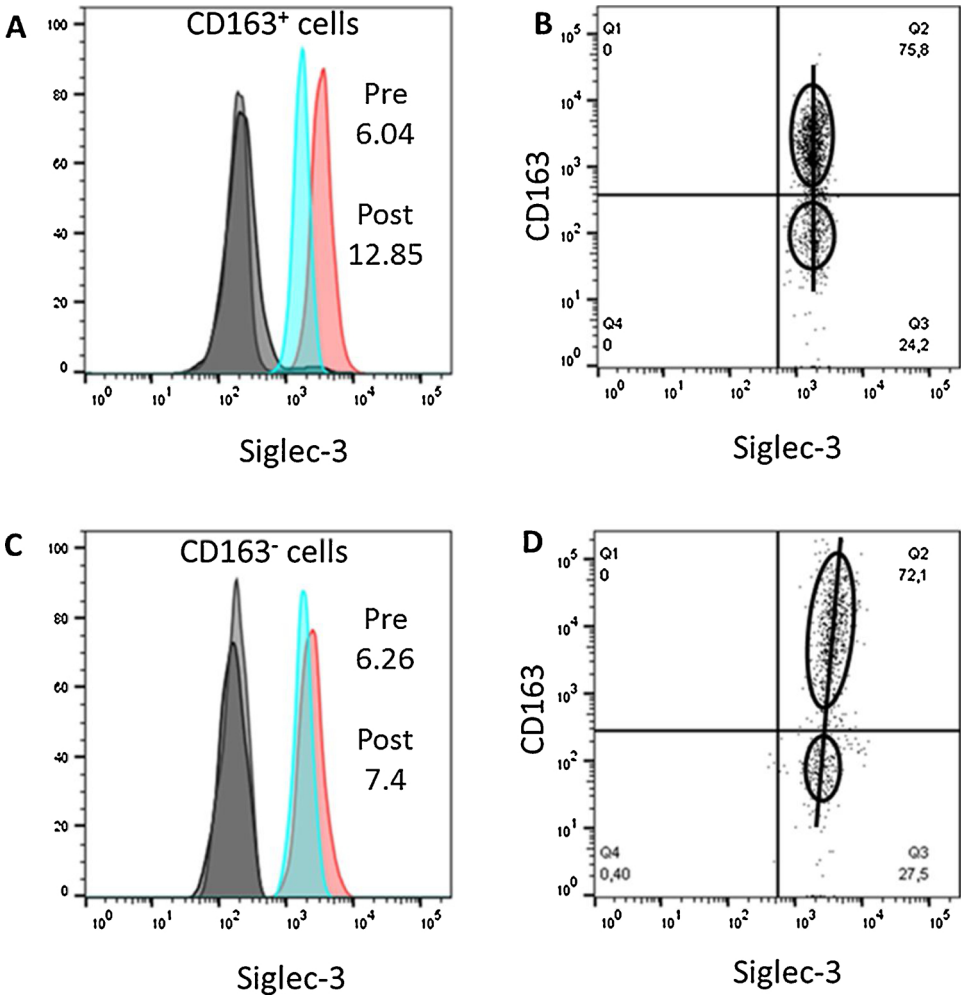

Fig. 5. Changes in expression of Siglec-3 on $CD163^+$ and $CD163^-$ monocytes.
Left: Changes in expression of Siglec-3 on $CD163^+$ (A) and $CD163^-$ (C) monocytes after infection (sepsis time). Blue histograms correspond to fluorescence intensity (FI) of Siglec-3 at the preinfection time and red histograms at the post-infection time. Gray peaks correspond to fluorescence intensity in negative controls using an irrelevant isotype antibody (light gray for preinfection and dark gray for post-infectio time). Figure A is representative of six independent experiments using cells from six different animals, while Figure C is representative of five.
Right: Dot plots where the expression of CD163 (Y axis) and Siglec-3 (X axis) is representative of monocytes at preinfection (B) and post-infection (D) times. Dot plots are representative of six independent experiments using cells from six different animals. NMFI preinfection (Pre) and post-infection (Post) are indicated.

[18]. Therefore, the CD163 shedding may explain the decrease in surface CD163 on *in vitro* bacteria-exposed monocytes.

Regarding the increase of CD163 levels observed *in vivo*, there are previous works in porcine model [19,20] that point to the recruitment of new monocytes with higher CD163 levels into the bloodstream as cause of it. These reports showed that *Actinobacillus pleuropneumoniae* infection caused the recruitment of monocytes with a high CD163 expression from the bone marrow into the bloodstream. In this way, *H. parasuis* infection has been shown to induce the production of CCL5, a ligand of CCR5 [21]. This chemokine could play an important role in the recruitment of the $CD163^+$ bone marrow monocytes because these cells expressed higher levels of CCR5 than of $CD163^-$ bone marrow monocytes [22].

CD163 plays an important role during sepsis because it has an anti-

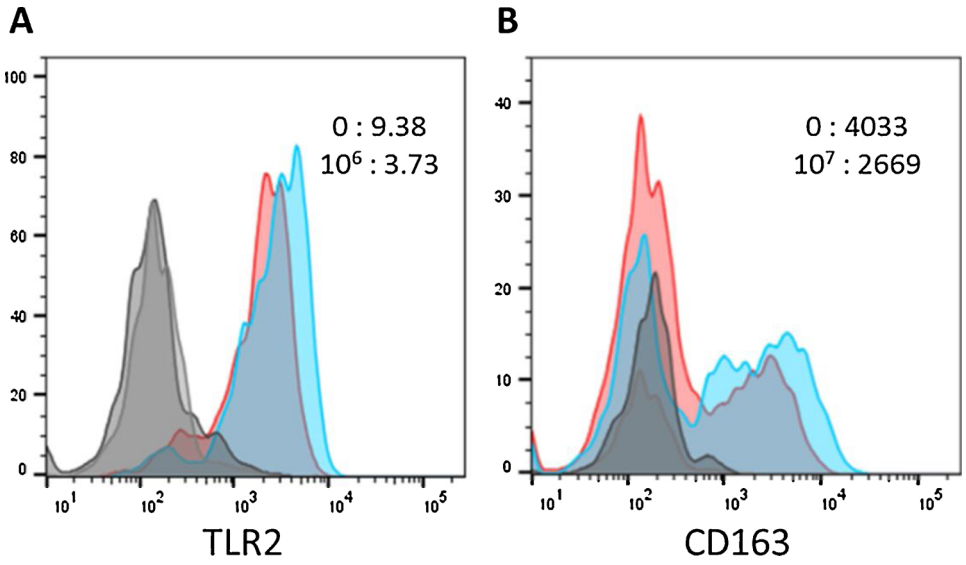

Fig. 6. Changes in TLR2 and CD163 expression in bacteria-exposed monocytes.
Changes in expression of TLR2 (A) and CD163 (B) between monocytes cultured for 24 h in complete medium with 10^6 CFUs (TLR2) or 10^7 CFUs (CD163) (red histograms) or control without bacteria (blue histograms). Gray peaks correspond to negative controls using an irrelevant isotype antibody (light gray, no exposed bacteria, and dark gray, exposed bacteria. Figures are representative of four independent experiments. NMFI (A) or MFI (B) are indicated.

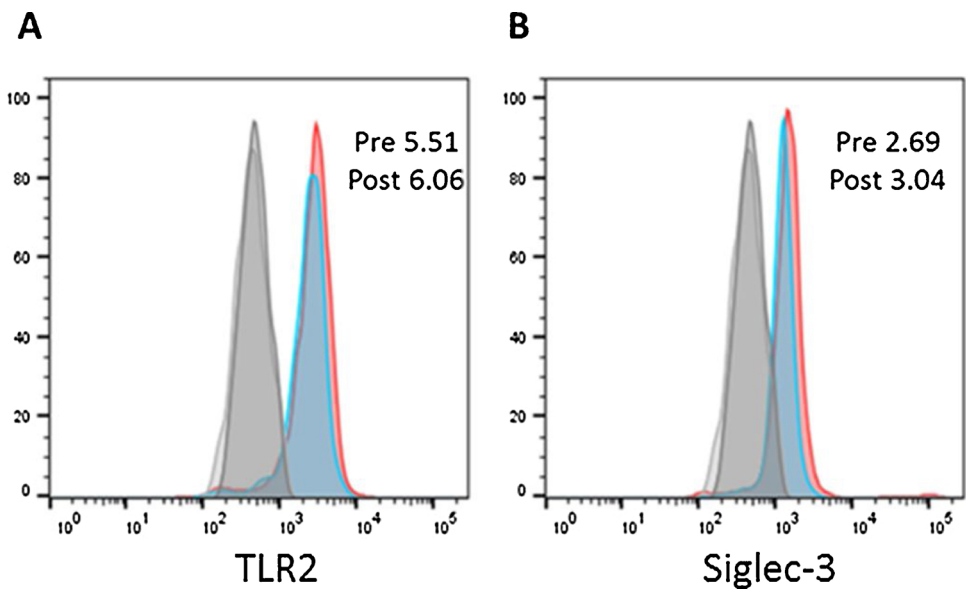

Fig. 7. Changes in TLR2 and Siglec-3 expression on sera-exposed monocytes.
Changes in expression of TLR2 (A) and Siglec-3 (B) between monocytes cultured 24 h in complete medium with sera from an infected pig (red histograms) or sera from the same pig before infection (blue histograms). Gray peaks correspond to negative controls using an irrelevant isotype antibody (light gray, sera pre-infection, and dark gray, sera post-infection). Figures are representative of four independent experiments. NMFI preinfection (Pre) and post-infection (Post) are indicated.

inflammatory function by means of the removal of circulating DAMPs, such as HMGB1 and free hemoglobin, for which it is necessary that haptoglobin participates [2], and its levels are also increased in the blood during infection caused by *H. parasuis* [23]. Although it has not been possible to demonstrate *in vitro* the hemolytic capacity of *H. parasuis*, several studies have found genes coding for hemolysis in virulent strains, so it is speculated that these bacteria could activate these genes in the host [24–26]. Therefore, high levels of CD163 and haptoglobin could be beneficial for the host during infections by hemolytic bacteria, such as *A. pleuropneumoniae* and presumably *H. parasuis*.

In the case of TLR2, the decrease observed *in vitro* may be due to a downregulation of its expression or to an endocytic internalization of the receptor. Results obtained for Dietrich et al. [27] suggested that murine macrophages exposed to TLR ligands can internalize TLR2. On the other hand, several studies performed in human monocytes showed the bacteria/LPS-induced up-regulation of this receptor [28]. Therefore, our *in vitro* results about bacteria-induced decrease of TLR-2 should be taken with caution and the interaction of monocytes with the blood environment during infection should not be completely ruled out to explain the increase in this receptor observed in pigs with sepsis.

Consistent with data from bone marrow monocytes under steady state conditions [22], we observed no differences in the expression of TLR2 and Siglec3 between $CD163^+$ and $CD163^-$ blood monocytes before infection. Conversely, the expression of these receptors was significantly higher on $CD163^+$ than on $CD163^-$ on blood monocytes during sepsis. In addition, after infection, a positive correlation between the expression of CD163 and the other two receptors was seen. As previously mentioned, monocytes with high CD163 expression appear in peripheral blood after infection presumably because of their recruitment from the bone marrow. The correlation between CD163 and TLR2 or Siglec-3 expression may suggest that the cells with high TLR2 and Siglec-3 expression exhibit the same origin that the cells with high CD163. A high expression of TLR2 and CD163 would enhance the capacity of circulating monocytes to interact with DAMP/PAMP (TLR2) or bacteria (a pro-inflammatory function of CD163) and produce pro-inflammatory cytokines in order to control the bacterial infection. In addition, $CD163^+$ cells seem to have a greater capacity to present antigens [22,29]. Moreover, the high expression of CD163 (with its anti-inflammatory functions) and Siglec-3 could compensate (or try to compensate) for the expected excess of pro-inflammatory signal. *H. parasuis* could take advantage of the inhibitory mechanism of Siglecs by covering its surface with sialic acid [7,9].

5. Conclusions

The expression of TLR2, Siglec-3 and CD163 on peripheral blood monocytes is significantly higher during *H. parasuis*-induced sepsis than before infection. In addition, a correlation between CD163 expression and TLR2 and Siglec-3 expressions during infection was observed. These changes may be due to a recruitment of new monocytes into the bloodstream rather than to changes in the expression profile of the blood monocytes. Further *in vivo* studies should be performed with sublethal doses to monitor *H. parasuis*-induced changes over a longer period of time, including comparisons with new time-paired *in vitro* studies. Pigs suffering *H. parasuis*-induced sepsis could be a good experimental model for future researchs in physiopahtology of sepsis.

Conflict of interest

The authors declare no conflict of interest.

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