Porcine Alveolar Macrophage-like cells are pro-inflammatory Pulmonary Intravascular Macrophages that produce large titers of Porcine Reproductive and Respiratory Syndrome Virus

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Lung inflammation is frequently involved in respiratory conditions and it is strongly controlled by mononuclear phagocytes (MNP). We previously studied porcine lung MNP and described a new population of cells presenting all the features of alveolar macrophages (AM) except for their parenchymal location, that we named AM-like cells. Herein we showed that AM-like cells are macrophages phagocytosing blood-borne particles, in agreement with a pulmonary intravascular macrophages (PIM) identity. PIM have been described microscopically long time ago in species from the Laurasiatheria superorder such as bovine, swine, cats or cetaceans. We observed that PIM were more inflammatory than AM upon infection with the porcine reproductive and respiratory syndrome virus (PRRSV), a major swine pathogen. Moreover, whereas PRRSV was thought to mainly target AM, we observed that PIM were a major producer of virus. The PIM infection was more correlated with viremia in vivo than AM infection. Finally like AM, PIM-expressed genes were characteristic of an embryonic monocyte-derived macrophage population, whose turnover is independent of bone marrow-derived hematopoietic precursors. This last observation raised the interesting possibility that AM and PIM originate from the same lung precursor.

Respiratory infections are one of the major sources of disease in swine husbandry, leading to economic losses in the pig industry. Since the aetiology of respiratory diseases is multifactorial, the term porcine respiratory disease complex (PRDC) is often used1. One of the main pathogens at the root of PRDC is the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), an enveloped, positive-stranded RNA virus of the Arteriviridae family. Indeed, PRRSV presents long-term infections due to its capacities to alter the immune response, a property that facilitates bacterial and viral superinfections. PRRSV main cellular target is thought to be alveolar macrophages.

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AM-like cells are Pulmonary Intravascular Macrophages (PIM). AM-like cells represented the second-most abundant population of lung MNP after AM, being 10 times less represented than AM, but at least 5 times more numerous than cDC1, cDC2 or moDC. In a first description of AM-like cells, we demonstrated that these cells were not located in the alveoli, and that our broncho-alveolar lavage procedure avoids the contamination of the parenchymal cell preparation by AM. However, we were uncertain whether AM-like cells were localized in the lung interstitium or on the endothelial wall, in contact with the vascular lumen as described for PIM. Since PIM have been described as highly phagocytic cells, an experiment was devised in order to label phagocytic cells having access to blood-borne particles using FITC-labelled pseudomonas bacteria injected in the jugular vein. Peripheral blood mononuclear cells (PBMC), broncho-alveolar lavage (BAL) and parenchyma were collected 10 minutes post-bacterial injection, cell suspensions were recovered and stained for cDC1, cDC2, moDC and AM/AM-like discrimination as previously described (Fig. 1a). In the parenchyma, 19% +/- 4% of AM-like cells presented a clear phagocytosis of FITC-labelled bacteria, no cDC1 and few cDC2 were FITC-stained. Interestingly, moDC presented a highly variable FITC staining, from 2% to 18% (mean 13% +/- 5%). In the BAL, no FITC staining either of AM (Fig. 1a,b) or of BAL DC (data not shown) was observed. In order to identify blood-circulating cells that might phagocytose bacteria and contaminate the parenchymal preparation, the same gating as for parenchymal cells was applied for PBMC. No events were observed that could correspond to AM-like cells (empty gate 4 in Fig. 1a), proving that AM-like cells were not blood circulating cells. Conversely, the PBMC gates 2 and 3 (Fig. 1a), corresponding respectively to cDC2 and moDC in the parenchymal gating, presented a strong phagocytosis of bacteria, since around 50% of these two cell types presented FITC staining. Thus, the FITC staining of parenchymal cDC2 and moDC might mostly be due to blood circulating cell contamination of the parenchyma. In order to demonstrate that the lower staining of AM, cDC1 and cDC2 was not due to an intrinsic defect in bacterial phagocytosis, BAL cells were incubated in vitro with FITC-stained pseudomonas. All the DC/Macrophage subtypes (cDC1, cDC2, moDC and AM) presented more than 45% bacteria phagocytosis (data not shown), in agreement with similar capacities of these cells to phagocyte bacteria, provided that they enter in contact with the pathogen.

Thus, AM-like cells are the only parenchymal residing MNP population consistently phagocytosing blood bacteria. Although only 20% of the AM-like cells appeared FITC-positive, we consider that the 3 to 4 hours needed for MNP extraction from lung tissue allowed a majority of PIM that had phagocytosed bacteria to quench the acid-sensitive FITC, eventually exhibiting no staining. Alternatively, PIM might be able to move across the blood barrier and locate temporarily in the interstitium, losing their access to blood. We then proceeded to tissue staining and confocal imaging in order to visualize blood-borne bacterial phagocytosis in situ. We observed the localization of FITC-stained bacteria inside CD163<sup>+</sup>/MHC-II<sup>+</sup> cells (AM-like cells in the parenchyma, in v-Cadherin (a blood vessel specific marker)<sup>21</sup> rich area (Fig. 1c). Altogether, we show here that AM-like cells are bona fide PIM.

PIM/AM-like cells share with AM differential expression of key chemokine receptors and transcriptional regulators suggesting that they could be derived from the same embryonic precursors. Our previous work showed that AM and PIM/AM-like cells were very similar, for instance they did not
Figure 1. AM-like cells phagocytosed bloodborne bacteria. Inactivated FITC-labelled pseudomonas bacteria were injected in the jugular vein of anesthetized pigs. Ten minutes after, blood was collected on heparinized tubes and proceeded to Ficoll isolation of peripheral blood mononuclear cells (PBMC), animals were euthanized and cells from broncho-alveolar lavages and parenchyma were collected and stained for FACS analysis of DC and Mφ. (a) Dot plot gating strategy of lung DC and Mφ as previously described and histograms of phagocytosed bacteria (FITC) signal in the different gated populations. The PBMC gate 4 contained no event. One representative out of 4 independent experiments. (b) Plotting of the 4 independent experiments. Statistics: Mann-Whitney test *p < 0.05. (c) Confocal imaging of parenchyma from FITC-bacteria injected animals. Upper left, DAPI (white), FITC-bacteria (green), CD163 (red) and MHC-II (blue) staining. Upper right, magnification of the squared zone of the upper left picture. Arrows indicate PIM/AM-like cells (extra-alveolar CD163pos/MHC-IIpos) having internalized FITC-bacteria. Lower left, DAPI (white), FITC-bacteria (green), ve-Cadherin (red) and CD163 (blue) staining. Lower right, magnification of the squared zone of the lower left picture. Arrows indicate PIM/AM-like cells (extra-alveolar CD163pos) localized in ve-Cadherin rich area (lung capillaries) and having internalized FITC-bacteria. Alveoli are visualized by dashed lines. Images are representative of 3 independent experiments.
measured using RT-qPCR on the cell pellet and virus titration on cell culture supernatant. PIM/AM-like cells and in the percentage of dead cells of these two populations (Fig. 4a) as previously described for AM 28. Moreover, in agreement with this result, the proportion of AM and PIM/AM-like cells among MHC-II high BAL and parenchymal cells respectively decrease (Fig. 4b). The amount of PRRSV viral RNA was then measured by RT-qPCR on sorted cells (Fig. 4c). Both AM and PIM/AM-like cells were positive for viral RNA. A correlation analysis was performed with the following parameters: AM and PIM/AM-like cells PRRSV infection titers (Fig. 4c), serum PRRSV titers (viremia, as depicted in 29) as well as whole lung tissue viral load (Fig. 4d). PIM, but not AM infection, appeared correlated with lung titer (0.99 and 0.0025 respectively), and with serum titer (0.76 and −0.53 respectively) (Fig. 4e). This correlation must be taken with caution because it mainly stands on one individual presenting high viral titer in blood, lung and PIM/AM-like cells but not in AM.

In vivo, PRRSV infection of PIM/AM-like cells correlates with lung and blood PRRSV titer. Our in vitro data prompted us to measure the infection of PIM/AM-like cells during in vivo PRRSV infection. We chose to measure AM and PIM/AM-like cells PRRSV infection at an intermediate time point of 10 days post-infection (dpi), at which LENA viremia had reached a plateau 28. We first measured the percentage of dead cells in PIM/AM-like cells and AM populations. At day 10 post PRRSV infection, we observed a significant increase in the percentage of dead cells of these two populations (Fig. 4a) as previously described for AM 28. Moreover, in agreement with this result, the proportion of AM and PIM/AM-like cells among MHC-IIhigh BAL and parenchymal cells respectively decrease (Fig. 4b). The amount of PRRSV viral RNA was then measured by RT-qPCR on sorted cells (Fig. 4c). Both AM and PIM/AM-like cells were positive for viral RNA. A correlation analysis was performed with the following parameters: AM and PIM/AM-like cells PRRSV infection titers (Fig. 4c), serum PRRSV titers (viremia, as depicted in 29) as well as whole lung tissue viral load (Fig. 4d). PIM, but not AM infection, appeared correlated with lung titer (0.99 and 0.0025 respectively), and with serum titer (0.76 and −0.53 respectively) (Fig. 4e). This correlation must be taken with caution because it mainly stands on one individual presenting high viral titer in blood, lung and PIM/AM-like cells but not in AM.

We finally measured by RT-qPCR, the in vivo cytokine response of AM and PIM/AM-like cells in mock and PRRSV-infected animals at 10 dpi. No cytokine presented a significant differential expression when conditions or cell types were compared (Fig. 5a). In order to assess the global AM/PIM responses, we ran a principal component analysis (PCA) on the cytokine data, comparing AM and PIM/AM-like cells in non-infected or infected conditions. PIM/AM-like cells produced more cytokine mRNA than AM except for IL-4 in mock infected animals, and for IFN-β, IL-12p35, IL-12p40/IL-23 and IL-4 in infected animals. In infected animals, the first axis of the PCA encompassed 39% of the total variability of the samples (Fig. 5a). It segregated IL-8 and TNFα from IFN-γ, IL-6, TGFβ, IFN-β, IL-12p35 and IL-12p40/IL-23 cytokines. Except for the inter-individual variations, no obvious interpretation for this splitting can be proposed. Interestingly the second axis still encompassed 30% of the variability and clearly segregated PIM/AM-like cells from AM. Indeed PIM/AM-like cells expressed more IL-8, TNFα, IL-6 and IFN-γ, whereas AM expressed more IL-12p35 and IL-4. Thus we could observe in vivo, a tendency of PIM to produce more cytokines than AM at steady state and upon infection. However globally, at 10dpi, both PIM/AM-like cells and AM responded weakly to PRRSV.

Discussion

Herein, we showed that the previously described porcine AM-like cells phagocytosed blood-borne bacteria and produced inflammatory cytokines such as TNFα, IL-8 and IL-6 28. Some teams succeeded in isolating PIM using perfusion of the pulmonary vasculature with collagenase solution 30. Using this technique, the best controlled experiment 31 measured a PIM/AM ratio of 1:10, in perfect agreement with the AM/like/AM ratio we previously observed 3. Despite our inability to stain all AM-like cells, this last data associated with the homogeneity of AM-like cell population phenotype 4 are in strong support with the whole AM-like cell population being bona fide PIM.

Thanawongnuwech observed 20 years ago that collagenase-isolated PIM could be infected by PRRSV 31, moreover in vivo PRRSV infection decreased the ability of the lung to retain copper particles 34, in agreement with a PRRSV-mediated PIM depletion. We demonstrated here that AM-like cells were as permissive as AM to PRRSV infection in vitro and in vivo. Thus viral replication is at least as high in PIM/AM-like cells than in AM, demonstrating that AM are not the sole source of virus in the lung. Moreover, because of PIM/AM-like cells localization...
in the blood vessel lumen, allowing them to shed virus directly in the blood circulation, they might likely be the major cell types responsible for viremia.

Interestingly, we also showed here that both porcine AM and PIM/AM-like cells harbored characteristics suggesting that they derive from embryonic monocytes rather than from adult bone marrow, in consistency with what has been demonstrated for mouse AM. Although surprising according to their location in contact with blood, PIM origin can be paralleled with the recently described resident arterial macrophages in mice. These cells are embryonic monocyte-derived, and self-renew in the aortic wall. It has been shown that PIM had an important role in xenogeneic graft failure, and that their depletion before pig lung graft to primate extended the duration of graft functionality. The embryonic origin of PIM implies that the lung vasculature wall of the

Figure 2. PIM/AM-like cells and AM present a gene expression profile characteristic of embryonically-derived macrophages. Conventional DC1, cDC2, moDC, PIM/AM-like cells and AM were gated as in Fig. 1 and sorted by flow cytometry. (a) mRNA expression levels of embryonic-monocytes derived Mφ-associated genes (HDAC10, PU1) and bone marrow precursors derived Mφ-associated gene (cKit); as well as of MAFB, assessed by RT-qPCR. For each gene, data were normalized to the reference gene RPS24 (ribosomal protein S24) expression and presented as relative expression (arbitrary units (AUs)): for each animal, the population with the highest expression for this gene was considered as 100 and the other populations were normalized to it. Each symbol represents one animal. Statistics: Mann-Whitney test *p < 0.05. (b) Heat map synthesizing the means of real-time PCR data from A and from Maisonnasse et al.
PIM-depleted-graft can be re-populated from progenitors present within the graft. Thus, the detrimental effect of PIM in the context of xenograft might only be postponed by PIM depletion before grafting.

PIM have been described in the species from the Laurasiatheria superorder whereas no PIM were observed in primates and mice, species belonging to the Euarchontoglires superorder. However, in rats and humans, evidence exist of a few constitutive PIM that increase in number upon liver dysfunctions. In the rat model, PIM induced upon bile-duct ligation were shown to be responsible for endotoxin-induced mortality. It would thus be interesting to re-examine the role of PIM in the liver-induced inflammatory medical conditions.

Interestingly, it has been shown in lambs that PIM and AM appeared in the developing lung within the same time frame, increasing progressively from 1 day to 3 weeks of age. It is tempting to speculate that AM and PIM originate from the same lung cell precursor that relocate after birth either in the alveoli or the lung endothelium. This precursor would have, even in Euarchontoglires, the potential to differentiate in PIM, provided the appropriate, still unknown, endothelial/blood stimulus is present.

It is worth noting that AM express lower MAFB mRNA than PIM/AM-like cells. MAFB has been described as a repressor of cell-renewal capacities in adult macrophages. Thus the higher MAFB expression in PIM/AM-like cells would be in agreement with a lower proliferation capacity compare to AM, raising the interesting possibility that PIM/AM-like cells would originate from AM.

Materials and Methods

Pseudomonas FITC-staining. 1.10^10 cfu of UV-inactivated *Pseudomonas* were resuspended at 2 mg/ml in 0.1 M Na_2CO_3, pH 9 buffer and 150 µg of fluorescein isothiocyanate (FITC) (SIGMA) per ml of bacteria were added and incubated for 2h at 4 °C. Bacteria (FITC-*Pseudomonas*) were then washed twice in PBS and frozen in glycerol before use.

Pig lung cells collection. For *in vitro* characterization and infection, lung tissue samples were obtained from 5- to 7-month-old Large White conventionally bred sows from UEPAO, Tours, France. A BAL procedure was performed twice on the isolated left lung with 250 ml of PBS supplemented with 2mM EDTA (PBS/EDTA), to collect AM. Next, a 1-cm slice of external lung parenchyma was dissected from the same lung. Tissues were minced and incubated in nonculture-treated Petri dishes, to avoid differential plastic adherence of MΦ and DC, for 2h at 37 °C in complete RPMI, consisting of RPMI 1640 supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine and 10% FCS.

Figure 3. PIM/AM-like cells produce virus and upregulate inflammatory cytokines expressions upon PRRSV infection *in vitro*. (a and b) Cell-sorted PIM/AM-like cells and AM were *in vitro* infected at 10^{-3} MOI with LENA PRRSV strain. After 24h infection, the cell pellet and the supernatant were collected. (a) mRNA were extracted from cell pellets, retrotranscribed and virus RNA contents was quantified by qPCR (n = 9). (b) Infectious viral particles (TCID50/ml) were titrated in the supernatant according to the OIE manual (n = 9). Mock infected cells gave no signal in (a) as in (b) (data not shown). In (c) Cell-sorted PIM/AM-like cells and AM were *in vitro* infected at 5 MOI, cell pellet mRNA were extracted, retrotranscribed and the contents in TNFα, IL-8 and IL-6 transcripts were quantified by qPCR (n = 4, c). For each animal, mock infected AM 2−ΔΔCt mRNA cytokines expression (normalized to the reference gene RPS24) is considered as reference 100, from which PRRSV AM as well as mock and PRRSV AM-like/PIM conditions are normalized. Statistics: Mann-Whitney test *p < 0.05.
streptomycin, 2 mM L-glutamine, and 10% inactivated foetal calf serum (FCS) (all from Invitrogen, Paisley, UK), containing 2 mg/ml collagenase D (Roche, Meylan, France), 1 mg/ml dispase (Invitrogen), and 0.1 mg/ml Dnase I (Roche). Cells were passed through 40 μm cell strainers and red blood cells lysed with erythrocytes lysis buffer (10 mM NaHCO₃, 155 mM NH₄Cl, and 10 mM EDTA). Next, cells were washed with PBS/EDTA, counted, and step-frozen in FCS/10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) before staining and flow cytometry analysis or cell sorting.

For in vivo PIM targeting, experiments were performed at INRA GABI (Jouy en Josas, France). The animal experiment protocol was approved by the French Ethics Committee for Animal Experiments (Comité d'Ethique en Expérimentation Animale du Centre INRA de Jouy-en-Josas et AgroParisTech) and authorized by the French Ministry for Research (authorization no. 2017051200952846). All methods were performed in accordance with the relevant guidelines and regulations. The right jugular vein of 30 to 40 weeks old Melanoma Libechov Minipigs41 conventionally bred from GABI (INRA, Jouy en Josas, France) were catheterized under gaseous anaesthesia in order to reach the right atrium. Before FITC-pseudomonas injection, 30 ml of blood was withdrawn in heparinized tube as negative control. 0.6 to 10 mg of FITC-pseudomonas were resuspended in 5 ml physiological serum and injected in the catheter. After 10 minutes, 30 ml of blood was withdraw in heparinized tube as positive control. The animal was euthanized and the lung was sampled. An extensive BAL was processed using 3 times 100 ml PBS/EDTA. Parenchyma was collected, a 1 cm piece was frozen in Tissue Teck (Sakura, Paris, France) and conserved at −80°C, and the remaining tissue was minced and digested in Collagenase/Dispase/DNase medium, and single cell suspension was retrieved as previously described 4. BAL and parenchymal cell suspensions were then frozen in FCS/10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) before staining and flow cytometry analysis or cell sorting.

Flow cytometry analysis and cell sorting. Pig cell surface staining was performed as previously described4, using anti-MHC-II (clone MSA3) and anti-CD172a (clone 74-22-15a) (monoclonal antibody center Washington State University - Pullmann, WA), unlabelled or Phycocerythrin (PE) coupled anti-CD163 (clone 2A10/11) from Serotec (Oxford, UK) and isotype matched secondary antibodies coupled to PE, Alexa-488, and Alexa-647 (Invitrogen). Infected cells were then fixed in 4% paraformaldehyde before flow cytometry analysis. Samples were acquired on a Fortessa (BD-Bioscience) or sorted on a MoFlo ASTRIOS (Beckman-Coulter). For sorting, preparations were enriched in DC/Macrophages by gradient (Optiprep; Nycomed Pharma). Acquired data were analysed using FlowJo software (version X.0.6).
Microscopy. Sections of 14 µm were obtained from the parenchyma frozen in Tissue-Tek using a cryostat (Leica CM3050S, Nanterre, France) and deposed on Superfrost® glass slides (ThermoFisher scientific). Cryosections were fixed in methanol/acetone (1:1) at −20 °C for 20 min. Fixed slides were saturated using PBS supplemented with 5% horse serum (HS) and 5% swine serum (SS) 30 min at room temperature (RT). The same primary antibodies anti-MHC-II, anti-CD163 as in flow cytometry were used as well as anti-FITC (Fluorescein/Oregon Green Polyclonal Antibody, Alexa Fluor 488, ThermoFisher scientific) and a poly-clonal rabbit anti-ve-Cadherin (H-72, Santa-Cruz Biotechnology, UK), and secondary antibodies were added at 4°C overnight or 30 min, respectively. Slides were analyzed using an LSM510/U700 confocal microscope (Zeiss, LePecq, France).

Figure 5. PIM/AM-like cells produce more cytokines than AM at steady state and upon PRRSV infection in vivo. In vivo infected pigs were euthanized 10 days post-infection, their lung collected and BAL and parenchymal cells extracted, stained, analysed and sorted as above. (a) Transcriptomic expression (RT-qPCR) of different cytokines of the innate and adaptive immune response. For each animal, mock infected AM 2−ΔΔCT mRNA cytokines expression (normalized to the reference gene RPS24) is considered as reference 100, from which PRRSV AM as well as mock and PRRSV AM-like/PIM conditions are normalized. (b) Principal Component Analysis of the cytokines transcriptomic expressions raw data (2−ΔΔCT) for AM and PIM/AM-like cells of the 3 mock infected (left) and 3 PRRSV infected (right) animals depicted in (a). Each symbol represents the projection on the PCA analysis of one of the 6 different animals cell types (AM in red and PIM/AM-like cells in blue).
**Cell infection and viral titration.** Sorted populations were cultured in complete RPMI for 24 h in flat-bottom 96-well plates with $3.10^5$ cells/well and then infected at a MOI of $10^{-1}$ with a PRRSV virus (LENA) in complete RPMI. At 24hpi, supernatants were collected and frozen at $-80^\circ$C. After washing, cells were lysed in 100µl of RNA extraction buffer from the Arcturus PicoPure RNA isolation kit (Life Technologies) for quantification of viral RNA by RT-qPCR. The same protocol was applied with a MOI of 5 for quantification of cytokine production by RT-qPCR. PRRSV titers (TCID50/ml) were assessed according to the OIE manual of diagnostic test.

**RNA extraction.** Total RNA from sorted cells or infected cells was extracted using the Arcturus PicoPure RNA isolation kit according to the manufacturer’s instructions. Contaminating genomic DNA was removed using a Qiagen RNase-free DNase set.

**Real-time quantitative PCR (qPCR).** RNA was reverse transcribed using random hexamers and the Multiscribe reverse transcriptase (Life Technologies) qPCR were performed as previously described. The primers used were: HDAC10 (F: CCGCCGCCGGAATTGG, R: GGGCATGCTTGGCTGCTA), PU1 (F: TCCCCTACGGCATTCA, R: GCGTTTGCCGTTGGAAG), ekB (F: TGGGTCGAGAAGTACATTG, R: ATGCCGGGAGACATTTT), MAPB (F: TGGGTTCTTATAGCAAAATAGTTG, R: CACCAATTACTCAGCCATCA), PRBS (F: ATGGGCACAGCAGCTCACTC, R: GGAACCTGTAGTCGCGTTGGA); TNFα (F: TGGTGGTGGCAGCAGATG, R: CAGCCTTGGCCCCTGAA), IL-6 (F: CTGGCTCTTGAGATGGCTACTG, R: GGATCATCCTTTGGGCTCACCTT), IL-8 (F: TCTGCTTCTTCTGCTGCTTCTC, R: GCCACTGCGTAGCTGCTTGG), RPS24 (Ribosomal Protein S24).

**Cell infection and viral titration.** The primers used were used in vivo infection and tissue collection. For in vivo PRRSV infections, experiments were performed at ANSES (Ploufragan, France). The animal experiment was authorized by the French Ministry for Research (authorization no. 201506011329743) and approved by the national ethics committee (authorization no. 07/07/15). Eight specific pathogen-free (SPF) pigs (free from PRRSV, Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae) were housed in biosecurity level-3 air-filtered animal facilities. Treatments, housing, and husbandry conditions conformed to the European Union Guidelines (Directive 2010/63/EU on the protection of animals used for scientific purposes). At 10 weeks of age, 4 pigs were inoculated intranasally with LENA strain (5.10^5 TCID50/per animal in 2.5 ml per nostril). Ten days post infection, animals were anesthetized (Zoletil, Virbac, France) and exsanguinated. BAL cells were collected using 500 ml PBS/EDTA for right and left diaphragmatic lobes. BAL and parenchymal cells were sampled as above (pig lung cells collection). Cells were washed in PBS/EDTA and frozen in FBS/10% DMSO. A part of the data issued from this in vivo infection has already been published in 29.

**Statistical analysis.** All data were analysed using Graph Pad Prism (version 6) for Mann-Whitney test; R software (version 3.4.0) and package FactoMineR (version 1.39) for Principal Component Analysis method (PCA) and Pearson correlation coefficient determinations. When scatter plots are used, the mean is depicted by a horizontal bar.

**Data availability.** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**
E.B.1., P.M., E.B.2., E.C., P.R. and C.B.C. performed the experiments, C.U. performed the confocal imaging, F.L. brought the skills for PRRS virus manipulations, D.D. brought the skills for *pseudomonas* manipulation, L.J., M.T. and E.B.1. performed the statistical analysis, I.S.C. provided financial support, thorough discussions and critical manuscript reading, J.J.L. and O.B. supervised the *in vivo* experiments, N.B. provided financial support, supervised the working program, performed experiments and wrote the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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