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Comparative Analysis of Monocyte Subsets in the Pig

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Human and mouse monocyte can be divided into two different subpopulations based on surface marker expression: CD14/16 and Ly6C/CX3CR1, respectively. Monocyte subpopulations in the pig were identified based on reciprocal expression of CD14 and the scavenger receptor CD163. The two populations, CD14hi-CD163low and CD14low-CD163hi, show approximately equal abundance in the steady-state. Culture of pig PBMCs in CSF1 indicates that the two populations are a maturation series controlled by this growth factor. Gene expression in pig monocyte subpopulations was profiled using the newly developed and annotated pig whole genome snowball microarray. Previous studies have suggested a functional equivalence between human and mouse subsets, but certain genes such as CD36, CLEC4E, or TREM-1 showed human-specific expression. The same genes were expressed selectively in pig monocyte subsets. However, the profiles suggest that the pig CD14low-CD163high cells are actually equivalent to intermediate human monocytes, and there is no CD14+ CD16+ "nonclassical" population. The results are discussed in terms of the relevance of the pig as a model for understanding human monocyte function. The Journal of Immunology, 2013, 190: 6389–6396.

The domestic pig has some advantages over commonly used rodent models for the study of innate immunity (1). Whereas there are major differences between humans and mice in terms of the patterns of pathogen-induced gene expression in macrophages, pigs share a much greater proportion of inducible genes with humans, corresponding to greater promoter sequence conservation (2, 3). The precursors of macrophages in the blood, the blood monocytes, are heterogeneous in terms of surface markers and gene expression. Monocytes in human and mouse have been divided into two populations, based on expression of CD14 and CD16 in humans and Ly6C and CX3CR1 in mice. In both species, "intermediate" monocyte populations between the two extremes have also been described (4, 5). Only a few markers appear specific for intermediate monocytes (e.g., GFRα2, CLEC10A), and these were expressed at low levels, making isolation difficult (5). A second FcR, CD64, has also been used to further subdivide human CD16+ monocytes (6), whereas an Ly6C intermediate population of mouse monocytes has been reported (7). A consortium of investigators has proposed that the CD14hi population, which is more numerous in humans, be referred to as "classical" monocytes, with the minor CD16+ expression being "nonclassical," and monocytes that express both CD14 and CD16 be termed intermediate (5, 8). The intermediate monocytes have been ascribed specific functional roles in disease (9). Studies in mice and humans suggest that these extremes actually represent a continuum of differentiation (5) regulated by the growth factor, CSF1 (10). A recent study suggested that the transcription factor NR4A1 (nur77) is required for generation of the ly6C− (nonclassical) monocyte in mice (11).

The proposed functional equivalence of the human CD16+ and mouse Ly6C− cells (nonclassical) is mainly supported by the differential expression of a few key markers (12–14) and more recently by gene expression profiling (4, 15). Several separate studies have compared array profiles of human monocyte subsets (5, 15–17). They found broad similarities between genes preferentially expressed by classical CD14hi monocytes, but differed somewhat in the profiles attributed to the nonclassical cells, due in part to differences in definition of the intermediate phenotype.

The markers that distinguish mouse and human monocyte subsets cannot readily be applied to the pig. The main focus in the pig has been on a distinct marker, CD163 (18), because of the role of CD163 in cellular entry into macrophages of the important porcine pathogen porcine reproductive and respiratory syndrome virus (19). The expression of this marker correlated with differential expression of the mouse/human markers CCR2 and CX3CR1 at the mRNA level (20). There has been no previous analysis of the gene expression profiles of pig monocyte heterogeneity. In this study, we use a new microarray platform to characterize two major populations of monocytes in pig peripheral blood distinguished by their expression of CD163.

Materials and Methods

Animals and cells

Monocytes were isolated from at least 2 animals from each of the following breeds: Landrace, Large White, Duroc, Pietrain, Hampshire, and Large White Landrace F1 cross. Large White Landrace cross piglets were used for the newborn/day 8/day 21 data. In addition, monocytes were isolated from a Large White Landrace F1 crossed with a Pietrain and used for RNA analysis. All pigs were sourced from local livestock farmers and were kept...
in our facilities for at least 1 wk. There was no difference in diet and management between all pigs, and no sign of any infection was found on the animals. The pigs were sedated with ketamine and euthanized using a captive bolt. Blood was collected by intracardiac puncture into a vacuum-sealed glass bottle containing 100 ml acid citrate dextrose buffer. Whole blood was separated into ten 50-ml Falcon tubes (Greiner Bio one) that were

**FIGURE 1.** Cytometry characterization of freshly harvested PBMCs. Freshly harvested PBMCs from young male Large White × Landrace (A–C) and from a young male Pietrain (D–F) were stained with Abs CD14-FITC and either CD16-PE (A–D), CD163-PE (B–E), or CD172a-PE (C–F). No specific staining was shown when using their respective isotype control. Figure is representative of three different experiments. Other breeds are shown in the supplemental figures.

**FIGURE 2.** Effects of CSF1 on differentiation of PBMCs. Freshly harvested PBMCs from young male Large White × Landrace pigs (A–E) were cultured in complete medium in presence of rhCSF1 (10e4 U/ml) and harvested after 3 (F–J) and 7 d (K–O). Cells were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control (in gray line) or Abs CD14-FITC (B, G, L), CD16-PE (C, H, M), CD163-PE (D, I, N), or CD172a-PE (E, J, O) in colored line. Figure is representative of three different experiments. A total of 15,000 events were collected.
centrifuged at 1200 × g for 15 min with no brake. The buffy coat was
removed, and 25 ml was mixed with 25 ml RPMI (Sigma). Thirty milli-
liters of the buffy coat/RPMI mix was layered on top of 15 ml endotoxin-
free lymphoprep (endotoxin: <1.0 EU/ml; Axis-Shield) and centrifuged
at 1200 × g for 25 min with no brake. PBMCs separate out into a distinct
layer that can be easily removed. This was washed twice with RPMI 1640
and centrifuged at 600 × g for 10 min, then 400 × g for 10 min. Red cells
were lysed using 5 ml erythrocyte lysis buffer (10 mM KHCO 3, 155 mM
NaCl, 0.1% EDTA, sterile 0.2 μm filtered) for 5 min; then the cells were
washed and centrifuged at 400 × g for 10 min. The pellet was collected,
resuspended in PBS (Mg 2+ and Ca 2+ free), and counted before slow
frozen at −80°C in an isopropanol bath and then moved to −155°C
for long-term storage. The cells were recovered from cryopreservation as
previously described (2). In short, cells were recovered from cryopreservation as
quickly thawed at 37°C and quickly thawed at 37°C, the cells in freezing medium were then slowly
diluted by dropwise addition of 40 ml warm PBS over 2–3 min. For the
maturation experiments, PBMCs were cultured as previously described for
the bone marrow cells (2). They were seeded in large 100-mm 2 sterile petri
dishes in 20 ml complete medium: RPMI 1640, Glutamax supplement
(35050-61; Invitrogen), 10% heat-inactivated FCS (PAA Laboratories),
penicillin/streptomycin (15140; Invitrogen, Paisley, U.K.) in the presence
of NH4Cl, 0.1M EDTA, sterile 0.2

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Effects of CSF1 on expression of growth and marker expression

CSF1 has been implicated in monocyte maturation in both mouse
and human. We therefore investigated the effect of this factor on pig
monocytes in culture. rhCSF1 caused pig PBMCs to increase in
size and granularity (Fig. 2A, 2F, 2K) and to increase in auto-
fluorescence (Fig. 2L–O) compared with freshly isolated PBMCs
and Duroc) are shown in Supplemental Fig. 1. Other breeds (Large
White Landrace, Hampshire, and Duroc) are shown in Supplemental Fig. 1.

Results

Identification of monocyte subpopulations in the pig

Previous studies have indicated that there is significant interbreed
variation in monocyte function in pigs that could be important
in innate immunity (22). Accordingly, we sampled PBMCs from at
least two individual animals from six different breeds. We initially
examined CD14 and CD16 expression, although unlike humans,
the population expressing different levels of CD14 and CD16. By
contrast with humans, where the CD16+ population is a minor
subpopulation, these cells in pigs represented around half of the
CD14+ monocytes.

In each of the breeds, there was also variable expression of
CD163 on the CD14+ monocytes. In cross-bred animals (Large
White × Landrace), CD163 expression divided the monocyte pool
clearly in two with CD163, like CD16, being inversely related to
the level of CD14 (Fig. 1B). In others, for example, the Pietrain
(Fig. 1E), there was no clear separation of a CD14hi-CD163lo
population. CD14+ monocytes in all breeds were also uniformly
strongly positive for the CD172a marker, also known as SIRP α or
the macrophage fusion receptor, and there was also no obvious
variation in the expression of CD172a among the breeds or indi-
vidual animals. Other breeds (Large White, Landrace, Hampshire,
and Duroc) are shown in Supplemental Fig. 1.
showed increased expression of CD16 at day 7 (Fig. 2C, 2H, 2M), but surprisingly, CD163 was undetectable (Fig. 2D, 2I, 2N).

**Microarray analysis of porcine monocyte subsets**

To examine the gene expression profile of the monocyte subsets, we sorted by flow cytometry PBMCs from three individual Large White Landrace F1 pigs. The monocyte population was gated based on size and granularity (Fig. 3A), and dead cells were excluded using Sytox blue dye (Fig. 3B). Cells were stained with CD14 and CD163 Abs (Fig. 3D) or their corresponding isotype (Fig. 3D) and sorted into CD14hiCD163lo and CD14loCD163hi populations. RNA was extracted and analyzed using a newly developed and annotated pig whole-genome microarray (21). Statistical analysis of the microarray showed 4239 probe sets of 47,846 were differentially expressed between the CD14hiCD163lo and CD14loCD163hi populations. Probes were sorted and only those that were differentially expressed by \( \geq 1.5 \)- or \( \leq 0.67 \)-fold change between the monocyte subsets were included. These data were analyzed using Biolayout Express3D with a Markov cluster (MCL) of 1.7, \( R = 0.95 \), and smallest cluster = 3 (25). This generated 69 clusters that were grouped into two distinct shapes, one of which was composed of clusters of probes that were expressed more highly in CD14hiCD163lo monocytes and 1 composed of clusters that were more highly expressed in CD14loCD163hi monocytes (Fig. 4). The expression profiles of CD163, CD16, and CD14 shown in Fig. 4 confirmed the successful separation based on the markers and the differential expression of CD16 seen previously in Fig. 1. The top list of most differentially expressed genes is summarized in Fig. 5. A total of 48 genes had a fold change \( > 3 \) or \( < -3 \). Genes such as KMO or CLEC7A were more expressed in the CD14hiCD163lo subset, whereas IL-8, CXCXR2, or IL-18 was greater in CD14loCD163hi monocytes. A total of 2082 genes were relatively more highly expressed in CD163hi monocytes. Analysis of these genes for Gene Ontology terms using DAVID (26) showed enrichment for mitochondrial components, translation, cellular metabolic processes, intracellular protein transport, localization and binding, nuclear parts, cytoplasmic membrane-bound vesicles, and RNA processing (data not shown). Conversely, 1485 genes were expressed more highly in CD14hiCD163hi monocytes. Analysis of this list with DAVID showed enrichment for genes involved in leukocyte activation, differentiation, and inflammatory response.

**Comparison of monocyte subsets in pig, mouse, and human**

We compared the pattern of differential expression with the published human and mouse data from Ingersoll et al. (15) (Fig. 6). A total of 132 genes were differentially expressed in the same subsets in both human and mice monocytes, and 33 genes were differentially expressed but in a converse pattern between the 2 species. Among the genes more highly expressed on CD14hi populations of pig, in common with both human and mouse, were CD14, S100 calcium binding protein A8 (S100A8), C-C chemokine receptor type 1 (CCR1), IL18, lamin-B receptor (LBR), and CSF3R. Higher relative expression of JAG1, TREM1, SGK1, PDE4B, SERBINB2, CLEC4E, and CD36 was conserved in hu-

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**FIGURE 4.** Microarray data clustered into two distinct groups containing genes that were more highly expressed by CD163hi or CD163lo monocytes. Three-dimensional visualization of a Pearson correlation (\( R = 0.95 \)) from the analysis of the microarrays. Each sphere represents an individual probe set. Clustering of the graph, using the MCL algorithm (MCL = 1.7), gave a total of 69 clusters. These clusters were grouped into two distinct shapes, one of which was composed of clusters of probes that were expressed more highly in CD14hiCD163lo monocytes (A) and one composed of clusters that were more highly expressed in CD14loCD163hi monocytes (B). Expression of the surface markers CD163 (C), CD14 (D), and CD16 (E) is shown to verify successful sorting of monocytes.
man and pig, but not mouse. CD14<sup>lo</sup> pig monocytes shared higher relative expression of CD16, ITGAL, and LTB with the corresponding mouse and human cells. Human and pig CD14<sup>lo</sup> monocytes shared high expression of IFN-induced proteins with tetratricopeptide repeats 2 and 3 (IFIT2, IFIT3) and the scavenger receptor MSR1. Ingersoll et al. (15) noted a PPARγ signature in mouse Ly6C<sup>lo</sup> monocytes, but like human monocytes, pig monocytes expressed very little PPARG.

**Discussion**

This study has demonstrated that there is a significant set of genes that is coregulated with CD14 and CD163 in pig peripheral blood monocytes. The differential expression of CD16, inversely with CD14, as was conserved in pigs (15). In keeping with earlier studies, there were no major differences among breeds (27).

All monocytes in the pig, defined by expression of CD172a, were also clearly strongly positive for both CD14 and CD163; the differences in expression that define subpopulations are relative and small. As in mice, there is no population that has almost undetectable CD14, hence no equivalent of the human CD16<sup>+</sup> monocyte. Because these cells are only 10% of monocytes in humans, it may be that if they exist in pigs or mice, they actually have a shorter half-life and leave the circulation. Alternatively, because they are involved in patrolling behavior in humans, they could be in a marginal pool and not harvested when pigs are exsanguinated (4). Correspondingly, there is no way to segregate an “intermediate” monocyte population in the pig without a completely arbitrary gate setting. Nevertheless, CD14 did vary across a significant range in pig monocytes, and CD163 was detected at the highest levels on CD14<sup>hi</sup> monocytes. In humans, CD163 was reported to be more highly expressed in the CD14<sup>hi</sup>CD16<sup>hi</sup> nonclassical monocyte subset in human blood and inducible in the CD14<sup>hi</sup> monocytes during CSF1-induced differentiation in vitro (28). Conversely, later studies (4, 15) indicated quite the reverse, and expression can be induced in human CD16<sup>+</sup> monocytes by CSF1 (29). We have confirmed this finding independently at the mRNA level; CD14<sup>+</sup>, CD16<sup>+</sup> human monocytes have almost undetectable CD163 mRNA (J. K. Baillie and D. A. Hume, unpublished observations). CSF1 is present in the circulation and is important for macrophage differentiation, growth, and survival (30).

With the newly created and annotated pig cDNA “snowball” microarray, we examined the expression profile of the two monocyte populations. Broadly speaking, pig CD14<sup>hi</sup> monocytes resemble human CD14<sup>hi</sup> monocytes. As noted previously, and in common with human CD14<sup>hi</sup> monocytes, they express higher levels of the chemokine receptor CCR2 and are likely recruited to inflammatory sites by CCL2 (20). The pig CD14<sup>hi</sup> (CD163<sup>lo</sup>) monocytes express between 6- and 12-fold higher levels of the chemokine IL-8 and its receptors (IL8RB, IL8R2, CXCR2) than CD14<sup>lo</sup> monocytes, suggesting that they may have an important role in recruiting neutrophils to sites of inflammation or infection. Selective expression of G-CSF (CSF3) receptor was also shared by pig and human CD14<sup>hi</sup> monocytes. CSF3 controls the production, differentiation, and function of granulocytes (34), and has also been described as a maturation factor for monocytes (35, 36).

Production of CD14<sup>lo</sup> monocyte subset in the bone marrow may be coordinated with granulocytes (15, 37). CD14<sup>lo</sup> monocytes in both pig and human share with granulocytes the high expression of S100A8 (5). S100A8 has also been implicated in inflammation, and excessively high levels have been associated with recurrent infections (38, 39).

Because there is no obvious separation of intermediate monocytes, the functional equivalent of the CD16<sup>+</sup> human monocyte in pigs (and mice) is less obvious. In humans and pigs, CD36 was
more highly expressed by CD14^{hi} monocytes, whereas MSR1 was expressed by CD14^{lo} monocytes. The lysosomal asparaginyl endopeptidase legumain (LGMN) showed the most selective expression in CD14^{lo} pig monocytes. LGMN can be found in lysosomes and degrades antigenic proteins for presentation to CD4^{+} cells, so it is responsible for a key step in Ag presentation (40, 41). CD14^{lo}, CD16^{hi} monocytes also expressed the highest levels of the swine leukocyte Ag (SLA) class II genes (SLA-DRB1, SLA-DQA, SLA-DQB1, SLA-DRA, SLA-DRB1, SLA-DRB2), suggesting a role in Ag processing and presentation (42). A similar role in MHC processing has been described for human “intermediate” CD14^{+} CD16^{+} monocytes (5). The DAVID analysis suggests that, like human CD16^{+} monocytes, they have greater respiratory rates. Previous studies indicate that CD16^{+} cells share with CD16^{+} human monocytes an increased potential for inducible expression of proinflammatory genes including TNFa (42, 43).

Overall, the data suggest that the human and pig CD14^{hi} populations (or more accurately, the set of genes with expression

FIGURE 6. Comparison of monocytes subsets in pig, mouse, and human. Data from Ancuta et al. (16), Ingersoll et al. (15) and Zhao et al. (17) were reanalyzed to find genes that were preferentially expressed in the same pattern in human monocyte subsets in all studies.
correlated positively with CD14) are similar, and rather more different from the mouse. This view is in keeping with our recent studies of LPS-inducible genes in macrophages from the three species, where pig is much more closely related to human (1–3). These monocytes have also been referred to as “inflammatory” on the basis of their selective recruitment into inflammatory sites, and by contrast with the “resident” fate of the CD14+ monocytes (12, 44). That view is consistent with their preferential expression of inflammatory genes in the pig. On the basis of shared gene expression profiles, the CD14++, CD163+ pig monocytes resemble “intermediate” monocytes. Interestingly, and in keeping with the likely role of CSF1 in the maturation of monocytes in humans and mice (10), we were able to generate CD163+ monocytes after culturing the CD14+CD16++ subset, from PBMCs before cell culture did not affect the generation of these studies, CSF1 was found to promote the rapid appearance of the breed of pig, but based on the profiles in Fig. 1 and Supplemental Fig. 1, it would be difficult to identify a genuinely CD163+ population in any breed. Purification protocols used by others must somehow remove the CD163+ cells. Accordingly, it is unlikely that CD163+ is the only variable that controls virus susceptibility in pig monocytes. The >2000 genes that appear coregulated with CD163 are likely to contribute, alongside the >1400 that are downregulated.

The deficiencies of the mouse as a model for human macrophage biology (3) have been reinforced by a recent comparative analysis of gene expression profiles in mouse inflammatory models and the corresponding human diseases (48). Our analysis of pig monocyte subsets confirms our earlier data (2) in that pigs are significantly more human-like. Nevertheless, the use of the pigs as disease models (1) must also take account of the differences between the species.

Disclosures

The authors have no financial conflicts of interest.

References


