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Pig Skin Includes Dendritic Cell Subsets Transcriptomically Related to Human CD1a and CD14 Dendritic Cells Presenting Different Migrating Behaviors and T Cell Activation Capacities

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Swine skin is one of the best structural models for human skin, widely used to probe drug transcutaneous passage and to test new skin vaccination devices. However, little is known about its composition in immune cells, and among them dendritic cells (DC), that are essential in the initiation of the immune response. After a first seminal work describing four different DC subpopulations in pig skin, we hereafter deepen the characterization of these cells, showing the similarities between swine DC subsets and their human counterparts. Using comparative transcriptomic study, classical phenotyping as well as in vivo and in vitro functional studies, we show that swine CD163pos dermal DC (DDC) are transcriptomically similar to the human CD14pos DDC. CD163pos DDC are recruited in inflamed skin, they migrate in inflamed lymph but they are not attracted toward CCL21, and they modestly activate allogeneic CD8 T cells. We also show that CD163low DDC are transcriptomically similar to the human CD1a pos DDC. CD163low DDC migrate toward CCL21, they activate CD8 T cells and, like their potential human lung counterpart, they skew CD4 T cells toward a Th17 profile. We thus conclude that swine skin is a relevant model for human skin vaccination. The Journal of Immunology, 2014, 193: 5883–5893.

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side in the epidermis, originate from a local precursor, and have been controversially described as inducing either tolerance or CD8 T cell responses (16–18). A monocytodeviated DC (moDC) population called inflammatory DC (inDC) that differentiate from monocytes upon inflammation has also been described in different mouse tissues, and is characterized by the expression of DCSign (CD209), mannose receptor (MR/CD206), CD14, CD64, and FcεRI (19–22). These cells traffic in the lymph node (LN) in a CCR7- and CCR2-dependent manner; however, their abilities to present Ags remain controversial (19, 22–25).

Human skin DC classification has been revisited recently (26, 27) leading to the distinction of four different populations. Two conventional DDC have been described: the DC1c/BDCA1 DDC and the BDCA3 DDC, related to the BDCA1 and BDCA3 blood DC (28) and phenotypically and functionally equivalent respectively to the mouse CD11b and CD103 DDC (26). Formerly, a CD1a DDC population, distinct from LC, was acknowledged (29). CD1a DDC and BDCA1 DDC express a similar phenotype (CD11chigh/BDCA1pos/CD14pos/CD163neg/CD172aneg) (26, 29) and are considered by some authors as the very same population (30). Thus, CD1a and BDCA1 DDC represent, at least, overlapping CD172apos conventional DDC populations which, to allow the hereafter transspecies comparisons will be considered as bona fide equivalent. The third human skin DDC population is the LC, present in the epidermis, which strongly activates the proliferation and the cytotoxicity of CD8 T cells in vitro (29), whereas the fourth is the CD14 DDC, which expresses monocytic markers, is specialized in the induction of Ab-secreting B cells (29) and appears related to monocytoc-derived macrophages (27).

In a previous work (31), we proposed to segregate swine skin DC in different subpopulations, namely the LC, present in the epidermis, the CD172a low/DCD, resembling CD103pos murine DDC, and two other DDC populations called CD163pos (formerly named CD163high) and CD163low DDC that we had not been able to assign to their human or murine counterparts. In this study we refine the characterization of these cells through complementary methods such as multiparameter flow cytometry, quantitative RT-PCR (qRT-PCR) and comparative transcriptomics with murine mouse tissues, and is characterized by the expression of DCSign (CD209), mannose receptor (MR/CD206), CD14, CD64, and FcεRI (19–22). These cells traffic in the lymph node (LN) in a CCR7- and CCR2-dependent manner; however, their abilities to present Ags remain controversial (19, 22–25).

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Materials and Methods

Abs

The following Abs were used: anti-swine CD1 (76-7-4), CD172a (74-22-15, 74-22-15a), MHC class II (MHC-II) (Th2la, MSA3), IgM (PG145A), and TCRγδ (PGBL22A and 86D) from mAb Center Washington State University (Pullman, WA); anti-human CD21 (B-ly4) and PE-coupled CD14 (UTK4) from BD Pharmingen; anti-swine CD16 (G7), IgL (K139-3E1), and unlabeled or PE-coupled anti-swine CD163 (2A10/11) from AbD Serotec (Oxford, U.K.); and anti-human CD206 (122D2.08) from Dendritics (Lyon, France). The anti-swine CD209/DC-Sign is a gift from X.J. Meng (College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA) (32); matched isotype controls for mouse IgG1, IgG2b, and IgG2a were purchased from Invitrogen (Saint Aubin, France). Isotype-specific secondary reagents coupled to Alexa 488, PE or Tricolor, Alexa 647 were from Invitrogen.

Skin DC collection and purification

Biopsy punches were obtained from betadine disinfected large skin samples from either specific pathogen-free (SPF) Large White pigs from Anses Ploufragan or conventional Large White pigs from Institut National de la Recherche Agronomique (Nouzilly, France) and conventional miniature pigs from Institut National de la Recherche Agronomique (Jouy-en-Josas, France). Skin biopsies were sampled using 8-mm punches (Stiefel, Wächtersbacher, Germany) and incubated overnight at 4°C in complete RPMI 1640 medium plus 2 mg/ml Dispase (Invitrogen, Cergy Pontoise, France). The next day, biopsies, still in dispace, were incubated 2 h at 37°C allowing the separation of dermis and epidermis with tweezers. Epidermis and dermis were then incubated separately overnight at 37°C in complete RPMI 1640 medium. Collagendase D (Roche, Meylan, France) was added at 1 mg/ml on dermis cultures to extract dermal DC independently of their migration capacities. All the incubations were processed in non-culture-treated petri dishes to avoid plastic adherence of macrophages and DC. Biopsies were filtered on 80 μm nylon filters, and cells were then treated petri dishes to avoid plastic adherence of macrophages and DC. Biopsies were filtered on 80 μm nylon filters, and cells were then processed for FACS sorting. In vivo triggered skin inflammation on SPF pigs were performed in the Anses facilities (Ploufragan, France) in accordance with the animal welfare experimentation recommendations drawn up by the Directions Departementales de la Protection des Populations des Côtes d’Armor (Eniva/l’Université Paris-Est Créteil (UPEC). Briefly, acetone/ dibutylylphosphate (v/v) was epicutaneously applied on the leg of the animal. Animals were sacrificed at different time points, and their skin was sampled and processed as above.

FACS staining and DC sorting

Extracted skin cells were incubated in blocking buffer consisting in PBS, 2 mM EDTA plus 5% horse serum and 5% swine serum before proceeding to Abs staining. Primary Abs were incubated at 1/50 to 1/500 depending of the Ab. Secondary Abs were incubated at 1/200.

Cells were analyzed on a FACS Calibur (BD Pharmingen). At least 10⁶ events per DC subpopulations were acquired. For DC sorting, 800 punch skin biopsies from conventional Large White pigs (Institut National de la Recherche Agronomique) were digested as described above, 200–400 μm dermal cells and 30–60 μm epidermal cells were retrieved and FACS stained for CD172a, CD163, MHC-II, and DAPI Live, DAPI-negative, MHC-IIhigh/CD172apos/CD163high macrophages, MHC-IIhigh/CD172apos/CD163pos DC, MHC-IIhigh/CD172apos/CD163low DC, MHC-IIhigh/CD172apos/CD163neg DC from the dermis and MHC-IIhigh/CD172apos/CD163neg LC from the epidermis were cell sorted on a MoFlo ASTROs (Beckman Coulter) from the Imagif facility (Gif-sur-Yvette, France).

RNA extraction, microarray workflow, and analysis

Total RNA from conventional Large White pig cell–sorted subsets were extracted using the Arctopus PicPure RNA Isolation Kit (Arctopus Life Technologies) and checked for quality with an Agilent 2100 Bioanalyzer using RNA 6000 Nano or Pico Kits (Agilent Technologies). All RNA samples had a RNA integrity number > 8.5. When insufficient total RNA amounts for the downstream analysis steps were obtained (<50 ng), the RNAs from the sorted cell subsets of distinct animals were pooled. RNA amplification and labeling were performed using the One-Color Low Input Quick Amp Labeling kit (Agilent Technologies) following the manufacturer’s recommendations. First, 50 ng of each RNA sample were amplified and cyanin 3 labeled. The labeled cRNAs thus obtained were subsequently purified using RNeasy Mini Spin columns (Qiagen) and run onto a Bioanalyzer 2100 using RNA 6000 Nano chips (Agilent Technologies) to assess their profiles. cRNA yields and specific activities were measured using a NanoDrop ND-1000 (ThermoScientific).

Then, the cRNAs (600 ng) were fragmented and used for hybridization on custom-designed Agilient Gene Expression 4 × 44K porcinearrays (design ID: 039385). This design was based on the commercial porcine microarray (design ID: Polymers of 600 probes of ID) was replaced, based on quality analyses made by the Sgreganont program from the Sigenae group (33). New probes were designed from porcine expressed tags with the e-array software from Agilent Technologies. In addition, 214 pig probes corresponding to porcine orthologs of human or mouse transcripts expressed in DC (34) were added. For seven human DC transcripts, only bovine expressed sequence tags were available and were selected and submitted to the e-array probe design. For OAS3 and FPR2, human transcripts were used for the e-array probe design because expressed sequence tags from closer species were not available. After hybridization of the cRNAs on the custom-designed array, the chips were washed according to the manufacturer’s protocol.

Eventually, the slides were scanned by using a G2565CA Scanner System (Agilent Technologies) at the resolution of 5 μm and with a dynamic range of 8 bit. The resulting .tiff images were processed using the Feature Extraction Software version 10.7.3.1 (Agilent Technologies). All the protocols used can be obtained by contacting the CRB GADIE facility (http://crb-gadie.inra.fr/).
Raw data were background corrected using the "normexp" method and quantile normalized with the Limma package through Bioconductor in the R statistical environment (version 2.15.0). Quality control of the expression data were assessed by boxplots of raw expression data, density plots of normalized data, scatter plots and calculation of the Pearson's correlation coefficients between arrays, using the Ringo package. The microarray data have been assigned the Gene Expression Omnibus number GSE55300 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55300), and they are freely available in Gene Expression Omnibus (GEO).

The gene set enrichment analysis (GSEA) method from the Massachussetts Institute of Technology (http://www.broad.mit.edu/gsea [Cambridge, MA]) was used to statistically test whether a set of genes of interest (mouse and human cell–specific signatures) was distributed randomly in a larger list of genes sorted according to their relative expression values in a pairwise comparison of transcriptomic data between two porcine cell populations.

**Generation of murine and human cell specific signatures**

In our study, the mouse and human cell–specific signatures correspond to genes that were more highly expressed (>$1.5$-fold) in the cell population of interest (Supplemental Table I) as compared with all other cell populations considered in the preselected compendium of arrays (see list of public database array IDs taken into account in the human and mouse compendia). The cutoff of $1.5$-fold has been applied using a "min/max" procedure calculating fold change (minimum expression among all replicates for all cell types selected/maximum expression among all other replicates for all other cell types) as published previously (34).

To perform GSEA on porcine expression data using transcriptomic signatures composed of human or mouse genes, we used the annotations provided by the Sigene pipeline on the orthologs of the swine genes in human or mouse.

**qRT-PCR**

Skin cells from conventional Large white pigs were sorted and total RNA was extracted as above. To exclude DNA contamination, RNA samples were treated with RNase-free DNase (Qiagen). cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was done using 5 ng cDNA with 300 nM primers in a final reaction mixture volume of 25 μl 1× SYBR green PCR Mastermix (Applied Biosystems) using an Eppendorf Mastercycler ep realplex system (Eppendorf). The primers used were GAPDH (forward, 5'-CACACTTCTCCAGGAGCA-G3'-, and reverse, 5'-CCGACTCACCACCCATGT-3'), CPS2 (forward, 5'-AGGGAACCAAGACAAGAATAA-3', and reverse, 5'-TTTGGCC-AGCAACGACATGC-3'), CTNNB1 (forward, 5'-TTGTTAGCAAGCTGAGACGCT-3', and reverse, 5'-CTTCCAGTGAGAATTTAAAGA-3'), and reverse, 5'-GGAGGAGACG- CCTGACTTT-3'). RT values for each amplification were normalized against a housekeeping gene (GAPDH) using the 2^{(ΔCt)} formula (2^(-ΔΔCt) = 2^(CtGAPDH-CtTarget)).

Quantitative analysis was performed using the Realplex software (Eppendorf). The mRNA levels relative to the amount of GAPDH were calculated by analyzing the cycle threshold (Ct) values for each amplification curve, and the arbitrary units were established by the equation $2^{(-ΔΔCt)} = 2^{(CtGAPDH-CtTarget)}$.

**Pseudo afferent lumbar lymph duct cannulation in mini-pigs**

Lymph duct catheterization was carried out under licenses from the Direction of the Veterinary Services of Versailles (accreditation numbers A78-93, A78-15, and A78-730) and was approved by the Regional Paris South Ethics committee (number 08-001). Pseudo afferent lumbar lymph duct catheterism in miniature pigs was performed as described previously (35). Briefly, first, LN draining the flank, hindquarters, and inguinal areas were surgically resected. Two months later, after lymph vessel healing, a retroperitoneal surgery was performed for inserting a silicone catheter (4 french; Nutricia bSQ, Vygon, Ecouen, France) in the lumbar trunk. The catheter was led out through a skin opening into a flask containing 500 U heparin. To facilitate lymph draining, pigs were left free in 2-m² cages. Pigs received one i.m. injection of terramycin (20 mg/kg; Pfizer, Paris, France) and of low m.w. fluixin (1 mg/kg Finadyne; Schering-Plough, Levallois Perret, France). Heparin (exonaparin, Lovenox, Sanofi-Aventis, Paris, France) was injected intradermally every 12 h (1000 IU anti-Xa) to prevent cloting.

Lymph was collected twice a day. Cells were pelleted and step-frozen in FCS containing 10% DMSO. Supernatant (lymph) was frozen at −20°C.

**CCL21 cloning and production**

The full coding sequence of CCL21 was amplified from porcine macrophage RNA by RT-PCR using specific primers (CCL21, forward, 5'-TCACTC AGGCTTCTCTGC-3', reverse, 5'-CATGGCCAGTG- ACCAGC-3'). After agarose-gel electrophoresis purification, a 432-bp cDNA was cloned into the pcDNA3.1/CT-GFP-TOPO (Invitrogen, San Diego, CA). Integrity, fidelity, and orientation of insert were confirmed by sequencing. The clone CCL21 was submitted to European Molecular Biology Laboratory database and assigned accession number AJS5194 under http://www.ebi.ac.uk/ena.

CHO cells were transfected with plasmid coding pcCCL21-GFP, by using the Lipofectamine Plus Reagent (Invitrogen), and clones stably expressing CCL21 were isolated by limiting dilution after genetin selection (G-418; Life Technologies, Grand Island, NY) at a concentration of 800 μg/ml. Isolated clones were expanded and the expression of the fusion protein was assessed by direct flow cytometry detection of GFP and Western blotting using a biotin-conjugated goat anti-GFP polyclonal Ab (Rockland Immunochemicals, Gilbertsville, PA) and streptavidin-HRP (Thermo Fisher Scientific, Rockford, IL).

For protein production cells were grown in DMEM (BioWhittaker, Waltham, MA) supplemented with 5% FCS, 2 μg/ml t-glutamine, and 50 μg/ml gentamicin. When cells reached 80% confluence, the culture medium was replaced by medium without serum, and the supernatant was harvested 3–4 d later, aliquoted, and kept frozen at −20°C until use.

**In vitro migration**

For in vitro migration, 24 biopsy punches were deposited either on complete RPMI 1640 medium plus 1 mg/ml collagenase, or on pure CCL21 supernatant or mock supernatant or on pure lymphatic liquid from day 3/4 postimplantation (inflamed lymph) supplemented with 5 μg/ml LPS (Sigma-Aldrich) or on pure lymphatic liquid from more than 10 d post-surgery (steady-state lymph). Cells were left to crawl-out for 1 d, before removing the biopsies. Migrating cells were counted and stained for MHC-II/CD172a/CD163, before FACS determination of the percentage of each DC subpopulations. Absolute numbers of each migrating DC subpopulations were obtained by multiplying the percentage of each subpopulation by the absolute number of cells in the sample.

**Mixed lymphocytes reaction**

For the allogeneic global T cells stimulation experiments, PBMC from SPF pigs (Anses Ploufargan) were CSFE stained as described previously (36). Briefly, 5×10^6 cells were resuspended in RPMI 1640 medium containing 1 μM CSFE, and incubated 10 min at 37°C. Then PBMC were washed and 3×10^5 cells/ml were mixed in complete RPMI 1640/10% FCS, 10% γ-irradiated allogeneic naive CD8 T cells were added at a 1:10 ratio. Twenty-four hours later, cells were lysed, and RNA was extracted. qRT-PCR were then performed as stated above, on genes specific of the different Th differentiation pathways, namely IFN-γ for the Th1, IL-4 for the Th2, IL-17 for the Th17.
IL-10 for the regulatory T cells, and CXCL13 for the Tfh. DC without naive T cells were also processed to control for the T cells specificity of the cytokine signals.

Results

Identification of skin DC subsets

Swine skin DC from conventional Large White pigs were segregated into four different subpopulations according to our previous work (31), namely the dermal CD172a<sup>−</sup> DC, CD163<sup>−</sup> DC and CD163<sup>+</sup> DC and the epidermal LC. Like for mouse cross-priming CD103<sup>+</sup> DDC, swine CD172a<sup>−</sup> DDC specifically presented a high turnover, phagocytized apoptotic bodies in vivo and expressed Cadm1 (31). To ensure that our gating strategy did not result in cross-contamination between swine DC subsets, we next purified them and examined their expression of lineage-specific genes by qRT-PCR. Swine CD172a<sup>−</sup> DDC specifically expressed the mRNA encoding XCR1 (Supplemental Fig. 1A), a chemokine receptor recently identified as the best cross-species marker of CD8<sup>a</sup>-like cross-priming DC (37–40). Langerin mRNA was specifically expressed in swine epidermal LC (Supplemental Fig. 1A). Hence, we confirmed that swine CD172a<sup>−</sup> DDC are similar to mouse CD8<sup>a</sup>-like CD103<sup>+</sup> DDC. Moreover, the low levels of XCR1 or Langerin signals in the other populations than CD172a<sup>−</sup> and LC, respectively, showed the absence of contamination of CD163<sup>−</sup> and CD163<sup>+</sup> DC by CD172a<sup>−</sup> DDC or LC.

Skin DC transcriptomic profiles

To better characterize the CD163<sup>−</sup> and CD163<sup>+</sup> DDC, we next analyzed their transcriptomic profiles together with the one of epidermal LC, blood monocytes, and B lymphocytes. By performing pairwise comparison of swine immune cell types, we examined the distribution bias of transcriptomic signatures specific for putatively equivalent mouse or human immune cells, using the GSEA methodology as previously published for the comparison of human and mouse cell types (41, 42). As controls, we confirmed the significant enrichment of the mouse and human B cell transcriptomic signatures specifically in swine B lymphocytes when compared with all the other populations of swine immune cells, and of human and mouse skin LC transcriptomic signatures in swine LC (Fig. 1A, 1B; data not shown).

Mouse “Skin LN CD11b<sup>+</sup>” and human “Skin CD1apos DDC” transcriptomic signatures were significantly enriched in swine CD163<sup>−</sup> DDC when compared with the four other swine cell types examined, except for swine LC concerning the mouse signature where no significant enrichment was observed (Fig. 1A). The genes belonging to the human CD1a DDC transcriptomic signature that contributed the most to its enrichment in swine CD163<sup>−</sup> DDC as compared with the other swine cell types included CCL19, EHF, and FAM49A (Supplemental Fig. 2A). These results support the hypothesis that swine CD163<sup>−</sup> DDC belong to the conventional DC (cDC) and not to the monocytic lineage and are equivalent to human CD1a<sup>+</sup> DDC.

Mouse and human “Mono/Macro versus cDC” and “moDC” transcriptomic signatures were significantly enriched in swine CD163<sup>+</sup> DDC when compared with the four other swine cell types examined except in swine monocytes for the mouse “Mono/Macro versus cDC” signature, where no significant enrichment was observed (Fig. 1B). Moreover, the transcriptomic signature specific to human CD14 DDC was strongly and significantly enriched in swine CD163<sup>+</sup> DDC when compared with the four other swine cell types examined. The genes belonging to the human CD14 DDC transcriptomic signature that contributed the most to its enrichment in swine CD163<sup>+</sup> DDC as compared with the other swine cell types included DCSign (CD209), IL1A,
CH25H, PLAU, SLC1A3, MMP9 (Supplemental Fig. 2B). The other genes belonging to the human and mouse moDC/mono/mac transcriptomic signatures enriched in CD163<sup>pos</sup> DDC included LDLR, IGF1, CSAR1, MAFB and TLR4 (Supplemental Fig. 2B, 2C). MAFB and TLR4 were recently described to be specifically and strongly expressed in the monocyte/macrophage lineage and not to be expressed in the cDC lineage in mice (34, 43). Finally, when we analyzed the expressions of key cytokines present in the swine skin from the microarray data (Supplemental Fig. 1B), we could observe that CD163<sup>pos</sup> DDC expressed mRNA levels of IL-12A, IL-17, and IL-1β equivalent to those of other skin DC, but higher levels of IL-10, IL-7, IL-1α, and TNF-α, and lower levels of IL-15. This cytokine profile is very similar to the profile previously described for the human CD14<sup>pos</sup> DDC (29, 44). Thus, these results support the hypothesis that swine CD163<sup>pos</sup> DDC are of monocytic origin and similar to human CD14<sup>pos</sup> DDC.

In conclusion, our gene expression profiling analysis suggests that swine CD163<sup>low</sup> DDC are bona fide cDC, whereas CD163<sup>pos</sup> DDC resemble monocytes/macrophage, moDC and CD14<sup>pos</sup> human DC. To further characterize these CD163<sup>pos</sup> DDC, we compared them, phenotypically and functionally, side by side, with pig skin macrophages and DC. In human skin, the dermal macrophages have been previously described as MHC-II<sup>low</sup>/CD163<sup>high</sup> (45), a population also observed in pig dermis. We thus sorted these cells along with CD163<sup>low</sup> DDC, CD163<sup>pos</sup> DDC and LC and colored them with May-Grünwald Giemsa. We could observe that the putative pig dermal macrophages were large cells with a very sharp cell limit, oval nucleus, and a cytoplasm containing numerous large vesicles, typical of macrophages. In contrast, CD163<sup>low</sup> DDC and LC were round cells frequently presenting a bean shape nucleus and a “fuzzy” cell limit because of dendrite display (Fig. 2A). Finally CD163<sup>pos</sup> DDC were bigger, with more various nucleus forms, from bean shape to oval, a fuzzy cell limit and a cytoplasm containing small vesicles. Thus, CD163<sup>pos</sup> DDC are morphologically different from dermal macrophages.

Using FACS phenotyping, we observed that CD163<sup>pos</sup> DDC as well as CD163<sup>low</sup> DDC and LC expressed high levels of CD1<sup>low</sup> whereas macrophages are CD1<sup>low</sup>. Conversely, when we tested monocyte/macrophage markers, CD163<sup>pos</sup> DDC presented a phenotype very similar to macrophages with the expression of higher levels of CD14, CD16 (FcγR3a), DC-Sign, and MR than CD163<sup>low</sup> DDC. It is important to note that in previous studies, using the CAM36A anti-CD14 Ab (31, 35), we did not detect CD14 expression on swine skin DC. Conversely, in this study, using the Tük4 anti-CD14 clone, we could observe differential CD14 expression on DDC populations as depicted here.

To further refine the comparison of CD163<sup>pos</sup> DDC with skin macrophages as well as to confirm the expression of key genes involved in the differentiation of DC and macrophages, we tested their expression by qRT-PCR using purified skin cells from conventional Large White animals. It has been previously shown that high expression of CSF1R (46, 47) and MAFB (48) was characteristic of monocyte/macrophage and infDC (49), whereas high

FIGURE 2. CD163<sup>pos</sup> DDC are phe

notypically distinct from resident macrophages. (A) Cells extracted from dermis and epidermis upon dispase and collagenase digestion were stained with anti–MHC-II and anti-CD163 Abs cell sorted, deposed on the slide, and then colored using May-Grünwald Giemsa reagents. (B) Epidermal LC, DDC, and macrophages were stained with anti–MHC-II and anti-CD163 Abs cell sorted and one of the markers noted above the histogram and their relevant isotype-specific secondary Abs. Data are representative of at least three independent experiments.
expression of FLT3 (50, 51) and ZBTB46 (13, 52) was characteristic of cDC. We thus tested the expressions of these four molecules in the swine skin DC populations we sought to better characterize.

Strikingly, CD163pos DDC expressed as much CSF1R and MAFB transcripts as dermal macrophages, whereas CD163low and LC expressed low amount of CSF1R and MAFB (Fig. 3A). Thus CD163pos DDC appeared related to the monocyte/macrophage lineage. Moreover, when we tested the DC markers, we observed that CD163pos DDC significantly express these markers, although at a lower level (6 times less) than LC (Fig. 3B), whereas CD163pos DDC express FLT3 and ZBTB46 transcripts in equivalent amounts as macrophages and significantly less than CD163low DDC (110 times less). Thus, whereas CD163low DDC present a profile compatible with cDC, it appears that CD163pos DDC present a phenotype more similar to skin macrophages or moDC. Because in vitro–derived moDC are considered as the equivalent of infDC (19, 53, 54), we decided to test the mRNA expression of previously proposed markers of human and mouse inDC from different tissues, namely CCR2, CD64 (FCGR1A) and FCER1 (20, 54–56). CCR2 and FCERIA expressions were significantly stronger in CD163low DDC compared with LC and macrophages, whereas their expressions appeared variable in CD163pos DDC. Conversely the common γ-chain for FCR (FCERIG/FCRγ) coexpressed with FCERIA, CD64/FCGR1A and CD16/Fc γR3A presented a significantly stronger expression in CD163pos DDC than in CD163low DDC and macrophages, both cell types expressing it more than LC, CD163pos DDC expressed equivalent levels of CD64 to dermal macrophages, and they both express higher levels than CD163pos DDC and LC (Fig. 3C). Finally macrophages and CD163pos DDC expressed (although not significantly for the latter) BAFF (Fig. 3A), a key molecule by which macrophages and DC directly regulate B cell proliferative responses and isotype switching (57, 58), one of the main recognized property of human CD14pos DDC (29, 59). Thus, phenotypic and gene expression analyses confirmed that CD163pos DDC are conventional DC related genes (19, 53, 54), we decided to test the mRNA expression of previously proposed markers of human and mouse CD14pos DDC related genes (29, 59), and human CD14 DDC related genes (A), human CD14 DDC related genes (B), and mRNA expression levels of inflammatory DC related genes (C). Each symbol represents one animal. At least three independent experiments have been realized per gene and per cell population. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t tests.

To probe the migrating capacities toward LN of the different skin myeloid cells described above, we first measured their in vitro capacities to crawl out of skin biopsies spontaneously or toward a CCL21 gradient. After 24 h in vitro, cells having crawled out from skin biopsies were counted, stained and FACS analyzed to determine the absolute number of each migrating DC populations. In parallel, fresh samples were collagenase digested to determine how much of each subpopulation was originally present in the tissue (Fig. 4). We observed that LC and CD163low DDC presented a highly mobile behavior, even in absence of added chemokine, because ~30% of the collagenase extracted cells can be retrieved upon a mere crawl out. This amount increased further in presence of CCL21, reaching >70% of the extracted cells. Conversely, very few CD172aneg DDC (~1%) crawl spontaneously out of the skin. However, this amount was significantly increased in presence of CCL21 (Fig. 4A). CD163low DDC migratory behavior appeared similar to the one of CD172aneg DDC, although their skin emigration upon CCL21 stimulus was not significantly different from their spontaneous crawl out (Fig. 4A), in agreement with their low CCR7 expression as observed in the transcriptomic study (data not shown). Finally, as expected, macrophages presented a highly sessile behavior not significantly affected by the presence of CCL21.

FIGURE 3. CD163pos DDC express monocyte/macrophage genes whereas CD163low DDC express cDC genes. DDC, macrophages, and epidermal LC were stained with anti–MHC-II and anti-CD163 Abs and cell sorted as in Fig. 2B before lysis, mRNA extraction, and qRT-PCR using GAPDH as housekeeping gene. The mRNA levels relative to the amount of GAPDH were calculated by the equation 2^(-ΔΔCT(GAPDH)). Depicted in this paper are the relative expressions normalized to 100 for the highest value observed across all samples for each gene. mRNA expression levels of monocytes/macrophages and human CD14 DDC related genes (A), mRNA expression levels of conventional DC related genes (B), and mRNA expression levels of inflammatory DC related genes (C). Each symbol represents one animal. At least three independent experiments have been realized per gene and per cell population. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t tests.
Having previously set up a protocol of pseudo-afferent cathe-
erism allowing us to harvest in vivo DC that physiologically migrate
from cutaneous territories (35), we had observed that very few
CD163pos DDC were present in steady state lymph (31), in agree-
ment with their low in vitro migrating behavior described above.
However, during the first days following the surgical operation for
lymphatic catheterism, we could observe an increase in CD163pos
DDC in the lymph, with a peak at day 3/4 postsurgery, followed by
a progressive decrease. This lymphatic CD163pos DC increase was
paralleled with an increase of all the skin DC subpopulations
(Supplemental Fig. 3A, 3B). Monocytes/macrophages were present
in the lymph at early time points postsurgery, decreasing rapidly
after day 4. Interestingly, concomitantly with CD163pos DC ap-
pearance in the lymph, we observed an influx of granulocytes after
surgery, with a decrease of these cells upon day 10 postsurgery
(Supplemental Fig. 3A, 3B). We interpreted this influx as the con-
sequence of the surgery-triggered inflammation, which was
maximal at day 4 and reverted to normal by day 10. We thus
postulated an inflammation triggered migration of CD163pos DC.
To further test whether skin residing CD163pos DDC were ca-
pable of lymph migration in inflammatory conditions, we tested
again the in vitro crawl out capacities of skin myeloid cells, but
this time in lymphatic liquid harvested either more than 10 d
postsurgery (migrating steady-state lymph) or at the peak of inflam-
mation, 3–4 d postsurgery (inflamed lymph). Absolute numbers of
each migrating DC subpopulation is depicted. Mean ± SD of one
triplicate experiment representative of three independent
experiments. (B) Same experiment as (A). Dot blots depict the
percentage among DC of migrating cells upon each culture
condition. Histograms depict the number of cells migrating in
lymph from day 3/4 postsurgery (migrating inflamed lymph)
supplemented with 5 μg/ml LPS or in lymph coming from
more than 10 d postsurgery (migrating steady-state lymph).
Absolute numbers of each migrating DC subpopulation is
depicted. Mean ± SD of seven independent experiments.
*p < 0.05, **p < 0.01, ***p < 0.001, unpaired t tests.

**FIGURE 4.** LC, CD172a–, and CD163ppm migrate toward
CCL21, whereas CD163ppm DDC only migrate in inflamed
lymph. (A) Skin biopsies were deposed either on complete
RPMI 1640 medium plus 1 mg/ml collagenase (collagenase
extracted cells) or on mock CHO supernatant or on CCL21-
transfected CHO supernatant. Twenty-four-hour migrating
cells were counted and stained for MHC-II/CD172a/CD163
before FACS determination of the percentage of each DC
subpopulations (dot plots). Absolute numbers of each mi-
grating DC subpopulation is depicted. Mean ± SD of one
triplicate experiment representative of three independent
experiments. (B) Same experiment as (A). Dot blots depict the
percentage among DC of migrating cells upon each culture
condition. Histograms depict the number of cells migrating in
lymph from day 3/4 postsurgery (migrating inflamed lymph)
supplemented with 5 μg/ml LPS or in lymph coming from
more than 10 d postsurgery (migrating steady-state lymph).
Absolute numbers of each migrating DC subpopulation is
depicted. Mean ± SD of seven independent experiments.
*p < 0.05, **p < 0.01, ***p < 0.001, unpaired t tests.
inflammatory conditions was not due to an upregulation of CD163 expression on CD163\textsuperscript{pos} DDC, we checked that LPS stimulation did not modify the relative proportion of the different collagenase-extracted DC subpopulations (data not shown).

To test the possibility that CD163\textsuperscript{pos} DDC were inflDC that differentiate upon inflammation in skin, we measured the percentage of CD163\textsuperscript{pos} DDC among total DC in the dermis of pigs from animal husbandries with different rearing conditions (Supplemental Fig. 3C). We observed that whereas CD163\textsuperscript{pos} DDC represented <10\% of total dermal DC in SPF Large White pigs, they reached 20\% of the DDC in conventional Large White as well as in conventional mini pigs (used for lymphatic cannula- tion), exceeding 40\% of the DDC in some conventional individuals. To confirm the link between the presence of CD163\textsuperscript{pos} DC in the dermis and the inflammation of the tissue, we then submitted SPF pigs to acetone:dibutyl phtalate painting and looked at DC composition in the dermis. Whereas untouched dermis presented 

\[
\begin{align*}
\text{CD163pos DDC} \quad &\text{skin} \quad \text{induced} \quad \frac{1}{2} \% \quad \text{CD163pos DDC} \\
\end{align*}
\]

expression on CD163\textsuperscript{low} DDC, we checked that LPS stimulation of inflammatory conditions was not due to an upregulation of CD163 expression on CD163\textsuperscript{pos} DDC, we checked that LPS stimulation did not modify the relative proportion of the different collagenase-extracted DC subpopulations (data not shown).

To test the possibility that CD163\textsuperscript{pos} DDC were inflDC that differentiate upon inflammation in skin, we measured the percentage of CD163\textsuperscript{pos} DDC among total DC in the dermis of pigs from animal husbandries with different rearing conditions (Supplemental Fig. 3C). We observed that whereas CD163\textsuperscript{pos} DDC represented <10\% of total dermal DC in SPF Large White pigs, they reached 20\% of the DDC in conventional Large White as well as in conventional mini pigs (used for lymphatic cannulation), exceeding 40\% of the DDC in some conventional individuals. To confirm the link between the presence of CD163\textsuperscript{pos} DC in the dermis and the inflammation of the tissue, we then submitted SPF pigs to acetone:dibutyl phtalate painting and looked at DC composition in the dermis. Whereas untouched dermis presented 

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\end{align*}
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CD163\textsuperscript{low} DDC induce IL-17–producing Th cells

To obtain information on the capacities of skin DC subpopulations and macrophages to stimulate the proliferation of CD4 and CD8 T cells, we cultured whole allogeneic CFSE-stained PBMC from SPF pigs in presence of cell-sorted skin DC. After a 5-d coculture, we stained them with CD3, CD4, and CD8 Abs to distinguish CD3\textsuperscript{+}/CD8\textsuperscript{+} of triplicate experiment are depicted. The figure is representative of three independent experiments. \( ^\star p < 0.05, ^\star\star p < 0.01, ^\star\star\star p < 0.001 \), unpaired \( t \) tests. (B) Naïve peripheral blood CD4 T cells were cultured in presence of cell sorted DC. The transcriptomic expression of IL-17 was measure by qRT-PCR at 24 h. Two independent experiments are depicted.
ward CCL21, a functional characteristic of peripheral cDC in mice and humans. Finally, it has been shown in mice that, whereas CD8α-like cDC induced preferentially a Th1 response, the CD11b-like/IRF4-dependent/CD301bpos cDC were prone at promoting Th2 (64, 65) or inducing Th17 (66) responses depending on the pathogen and the tissue of origin of the DC. In humans, two papers (67, 68) described the migration of IL-12 secreting/Th1 promoting CD1a DC, although without distinguishing CD1aα/β dermal DC from CD1aβhigh epidermal LC (29) and CD1aβlow BDCA3pos DDC (26). Conversely, it has been shown recently that human lung BDCA1/CD1c DC induced a Th17 immune response (66). Thus, our finding that CD163pos DDC functionally induce the differentiation of IL-17-producing Th cells is in agreement with the phenotypic and transcriptomic parallelism between CD11b-like/IRF4-dependent/CD301bpos mouse cDC, the BDCA1/CD1c human cDC, and the CD163low/CD11bhigh swine cDC (28).

In contrast, CD163pos DDC shared numerous common phenotypic and transcriptomic features with the human CD14pos DDC. These are strengthened by their similar cytokine expression profiles (IL-10, IL-1α, IL-1β, TNF-α, but not IL-15) (29) and by the expression of BAFF, a molecule expressed by human moDCs (58) that directly regulates B cell proliferative responses and isotype switching (57, 58), a key property of human CD14pos DDC (29, 59).

CD163pos DDC phenotype (expression of CD206/MR, CD16, CD209/DC-SIGN, CSF1R, and MAFB) is in agreement with a monocytic origin. Moreover, the transcriptomic analysis strikingly highlights the common features of CD163pos DDC with the monocytic/macrophage lineage. These data question us about the identity of CD163pos cells as DC or macrophages. Indeed, their microscopic features were clearly different from skin macrophages, although they did not express the DC markers FLT3 and ZBTB46 (12, 13, 34), and were only slightly better at inducing CD8 T cell proliferation than skin macrophages. Finally, CD163pos DDC migrated in lymph in inflammatory conditions but not in a CCR7/CCL21-dependent manner. In vivo capacity of moDC to migrate in lymph is a long lasting debate (69). In mice, it has been shown that upon strong inflammatory stimulation, LPS induced moDC could localize in the LN in a CCR7-dependent manner (19), and that inflammatory lung DC could migrate to the draining LN and initiate an immune response although with a much lower efficiency than cDC (21). In human, it has been shown that in vitro-derived moDC were able to migrate in vitro (36, 70) and in vivo (71), although with a relatively low efficiency. However, the migrating potential of CD14pos human DDC remained questionable (26, 72). Pig CD163pos DDC do not migrate in vivo at steady state (31). They do not respond to CCL21 but they can migrate toward inflammatory lymphatic factors yet to be determined, indicating that these cells have migratory abilities different from cDC.

Because CD163pos DDC accumulate in inflamed tissue and migrate in lymph upon inflammation, these cells might correspond to the in vivo described in mice and more recently in humans (for review, see Ref. 69). We tested their expression of inflammatory DC markers. CD163pos DDC express CCR2 mRNA at a similar level as CD163low DDC and CD64 mRNA as much as dermal macrophages. Interestingly, whereas CD163pos DDC express FcERIα mRNA at lower levels than CD163low DDC, they express significantly more FcRy mRNA than all other DC/macrophage populations including the CD163low DDC. In human moDC, the FcERIα protein chain, when expressed without the FcRy-chain, accumulates in the cytoplasm without cell surface expression and the FcRy-chain expression is mandatory for surface export of the complete FcERI receptor (73). Thus, CD163pos DDC, which expressed both FcERIα and FcRy-chains mRNA, may be the only skin DC displaying a functional FcERI receptor, in agreement with an inflammatory phenotype. Interestingly, a recent paper described that MHC-IIpos/CCR7pos/CD14pos/FcERIpos human afferent lymph cells from seroma fluid were only present during post-surgical tissue inflammation but not in healed tissue (74). We tested the CD163pos DDC for a potential enrichment of the human inDC (54) transcriptomic signature when compared with the four other swine cell types but did not get any significant result (data not shown), precluding the possibility to be assertive about the inflammatory nature of both porcine CD163pos DDC and human CD14pos DDC. Thus, as stated recently for human CD14pos DDC (75), the belonging of swine CD163pos DDC to macrophages, moDC or inDC cell types remains an open question.

It’s worth noting that the leading edges (i.e., the list of genes contributing the most to the enrichment of a given signature in a pairwise comparison) analysis of swine CD163pos DDC (Supplemental Fig. 2B, 2C) emphasized the high expression in these cells of several genes linked to metabolism control such as IGF1, CH25H, or LDLR, opening the question about their potential role in adipose tissue maintenance as stated for M2 macrophages (76).

Finally, a recent paper (77) showed that skin resident commensals tuned the function of local T cells in an IL-1α–dependent manner. Interestingly, we observed that CD163pos DDC were underrepresented in SPF pigs, which we may assume have a less diverse skin microbiota compared with conventional pigs, and that IL1A is one of the most expressed gene in CD163pos DDC (see Supplemental Fig. 2B), as well as in human CD14pos DDC (29). It is thus tempting to propose a role of these cells in the dialogue between skin commensals and the immune system.

Interestingly, a new population of CD11bpos/CCR2pos/MHC-IIβhigh moDC has been described in mouse skin (22). This population presents phenotypic and functional properties intermediate between cDC and macrophages, is underrepresented in germ-free mice compared with SPF mice, and is thus reminiscent to the swine CD163pos DDC described in this study and the human CD14pos DDC.

Altogether, we propose to include pig skin DC populations into a unified mammalian DC classification (78): 1) the LC, 2) the human BDCA3pos (and CD103+ like-in mouse) DDC, characterized as CD172α+ and XCR1+ 3) the CD172apos/XCR1neg human BDCA1pos (CD11b-like/IRF4-dependent/CD301bpos mouse DDC, corresponding to the swine CD163low DDC, and 4) finally the monocyte-derived human CD14pos DDC (moDC CD11bpos/CCR2pos in mouse) corresponding to the swine CD163pos DDC.

The overall toolbox for the analysis of pig immune response has greatly increased these last decades, for instance the development of ELISA, ELISPOT (CD4, CD8, and Ab-secretory cells) (79, 80) and multiparametric cytometry analysis (79, 81, 82). These tools can now be combined with the use of SPF (83), inbred (84), and transgenic animals (85–87) and the complete genome sequence publication (88), to accurately monitor the vaccine responses of pigs.

We thus would like to emphasize that in addition to its structural similarities with human skin, swine skin presents a DC network very similar to the human one, making pigs a good animal model for skin vaccination studies.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental FIGURE 1A: CD172a<sup>neg</sup> DDC express XCR1 whereas LC express Langerine.
Dermal DC and epidermal LC were stained with anti-MHC-II and anti-CD163 antibodies and cell sorted before lysis, mRNA extraction and qRT-PCR using GAPDH as housekeeping gene. The mRNA levels of XCR1 (A) and Langerine (B), relative to the amount of GAPDH, were calculated by analyzing the cycle threshold (C<sub>T</sub>) values for each amplification curve, and the arbitrary units were established by the formula 2<sup>−[(C<sub>T</sub>(target) − C<sub>T</sub>(GAPDH))]</sup>. Depicted here are the mean relative expressions +/- SD, normalized to 100 for the most expressed cell type for each sample, of at least 3 independent experiments.

Supplemental FIGURE 1B: Cytokines production by skin DC subpopulations
Linear expression of genes encoding cytokines extracted from the transcriptomic profile (n=3).
Supplemental FIGURE 2: CD163\textsuperscript{low} and CD163\textsuperscript{pos} DDC Leading edge.

A) Heatmap of the individual profiles of the genes belonging to the human “CD1a DDC” fingerprints and more highly expressed in pig CD163\textsuperscript{low} DDC as compared to CD163\textsuperscript{pos} DDC, B cells, LC and monocytes.

B and C) Heatmap of the individual profiles of the genes belonging to the human “moDC”, “CD14 DDC” or “mono/MP vs cDC” fingerprints (b) or of the mouse “moDC” or mono/mac vs cDC” fingerprints (C) and more highly expressed in pig CD163\textsuperscript{pos} DDC as compared to CD163\textsuperscript{low} DDC, B cells, LC (and monocytes, in the case of the moDC and CD14 DDC signatures). The color scale indicates the relative expression levels, from blue (lower expression) to red (higher expression). The figure was generated using the software Gene-E (version 2.1.71) from the Broad Institute.
Supplemental FIGURE 3: CD163<sup>pos</sup> DDC are overrepresented in conventional and inflammatory skin

A) and B) Lymph from pseudo-afferent canulated pigs (2 different pigs are shown in A and B) was collected at different days post surgery. Lymph cells were stained with anti-MHC-II, CD163 and CD172a antibodies. The percentage of each subpopulation among total lymphatic cells is depicted.

C) Percentage of CD163<sup>pos</sup> DDC in dermis of swine from different rearing conditions.

D) In vivo Acetone/dibutylphtalate (V/V) painting on SPF pigs skin. Animals were sacrificed at days 2 or 4 post-painting and skin DC were dispase and collagenase extracted before MHC-II/CD163 staining and FACS analysis. Mean +/- standard deviation of painting on 5 different animals.