Characterisation of the myeloid cell populations' resident in the porcine palatine tonsil

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Author contribution statement

FS and BC contributed to the performance of the experiments, FS also contributed to the design of the experiments, performed the data analysis and preparation of the manuscript. JE contributed to the design of the experiments, performed the cell sorts and assisted with the preparation of the manuscript. SG, HC, DW and FSt contributed to the design of the experiments and preparation of the manuscript. All authors reviewed the manuscript.

Keywords

Dendritic Cells, Macrophages, myeloid, porcine, Palatine Tonsil, immunology, pig

Abstract

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Tonsil tissue was supplied by a local abattoir

Blood was granted and approved by the Animal and Plant Health Agency Ethics Committee and all procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Project Licence PPL 70/8343

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Abstract: The palatine tonsil is the portal of entry for food and air, and is continuously subjected to environmental challenges including pathogens which use the tonsil and pharynx as a primary site of replication. In pigs, this includes the viruses causing porcine respiratory and reproductive syndrome, and classical and African swine fever; diseases which have impacted the pig production industry globally. Despite the importance of tonsils in host defence, little is known regarding the phenotype of the myeloid cells resident in the porcine tonsil. Here, we have characterised five myeloid cell populations that align to orthologous populations defined in other mammalian species: a $CD4^+$ plasmacytoid DC (pDC) defined by expression of the conserved markers E2.2 and IRF-7, a conventional dendritic cell (cDC1) population expressing $CADM1^{high}CD172a^{low}$ and high levels of XCR1 able to activate allogeneic CD4 and CD8 T cells; a cDC2 population of $CADM1^{dim}$ cells expressing FLT3, IRF4 and CSF1R with an ability to activate allogeneic CD4 T cells; $CD163^+$ macrophages (MΘs) defined by high levels of endocytosis and responsiveness to LPS and finally a $CD14^+$ population likely derived from a myelo-monocytic lineage, which showed the highest levels of endocytosis, a capacity for activation of $CD4^+$ memory cells, combined with lower relative expression of FLT3. Increased knowledge regarding the phenotypic and functional properties of myeloid cells resident in porcine tonsil, will enable these cells to be targeted for future vaccination strategies to current and emerging porcine viruses.

Introduction

Pigs are both an important source of meat globally and represent a valuable biomedical model. The porcine and human immune systems present evolutionary convergent features and, as such, pigs represent an important model for disease pathogenesis and vaccine development (Meurens et al., 2012). In pigs, however, the mononuclear phagocyte system (MPS) of dendritic cells (DCs) and macrophages (MΘs) is less characterised than those of either mice or humans. As a first line of defence to pathogen invasion, a clearer understanding of these cells, and how they might be identified, will facilitate our understanding of host-pathogen interaction in this species.

DCs are the sentinels of the immune system, they possess a distinct morphology and a unique capacity to activate naïve T cell populations (Steinman and Cohn, 1973, Steinman et al., 1978). They are also able to coordinate or regulate the adaptive immune system, depending on the antigenic signals and microbial environment at the time of antigen uptake. DCs are classified into two populations; plasmacytoid DCs (pDCs), specialising in the production of type I IFNs and conventional DC (cDCs), which are potent antigen-presenting cells (APCs) (Salio et al., 1999, Cella et al., 1999). Two sub populations of cDCs (cDC1s and cDC2s) have been described in mouse and human (Schlitzer and Ginhoux, 2013) and more recently in other mammalian species (Marquet et al., 2011, Marquet et al., 2014, Manh et al., 2013, Crozat et al., 2010a, Vu Manh et al., 2015a). Across species, these populations share expression of several conserved phenotypic markers, cytokine secretion profiles and specific functionalities. However, while cDC1s are presumed unique in their capacity to cross-present antigen to CD8 T cells in mice (den Haan et al., 2000), both cDC1 and cDC2 appear able to cross present in humans, depending on specific TLR stimulation and the local cytokine environment (Nizzoli et al., 2013, Jongbloed et al., 2010) indicating that these subsets may have some redundant functions. During an immune response an additional 'inflammatory' DC subset has been identified (moDC) in lymphoid tissue which in mice are recruited from circulating Ly6Chi monocytes (Leon et al., 2007). This population is capable of presenting antigen to both CD4 and CD8 T cells and inducing T_H1, T_H2 (Leon et al., 2007, Hammad et al., 2010) or T_H17 mediated responses (Segura and Amigorena, 2013, Segura et al., 2013).

MΘs are also resident in lymphoid (and non-lymphoid) tissues that have developed from either early erythro-myeloid progenitors from the extra-embryonic yolk sac or which have matured from circulating monocytes (Ginhoux and Guilliams, 2016). These cells are characterised by their variable expression of CD14, their responses to TLR4 stimulation, active phagocytic properties and their production of inflammatory cytokines such as TNF-α, IL1β, IL-6, IL-8 and IL-12 (Fairbairn et al., 2011).

In pigs, DCs and MΘ/monocyte populations have been characterised successfully in skin (Marquet et al., 2011, Marquet et al., 2014), blood (Auray et al., 2016, Edwards et al., 2017, Vu Manh et al., 2015b), lungs (Maisonnasse et al., 2016) and lymphoid tissue (Parra-Sanchez et al., 2018). In the skin, $CD172a^{neg}CD163^{neg}$ cells were identified as cDC1 cells given their high expression of CADM1 and XCR1. The $CD163^{low}$ $CD172a^{pos}$ cells expressed markers ZBTB46 and FLT3 aligning them with mouse and human cDC2s. A population of $CD163^{pos}$ porcine dermal dendritic cell were also classified as similar to human $CD14^{+}$ dermal DCs (Marquet et al., 2011, Marquet et al., 2014). Applying a similar panel of antibodies, cDC1 and cDC2 populations were identified in porcine lungs (Maisonnasse et al., 2016).

The palatine tonsil is positioned at the opening of the respiratory and gastrointestinal tract, providing an immunological barrier (consisting of macrophages, dendritic cells and lymphocytes) equipped to induce an immune response. In human tonsils, three populations of DCs have been described; pDCs, cDC1s and cDC2s (Segura et al., 2012) and more lately cDC1s and cDC2s have been identified in the porcine tonsil (Parra-Sanchez et al., 2018). However, the different populations that make up the milieu of myeloid cells which reside in the porcine tonsil has received little attention. It is these cells which form the first line of defence to air-borne pathogens and viruses and are tasked with ensuring an appropriate immune response is relayed following encounter with pathogen or commensal derived microbes. Here, we successfully employed multi-parameter flow cytometry to immunophenotype five distinct myeloid cell populations resident in porcine tonsil. To further characterise these populations, we localised these subsets *in situ* using confocal microscopy, sorted and assessed these cells functionally and, by way of RT-qPCR, evaluated the expression of conserved markers expressed by various myeloid cells populations. Through these analyses we identified three orthologous classical DC subsets (pDCs, cDC1s and cDC2s), MΘs, and a CD14 positive subset with characteristics interrelating with DCs and MΘs, consistent with a monocyte derived DC population.

Materials and Methods

Animals and tissue collection

Pig palatine tonsils were obtained from a local abattoir and transported at room temperature to the laboratory. Pigs were typically 6-12 month-old Large White or Large White cross breeds. For the mixed leukocyte reaction (MLR), peripheral blood mononuclear cells (PBMC) were isolated from blood obtained from animals kept at the Animal and Plant Health Agency (APHA) facilities under housing and sampling regulations approved by the APHA Animal Welfare and Ethical Review Board and conducted in accordance with the Animals (Scientific Procedures) Act, UK.

Tonsil cell isolation and lymphocyte depletion

Porcine palatine tonsils were dissected from the surrounding tissue and washed twice with PBS before being placed in a Petri dish. Tonsils were then cut into small fragments while submerged in PBS and further dissociated using the perforated end of a syringe plunger. The resulting cell suspension was filtered through a 40 μm cell strainer (Corning, Sigma-Aldrich, Gillingham, UK). Mononuclear cells were then separated over a Ficoll gradient (1.077 g/l, Sigma Aldrich). Myeloid cells were enriched by magnetic depletion of lymphocytes using anti-CD3 (clone 8E6), anti-CD8α (clone PT36A) (both from Washington State University Monoclonal Antibody Center, Pullman, USA), anti-CD21 (clone BB6-11C9.6, Cambridge Bioscience, Cambridge, UK), and anti-IgM (Clone K52 1C3; Bio-Rad AbD Serotec Ltd, Oxford, UK) mAbs followed by incubation with anti-mouse IgG1 magnetic beads and separation through LD columns (Miltenyi Biotech, Bisley, UK) according to the manufacturer's instructions.

Flow cytometry and cell sorting

For phenotypic analysis of tonsillar myeloid cells, cell surface staining was performed in three consecutive steps. Cells were initially incubated with the same lymphocyte lineage antibodies as described above (anti-CD3, anti-CD8α, anti-CD21 and anti-IgM, all of an IgG1 isotype) and anti-CD4-PerCP-Cy5.5 (clone 72-12-4; BD Pharmingen, Oxford, UK), CD14 PE Texas Red (clone Tük4; Fisher Scientific, Loughborough, UK), MHC class II-DR (clone 2E9/13; Bio-Rad AbD Serotec Ltd,) labelled with Zenon anti-mouse IgG2b PE (Life Technologies, Paisley, UK) and anti-Syn-CAM (TSLC1/CADM1) biotinylated antibody (Clone 3E1; MBL, Caltag Medsystems, Buckingham UK). Following incubation for 10 mins at room temperature (rt), cells were washed then labelled with a secondary anti-mouse IgG1 Brilliant Violet 421 (Clone RMG1-1; Bio Legend London, UK) and streptavidin Brilliant Violet 605 (BioLegend) again for 10 mins at rt. Finally, cells were stained with anti-CD172a FITC (clone BL1H7; Bio-Rad AbD Serotec Ltd) and anti-CD163 conjugated to anti-mouse IgG1 Zenon APC (Life Technologies), again for 10 mins at rt. Data was acquired on a LSRII Fortessa (BD Biosciences Oxford, UK) and collected in FACS Diva Software (BD biosciences). All analysis and compensation was performed using Kaluza Software (Beckman Coulter, High Wycombe UK).

For several downstream analyses the identified myeloid populations were stained as described above and sorted using a MoFLo Astrios (Beckman Coulter). Sorted populations were collected in RPMI-1640 medium supplemented with 40% foetal bovine serum and 100U/mL of penicillin, 100µ/mL streptomycin (Life Technologies). For mRNA extraction, cells were pelleted and supernatant removed before snap freezing in liquid nitrogen. Cells were stored at -80°C until RNA extraction. Typically, between 3-8x10^5 cells were analysed by flow cytometry (per sample) depending on the experiment. For sorting, between 5-10x10^6 cells were sorted depending on the pig.

TLR stimulation and intracellular cytokine staining

Lymphocyte-depleted tonsillar mononuclear cells from 8 pigs (obtained from an abattoir) and isolated as described above and seeded in round-bottom 96-well plates in 200µl of complete RPMI-1640 (cRPMI), supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Life Technologies). Cells were cultured for 10 hours with either CpG ODN21798 (to stimulate TLR9) (Miltenyi Biotec, Bisley UK) at 10µg/ml, Poly I:C HMW (To stimulate TLR3) (Invivogen, Toulouse, France) at 10µg/ml, LPS (to stimulate TLR 4) (Invivogen,) at 1µg/ml or media supplemented with recombinant IL-3 at 10ng/ml at 37°C + 5% CO_2 . Following 4 hours of culture GolgiPlug® (BD Biosciences) was added and cells incubated for a further 6 hours. Cells were then stained as described above with the exception of applying non-biotinylated anti-Syn-CAM (TSLC1/CADM1) (Clone 3E1; MBL) (Caltag Medsystem) labelled with anti-chicken IgY APC (Jackson ImmunoResearch, Newmarket, UK) and CD163 conjugated with Zenon anti-mouse IgG1 APC AlexaFluor750 (Life Technologies, Paisley, UK). For intracellular staining, cells were treated with BD Cytofix/Cytoperm™ (BD Biosciences, Oxford, UK) for 20 minutes at 4°C washed with BD Perm/Wash™ before staining with either biotinylated anti-IL-12 (R&D Systems, Abingdon, UK) or directly conjugated anti-TNF-α Brilliant Violet 605 (eBioscience, Hatfield, UK) in Perm/Wash™ buffer. IL-12 staining was detected by addition of streptavidin BV605 for 30 minutes at 4°C. Finally, cells were washed with Perm/Wash™ and resuspended in PBS supplemented with 2% FBS. Staining was assessed on the LSRII Fortessa.

OVA processing by tonsillar myeloid cells

Lymphocyte-depleted tonsillar mononuclear cells from 4 pigs were seeded at $2.5x10^5$ cells/well in cRPMI-1640. DQ-OVA FITC (Life technologies) at a final concentration of 2μg/ml, or media (as a negative control), were added in triplicate to the cells and cultured at either 37°C or 4°C (to confirm active uptake of antigen). After 1.5 hours incubation, cells were stained using the phenotypic staining protocol described above with the exception that CD172a-FITC was substituted with CD172a (non-conjugated) labelled with anti-mouse IgG1 APC Zenon labelling kit (Life Technologies). Samples were acquired on the LSRII Fortessa.

Mixed lymphocyte reaction

For allogeneic T cell stimulation experiments, PBMC were stained using CellTrace™ Violet Cell Proliferation Kit (Life Technologies) 10^6 cells were incubated with 5μM of dye in PBS (at 37°C for 20 minutes). Cells were washed and resuspended in cRPMI-1640 and incubated for a further 10 minutes. Sorted myeloid cells were seeded at $5x10^3$ cells/well, and added to ?stained PBMC at $5x10^4$ in a final volume of 200μl in either duplicate or triplicate depending on the number of myeloid cells successfully sorted. Negative and positive controls were included using CellTrace™ Violet stained PBMCs with media and pokeweed mitogen (PWM; Sigma-Aldrich) at a concentration of 10μg/ml respectively. After 5-day culture, cells were washed and stained with anti-CD4-PerCP-Cy5.5 anti-CD8a-PE (clone76-2-11; BD Pharmingen) and analysed by flow cytometry. Reduction of CellTrace™ Violet staining was evaluated as an indicator of cell proliferation, whereby $CD4^+CD8a^-$ (Naïve $CD4^+$ T helper cells), $CD4^-CD8a^+$ (cytotoxic T lymphocytes) and $CD4^+CD8a^+$ ($CD4^+$ T memory cells) were identified and the percentage of proliferating cells in each population determined. To identify the relative proliferation index of each of the T cell populations, a value of 100 was assigned to the myeloid cell population stimulating the maximum percentage of proliferating cells and other populations where normalised to this.

RNA extraction and quantitative RT-PCR (RT-qPCR)

Total RNA from FACSorted cell populations was extracted using the RNeasy Micro Kit (Qiagene, Manchester, UK) according to the manufacturer's instructions. Genomic DNA was removed using the RNase-Free DNase Set (Qiagene, Manchester, UK) during RNA extraction. RNA was reverse transcribed using random hexamers and the M-MLV reverse transcriptase (Promega, Southampton, UK). All qPCR reactions were performed using SYBR® Select Master Mix (ThermoFisher Scientific, Paisley, UK) in a final volume of 20μl. The primers used are listed in Table 1. Analysis was performed using the MxPro QPCR Software (Agilent Technologies, Stockport, UK) and the cycle threshold (C_T) values for each amplification curve were determined. Relative quantification was calculated using the ΔC_T method and normalized to the expression of β-actin mRNA. In order to compare data sets the arbitrary units (AU), for each animal the cell population with the highest level of expression of each gene was considered 100 and the remaining populations were normalised to it as shown by Maisonnasse and colleagues(Maisonnasse et al., 2016).

Confocal microscopy

Optimum Cutting Temperature (OCT, Tissue-Tek) treated tissue blocks were submerged in isopentane at -80°C until frozen and cut into 6-10µm thick sections by cryo-sectioning (Leica RM2135 cryotome). Sections were then transferred on to microscopy slides and fixed in absolute ethanol before storing at -80°C prior to processing.

The mounted tissue sections were placed into Sequenza clips (Shandon, Paisley, UK) and then incubated in 5% (w/v) normal goat serum (Sigma-Aldrich) in TBS-T either overnight (panel 1) or for 30 minutes (panel 2) at room temperature in the sequenza staining rack. For identification of $CD14^+$ cells and pDCs, panel 1 antibodies were applied, these included lineage antibodies (as described above; conjugated to biotin), Anti-CD4α AF488 (Clone MIL17; Bio-Rad) and Anti-CD14 dylight 550 (Clone Tük4;), all antibodies were conjugated to their respective protein/flourochome using Lightning link labelling kits (Expedeon). Panel 1 antibodies were applied to the slides for 120 minutes at 37°C then washed 3 times in TBST before incubation with streptavidin-APC (Bio-Rad) at 37°C for a further 90 minutes before washing again for 3 times in TBST. The slides were then incubated with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Thermo Fisher) diluted 1/10000 in deionised water at room temperature for 30 minutes before washing twice. For detection of cDC1s, cDC2s and macrophages, panel 2 antibodies were applied for 30 minutes at room temperature which included the lineage antibodies (unconjugated). After washing 3 times with TBST, anti-mouse IgG1-BV421 (Clone RMG1-1; Bio Legend) was applied for detection. After further washing steps, anti-CD172a FITC (clone BL1H7) anti-Syn-CAM (TSLC1/CADM1) biotinylated antibody (Clone 3E1) and anti-CD163 (clone 2A10/11; Bio-Rad) conjugated to anti-mouse IgG1 Zenon APC (Life Technologies) were added for 30 mins at room temperature. Slides were then washed before applying streptavidin Brilliant Violet 605 (BioLegend) for 30 minutes at room temperature.

Finally, all the slides were washed twice with deionised water before being removed from the Sequenza clips and coverslips mounted with Pro-Long Gold anti-fade mounting media (Thermo Fisher). The slides were allowed to dry in the dark overnight and then sealed with nail varnish. Slides were imaged using the Leica SP2 confocal microscope.

Statistical analysis

GraphPad Prism 6.0 (GraphPad software, La Jolla, California, USA) was used for the analysis of data sets. Statistical tests applied to each data set are indicated in the relevant figure legend.

Results

Identification of five distinct populations of myeloid cells in porcine tonsil

A panel of markers that had previously defined DCs and MΘ populations in various species (Crozat et al., 2010b, Contreras et al., 2010, Marquet et al., 2011) was selected to determine the presence of myeloid populations resident in porcine tonsil (Figure 1). Since myeloid cells are comparatively rare in secondary lymphoid tissues they were enriched by antibody-associated magnetic depletion of cells expressing the lymphocyte lineage markers CD3 (T cells), CD8α (NK cells), CD21 and sIgM (B cells). Following doublet discrimination (Figure 1A), live cells were identified and remaining lineage positive cells excluded. After selection of MHC class II positive cells, a candidate pDC population was identified as CD172a^{low}CD4^{+} MHC II^{low}, which corresponds to the defined porcine plasmocytoid dendritic cells (pDCs) (Summerfield et al., 2003) (Figure 1B). The remaining cells delineated into CD172a$^{neg/low}$, CADM1^{high}, MHC II^{hi} cells representing a putative cDC1 population. The CD172a^{high} cells could be divided into three distinct populations whereby CD172a^{hi}, CD14^{-}, CD163^{-}MHC II^{hi}, CADM1^{low} phenotypically resembled a cDC2-like population (cDC2); CD172a^{hi}, CD163^{-}, CD14^{+}, MHC II^{hi} cells were believed to be a monocyte derived population (CD14^{+} cells), while CD172a^{hi}, CD163^{+}, CD14^{-}, MHC II^{hi} cells represented a macrophage–like population (MΘs).

To assess the relative frequency of each of the five cell populations in the tonsil, the percentage of each subset was determined within the MHC-II positive fraction (Figure 1C). The CD14^{+} cells were the most frequent DC population at 6.75±2.20%, followed by cDC1s (2.54±1.49%), MΘs (1.52±0.71%), cDC2s at 1.00±0.74% and pDCs being the rarest population (0.32±0.34%).

Expression of conserved myeloid cell markers assessed by qPCR

The key aim of this study was to align the identified myeloid cell populations with their human and mice counterparts and to correlate them with similar populations in porcine skin (Marquet et al., 2011, Marquet et al., 2014), lungs (Maisonnasse et al., 2016) blood (Auray et al., 2016) (Edwards et al., 2017) and lymphoid tissue, including tonsil (Parra-Sanchez et al., 2018). However, not all proteins can be assessed by flow cytometry in pigs, due to lack of suitable antibodies. Several genes were therefore selected for further evaluation by gene expression. Expression of these markers is conserved across DC subsets and/or MΘ populations in different species (Maisonnasse et al., 2016) and their appearance on porcine tonsil myeloid cells was assessed by RT-qPCR. The putative pDC population were found to express high levels of E2.2, a gene important in pDC development (Auray et al., 2016), and also IRF7 and FLT3 (Figure 2). The cDC1-like cells expressed the cDC marker ZBTB46 (Satpathy et al., 2012) and the highest levels of the bona fide DC marker FLT3. These cells also demonstrated expression of XCR1, a gene considered to be a hallmark of cDC1 (Bachem et al., 2010). The cDC2-like cells expressed FLT3 and ZBTB46, and high levels of IRF4 and SIRPα, genes involved in cDC2 development. Monocyte/MΘ related genes including IL-1B, MAFB and CSF1R were also expressed by the cDC2-like population, albeit at lower levels than observed in MΘs. Notably, the CD14^{+} cells expressed DC related genes FLT3 and ZBTB46, but also macrophage related genes IL-1B, MAFB and CSF1R. IRF4 expression was also observed although at lower levels than associated with the cDC2-like cells. Finally, as expected, macrophages failed to express FLT3 but did express ZBTB46 but at slightly lower levels than the cDC and CD14^{+} cells. Unsurprisingly, the putative macrophage

population expressed the highest levels of IL-1B, MAFB and CSF1R and also the highest levels of SIRPα.

MHC-II and costimulatory molecule expression on isolated and cultured tonsillar myeloid cells

DCs are characterised by an ability to activate naïve T cells due to their constitutive expression of MHC class II and costimulatory proteins (Banchereau and Steinman, 1998). At steady state, and immediately after isolation, the highest levels of MHC class II were associated with cDC1s, closely followed by cDC2. The lowest levels of MHC class II were associated with pDCs (Figure 3). The highest levels of CD80/86 expression were associated with the MΘ-like populations while again the lowest levels were expressed by pDC. To evaluate whether these markers might increase in expression as the cells develop a more mature phenotype, cells were cultured for 4 hours in the absence of specific stimulation. For both pDCs and MΘs, there was only a modest increase in CD80/86 and MHC-II expression levels as demonstrated by the MFI (mean fluorescence intensity), which was not statistically significant. In contrast, $CD14^+$ cells, cDC1-like and cDC2-like cells all demonstrated a significant upregulation of both these markers. $CD14^+$ cells increased expression levels of CD80/86 from 2.111±0.172 to 5.232±0.941, and MHC-II expression from 47.141±13.775 to 234.164±53.693. cDC1-like cells increased their CD80/86 expression from 1.742±0.328 to 4.715±1.821 and MHC-II from 97.517±15.997 to 387.157±82.053 and finally cDC2-like cells increased CD80/86 from 3.065±0.460 to 7.013±2.514 and MHC-II from 72.528±13.334 to 337.820±71.097 (Figure 3).

Evaluation of TLR expression across the five populations of myeloid cells

Myeloid cells express a broad repertoire of pathogen recognition receptors including toll-like receptors (TLRs). Binding of the TLRs serves as a danger signal resulting in myeloid cell activation and ultimately a trigger for activation of the adaptive immune system. Others have demonstrated that DCs and MΘ cell populations express conserved TLR profiles; for example, human and mouse MΘs are associated with high levels of TLR4 expression (Vaure and Liu, 2014) while cDC1 cells express TLR3 (Reynolds and Haniffa, 2015). To further evaluate the phenotypes of the five myeloid cell populations the TLR profile (TLR1-10) of each of the populations was determined by RT-PCR (Figure 4). TLR5 expression was below the limit of detection and therefore the data is not shown. TLR1, 8 and 10 were expressed at comparatively similar levels across all cell populations with the exception of pDCs, which expressed much lower levels. TLR2, 4 and 6 were expressed at significantly higher levels on the MΘ-like cells compared to all other populations. TLR2, 4 and 6 were also expressed, albeit at a lower level, on the putative $CD14^+$ cells and the cDC2-like cells, while negligible levels of expression were seen on cDC1-like and pDCs. Similarly, to human and mouse pDCs, TLR7 and 9 were expressed at high levels on porcine tonsil pDC populations. However, TLR7 was not restricted to pDCs, but was also expressed by MΘ -like cells, cDC2 and $CD14^+$ cells (again at a lower level). This is consistent with a previous report demonstrating expression of TLR7 by porcine blood monocytes and cDC2s, in addition to pDCs (Auray et al., 2016). Another difference to human and mouse cells was the very high level of expression of TLR3 on pDCs, which is otherwise restricted to cDC1 cells, yet in pigs was expressed at very low levels on tonsillar cDC1s. Notably cDC1s expressed TLR9 at comparable levels to pDCs.

Evaluation of antigen processing, T cell stimulatory capacity and cytokine responses to TLR stimulation

One of the cardinal functions of myeloid cells is their ability to process and present antigen in order to activate T cells. To evaluate the antigen processing capacity of each of the five identified populations we assessed the uptake and processing of quenched DQ-OVA-FITC particles by the myeloid cell populations following 1.5 hours of culture (Figure 5A). Both pDCs and cDC1s were the least efficient at processing DQ-OVA-FITC particles followed by the cDC2 population. In contrast the $CD14^+$ cells where the most efficient followed by the MΘs. Next, to assess the relative ability of the myeloid cells to activate T cells we compared the capacity of the different sub-populations to activate allogeneic $CD4^+$ and $CD8^+$ T cells in a mixed leucocyte reaction (MLR). The cDC1-like and cDC2-like populations were most able to stimulate naïve $CD4^+$ T cells with the DC2 cells showing the highest stimulatory capacity (Figure 5B). The cDC1 and cDC2 like populations were most effective at stimulating $CD8^+$ T cell proliferation however, while cDC1-like cells showed a tendency toward the higher proliferation index, this was not found to be statistically significant. Finally, the cDC1s, cDC2s and the $CD14^+$ cells were equally able to stimulate memory $CD4^+$ T cells (shown by others to express a CD4/CD8 double positive phenotype) (Zuckermann and Husmann, 1996) while the pDCs and MΘs showed a relatively low capacity for stimulating allogeneic T cells. Also, since the PBMC were stained with antibodies to CD4 and CD8 (and didn't include CD3), we cannot discount the possibility that NK cells will also be included within the CD8 T cell population.

Finally, we evaluated how the myeloid cells might respond to TLR stimulation. Cells enriched in myeloid subsets (through depletion of cells expressing lineage markers) were cultured with CpG (ODN21798) which is a TLR9 agonist, Poly I:C as a TLR3 agonist and LPS as a TLR4 agonist and assessed for expression of IL-12 and TNF-α by flow cytometry. Due to decreased cell viability following 10 hours culture, pDCs had to be excluded from these analyses. The results showed that cDC1s secreted IL-12 following CpG stimulation (Figure 5C,D), most likely reflecting TLR9 expression on this cell population (Figure 4). However, $CD14^+$ cells also responded to CpG stimulation secreting both IL-12 and TNF-α despite their comparatively low levels of TLR9 expression. Similarly cDC1-like cells secreted the highest levels of IL-12 following Poly I:C stimulation despite an apparent low abundancy of TLR3 associated with these cells.

In situ localisation of myeloid cell populations in tonsils

To further evaluate the 5 myeloid cell populations identified in the tonsil, we investigated their sub-localisation *in situ*. To minimise spectral overlap between the fluorophores, the 5 myeloid cell populations were identified across two separate panels; panel 1to identify $CD14^+$ cells and pDCs and panel 2 to identify cDC1s, cDC2s and macrophages (Figure 6 and 7).

The tonsil regions assessed included the tonsillar crypts (C), lymphoid follicles (F), the interfollicular area (IFA) and the epithelia (E). Using panel 1, pDC's were detected in the IFA and less frequently in the follicles (data not shown) and beneath the squamous epithelia lining the crypt (Figure 6). Similarly, $CD14^+$ cells were located mostly in the IFA and

beneath the epithelia of the crypt. On occasion these cells were detected in the follicle. Neither of these populations could be detected in the epithelium or connective tissue.
Applying panel 2 (Figure 7), cDC1s were observed in the area surrounding the crypt, the follicles, the connective tissue and also the IFA. cDC2s were restricted to the crypt epithelium and the follicles. Finally, macrophages were found in the crypt lumen leading to the outside of the tissue and also in the area surrounding the crypt. They were particularly abundant on the edge of the tonsils, where they could be detected in the epithelum, the sub-epithelial connective tissue, sinoid and the adjacent lymphoid tissue. Clearly macrophages and $CD14^{+}$ cells were positioned close to areas were pathogens might be expected to enter the tonsil.

Discussion

Understanding the complexity of myeloid cell populations in pigs has become an important topic both in furthering our understanding on how these cells coordinate the adaptive immune system but also with respect to the opportunities that these cells offer as targets to modulate the immune response e.g. in vaccine development. Specialised subsets of DCs and monocyte/MΘs have now been comprehensively studied in various species and tissues and the evolutionary conserved patterns of cell phenotype and function established between human and rodents have generally remained true across livestock and companion animal species (Robbins et al., 2008, Crozat et al., 2010b, Contreras et al., 2010, Marquet et al., 2011) .

Porcine myeloid cell populations have recently been characterised in blood (Edwards et al., 2017, Auray et al., 2016), lung (Maisonnasse et al., 2016) and skin (Marquet et al., 2011, Marquet et al., 2014) however, little is known regarding their frequency and phenotype in secondary lymphoid organs, including the tonsil. A very recent study reported the presence of two resident dendritic cell populations; cDC1s and cDC2s in swine palatine tonsil (Parra-Sanchez et al., 2018), however, to our knowledge, this is the first study to apply multi-colour flow cytometry, confocal microscopy in addition to molecular and functional assays to delineate 5 distinct populations of myeloid cells resident in porcine tonsil.

The work presented here demonstrates clear homologies of porcine tonsillar myeloid cells with myeloid populations described in other porcine tissue and human tonsil. The myeloid cells were immature in the steady state, a feature consistent with the orthologous population in human tonsil (Segura et al., 2012). Porcine pDCs were identified as MHC-II^{low} $CD172a^{low/neg}$ $CD4^{+}$ $CADM1^{-}$ $CD14^{-}$ $CD163^{-}$ as previously described in porcine blood (Auray et al., 2016, Edwards et al., 2017). True to their assigned lineage, PCR analysis confirmed expression of FLT3, a tyrosine kinase receptor (necessary for development of DCs from progenitor cells (Karsunky et al., 2003)) and E2-2, a specific transcription regulator of pDCs development in mouse human and pig (Cisse et al., 2008, Auray et al., 2016). pDCs also expressed IRF7 and TLRs 7 and 9 as observed in human pDCs (Kastenmuller et al., 2014) and demonstrated a low antigen processing and T cells stimulatory capacity as shown in other porcine tissue (Summerfield et al., 2003) (Jamin et al., 2006, Edwards et al., 2017). The cDC1-like cell population was identified by the high expression of MHC-II, the low/neg expression of CD172a, negative expression of CD4, CD14 and CD163 and high expression of CADM1 as described in porcine skin (Marquet et al., 2014), lung (Maisonnasse et al., 2016) and blood (Auray et al., 2016, Edwards et al., 2017). This phenotype was also described by Parra-Sachez et al. (2018) in tonsil. High levels of FLT3 and XCR1 mRNA expression confirmed the definition of this subset, and homology across species (Reynolds

and Haniffa, 2015). (Summerfield et al., 2015). Secretion of IL-12 and a propensity to drive T_H1 responses have also been linked to this population (Jongbloed et al., 2010) and here, high levels of IL-12 were associated with cDC1s following CPG stimulation, also demonstrated in cDC1s from porcine lung (Maisonnasse et al., 2016). It was interesting that high levels of IL-12 was also produced by cDC1s in response to Poly:IC despite the low abundancy of TLR3. Furthermore, all tonsillar populations responded to TLR3 agonist with TNF-α. Clearly the interaction of DCs, with each other (and possibly any remaining lymphoid cells in the population) is influencing the cytokine secretion profile in addition to TLR expression. The assessment of sorted cell subsets may have revealed the genuine cytokine expression profile for each population, although evaluation of a mixed population of cells permits a more realistic approach for assessing cytokine secretion patterns *in vivo*.

The phenotype of the cDC2 lineage was confirmed as MHC-II^{high}CD172a^{high}CD4^{-} CADM1^{low}CD14^{-}CD163^{-} with a moderate ability to take up and process antigen but a superior capacity to activate allogeneic naïve CD4 T cells. Activation of CD4 T cells, moderate CADM1 expression and induction of T_H2 responses are hallmarks of this cell population in porcine lung (Maisonnasse et al., 2016) and blood (Auray et al., 2016, Edwards et al., 2017). PCR analysis of this sorted cell subset revealed FLT3 and ZBTB46 expression and the highest levels of the transcription factor IRF4, necessary for development of cDC2s from CD11c progenitor cells in lung and spleen in the mouse (Bajana et al., 2016) and for promoting CD4^{+} T cell responses in humans (Vander Lugt et al., 2014). Finally, TLR expression across these cells was consistent with what has been observed in porcine blood cDC2s (Auray et al., 2016) suggesting a conserved TLR expression profile in this population across several tissues.

The lineage of the fourth, most prominent CD14^{+} cell subset identified in the tonsil (MHC-II^{high}CD172a^{high}CD4^{-}CADM1^{low}CD14^{+}CD163^{-}) was less clear. Expression of FLT3 and ZBTB46 would classify them as cDCs rather than pDCs, monocytes and MΘs (Satpathy et al., 2012) yet the low expression of MAFB (Bakri et al., 2005) and CSF1R (Sasmono et al., 2003) and variable expression of IL-1β (Beuscher et al., 1990) favours a myelo-monocytic cell lineage. Notably, a population of CD163^{+} DDC in porcine skin which transcriptomically aligned to moDCs in human and mouse (Marquet et al., 2014) expressed CSF1R, MAFB and ZBTB46 suggesting this fourth population may also align with this subset. Furthermore, expression of CD14 (Boltjes and van Wijk, 2014), FLT3 (Menezes et al., 2016), TNF-α secretion (Segura et al., 2013) and a role in pathogen clearance (high uptake of DQ-OVA) (Serbina et al., 2003) are consistent with an inflammatory dendritic cell lineage generated from circulating myelo-monocyctic cells as shown by others (Lindstedt et al., 2005). However, we cannot discount the possibility that these cells might also contain cDC2s, given that different levels of CADM1 were expressed on these cells and that CD14 has also been associated with human blood cDC2s (Reynolds and Haniffa, 2015). However, CD14 remains a marker used to indicate a likely monocytic origin (Collin and Bigley, 2018) and is yet to be demonstrated as a specific marker for cDC2s in pigs (Maisonnasse et al., 2016, Summerfield et al., 2003, Auray et al., 2016, Edwards et al., 2017). It is also plausible that the two levels of CADM1 represent two populations of moDCs which have yet to be fully delineated and therefore assumed to be a single DC subset (Chow et al., 2016). Notably, the dominance of such a cell population in healthy pigs at slaughter seems counter-intuitive, but while these pigs are clinically healthy, they are not SPF at slaughter age and as such are subject to challenge by both environmental stimuli and pig specific pathogens (Crossan et al., 2015).

Furthermore, the variety of husbandry practises adopted between farms might explain the variability in frequency of this population between animals.

Finally, we detected a population of tonsillar cells expressing MHC-II^{high}CD172a^{high}CD4^{-} CADM1^{low},CD14^{-} and CD163^{+}, characterised by a high capacity to capture and process antigen, a low capacity for naïve T cell stimulation and an absence of FLT3 expression suggesting these cells to be MΘs. This is further supported by the high relative abundancy of CSF1R, MAFB, SIRPα and IL-1β transcripts which is consistent with a MΘ lineage. Notably, the highest level of SIRPα (CD172a) transcripts were associated with MΘs, which is in contrast to the flow cytometry data which demonstrated similar levels of SIPRα surface expression across macrophages, cDC2s and CD14^{+} cells. The reason for this inconsistency is unclear but might relate to the higher detection sensitivity of RT-qPCR, or that surface expressed SIRPα expression changes in response to various immune mechanisms following cell activation (Kong et al., 2007). This subset also showed the highest level of expression of TLR2, TLR4 and TLR6 (Roy et al., 2014), again consistent with a MΘ identity. Interestingly this was the only cell population which appeared to be present in the crypt, the connective tissue and the epithelium and is therefore likely to play a significant role in the uptake of antigens and host defence. Notably, all of the myeloid cell populations were observed in the area beneath the crypt epithelium indicating that all these cells are well positioned to assist in the uptake of antigens which have translocated the crypt epithelium, for subsequent T lymphocyte activation. This is consistent with previous reports reviewed in (Horter et al., 2003).

Despite the clear alignment of the myeloid populations with their human and mouse counterparts, differences were observed. For example, pDCs expressed TLR3 which is otherwise restricted to the cDC1 cell subsets in mice (Segura et al., 2010) and humans (Jongbloed et al., 2010) although this has also been reported in porcine blood pDCs (Auray et al., 2016) and might imply a porcine specific pDC response to a wider set of pathogens. Furthermore, we demonstrated that cDC1 and cDC2 subsets share a similar capacity to activate allogeneic CD8 T cells, which is consistent with cDC populations in lung (Maisonnasse et al., 2016) while others have shown that in porcine blood (Edwards et al., 2017) and lymph DCs in sheep (Contreras et al., 2010), cDC1s are superior at activating CD8 T cells. Perhaps in tonsil and lung, being two of the main portals of pathogen entry, a shared ability between cDCs populations to stimulate CD8 T cells may be advantageous. We also report that CD14^{+} cells were the most frequent population in the tonsil, closely followed by cDC1s which is in contrast to other tissues, where cDC2s are typically found to be more widespread than cDC1s (Maisonnasse et al., 2016, Ginhoux et al., 2016). A higher frequency of cDC1 (compared to cDC2) was also reported in porcine lymphoid tissue (Parra-Sanchez et al., 2018). The reason for this altered balance of DC subsets remains unclear but could reflect a local presence of DNA-associated pathogen and thus a requirement for TLR9 expression, a receptor which appears to be specific to cDC1s. Alternatively, this disparity might just reflect inherent differences between different tissues. Our results demonstrate that cDC1s populations secrete IL-12 as also shown in the porcine lung (Maisonnasse et al., 2016) while others have shown that in the blood, pDCs secrete the highest levels of IL-12 (Auray et al., 2016) (Edwards et al., 2017). Due to the scarcity and limited survival of pDC cells outside the tonsil we were unable to include these cells in our analyses, and therefore can neither refute nor confirm this for porcine tonsil. Finally, the CD14^{+} cells population in the porcine tonsil was found to be CD163$^{neg/low}$ with levels of FLT3 mRNA comparable to cDC2s. This is in contrast to CD163^{low} cells (believed to be moDCs in the lung) which were negative for FLT3 (Maisonnasse et al., 2016). CD163^{low} cells in skin were also shown to be

negative for FLT3 although expression was still 100 times higher than observed in macrophages (Marquet et al., 2014). The reasons for this difference are currently unclear and require further investigation.

This study has demonstrated a distinction and specialisation between myeloid cell populations as shown previously by others. However, there is also clearly a degree of plasticity in both cell phenotype and function. For example CADm1 is expressed on cDC2s, albeit at levels significantly lower, than associated with cDC1s. We also reported co-expression of TLR2 4 and 6 across $CD14^{+}$ cells cDC2s and macrophage populations indicating that all three cell subsets are able to recognise and respond to similar invading pathogens. Clearly as this area of work develops and techniques to delineate myeloid cell populations become more sophisticated, the association between these identified myeloid cells may become more apparent and additional cell populations may also emerge. It is likely that both the anatomical and pro-inflammatory environment will add a further layer of complexity both to the classification of these cells and their ontogeny. How this added 'flexibility' might then influence DC responses to pathogens entering the tonsil remains to be explained.

In summary, this study dissected the myeloid cells present in the porcine palatine tonsil and identified five distinct populations of the myelo-monocytic and DC lineages; two subsets of conventional DCs (cDC1s and cDC2s), pDCs, as well as a putative moDC population and macrophages, with clear homology to human subsets. As such the interaction of tonsillar myeloid cells with viruses such as classical swine fever, which share many characteristics with human viral haemorrhagic fever (Meurens et al., 2012), might assist in furthering our understanding of host-pathogen interaction.

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Author contributions

FS, BC and LS contributed to the performance of the experiments, FS also contributed to the design of the experiments, performed the data analysis and preparation of the manuscript. JE contributed to the design of the experiments, performed the cell sorts and assisted with the preparation of the manuscript. SG, HC, DW and FSt contributed to the design of the experiments and preparation of the manuscript. All authors reviewed the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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Target mRNA	Primer sequence	Reference
CSF1R	Fwd: 5'-TGAACGACTCCAACTACATTGTCA-3' Rev: 5'-TGTAGACGCAGTCGAAGATGCT-3'	(Marquet et al., 2014)
E2.2	Fwd: 5'-CCTTCTCTCTCAGCAGGCAC-3' Rev: 5'-CAGACGACCCTTTGCTCCAT-3'	Designed
IRF7	Fwd: 5'-TGGCAGCACATACTGGTGAG-3' Rev: 5'-AGTGGGCCTGCATATGGAAC-3'	Designed
XCR1	Fwd: 5'-CGATGCCGTCTTCCACAAG-3' Rev: 5'-GGAACCACTGGCGTTCTGA-3'	(Marquet et al., 2014)
IL-1b	Fwd: 5'-AGAGATGAAGTGCTGCACCC-3' Rev: 5'-ACAGACAAAGTCATCATTGCACG-3'	Designed
IRF4	Fwd: 5'-CCGGCCTGTGAAAATGGTTG-3' Rev: 5'-GGACGTGGTCAGCTCTTTCA-3'	Designed
Zbtb46	Fwd: 5'-GCTGGTGCACAGCAAGGA-3' Rev: 5'-GCGGCCGACATGAACAC-3'	(Marquet et al., 2014)
MAFB	Fwd: 5'-TGCGTTCTTTAGACCAATATGTTATGT-3' Rev: 5'-CACCAATAACTCGCCCGCTAT-3'	(Marquet et al., 2014)
FLT3	Fwd: 5'-	(Marquet et al.,

	TGTTCACGCTGAATATAAGAAGGAA-3’ Rev: 5’-GGAGCAGGAAGCCTGACTTG-3’	2014)
SIRPα	Fwd: 5’-CTGAGACCATCCGAGTTCCG-3’ Rev: 5’-CACGCCCACCGTGATAAAGA-3’	Designed
β-actin	Fwd: 5’-GACTCAGATCATGTTCGAGACCTT-3’ Rev: 5’-CATGACAATGCCAGTGGTGC-3’	Designed
TLR1	Fwd: 5’-AGATTTCGTGCCACCCTATG-3’ Rev: 5’-CCTGGGGGATAAACAATGTG-3’	(Uddin et al., 2013)
TLR2	Fwd: 5’-TGCTATGACGCTTTCGTGTC-3’ Rev: 5’-CGATGGAGTCGATGATGTTG-3’	(Uddin et al., 2013)
TLR3	Fwd: 5’-GAGCAGGAGTTTGCCTTGTC-3’ Rev: 5’-GGAGGTCATCGGGTATTTGA-3’	(Uddin et al., 2013)
TLR4	Fwd: 5’-TCATCCAGGAAGGTTTCCAC -3’ Rev: 5’-TGTCCTCCCACTCCAGGTAG-3’	(Uddin et al., 2013)
TLR5	Fwd: 5’-GGTCCCTGCCTCAGTATCAA-3’ Rev: 5’-TGTTGAGAAACCAGCTGACG-3’	(Uddin et al., 2013)
TLR6	Fwd: 5’-TCAAGCATTTGGACCTCTCA-3’ Rev: 5’-TTCCAAATCCAGAAGGATGC-3’	(Uddin et al., 2013)
TLR7	Fwd: 5’-TCTGCCCTGTGATGTCAGTC -3’ Rev: 5’-GCTGGTTTCCATCCAGGTAA-3’	(Uddin et al., 2013)
TLR8	Fwd: 5’-CTGGGATGCTTGGTTCATCT-3’ Rev: 5’-CATGAGGTTGTCGATGATGG-3’	(Uddin et al., 2013)
TLR9	Fwd: 5’-GGCCTTCAGCTTCACCTTGG-3’	(Auray et al., 2016)

	Rev: 5’-GGTCAGCGGCACAAACTGAG-3’	
TLR10	Fwd: 5’-GCCCAAGGATAGGCGTAAAT -3’ Rev: 5’-CTCGAGACCCTTCATTCAGC-3’	(Uddin et al., 2013)

Abbreviations: Il, interleukin, qPCR, quantitative PCR, TLR, toll-like receptor

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Legends to the figures

Figure 1. Phenotype of porcine tonsillar myeloid cells. (A) Dissociated tonsillar cells were depleted of cells expressing specific lineage markers (CD3, CD21, CD8α and IgM), and the subsequent enriched myeloid cells were stained with mAbs and examined by flow cytometry. Illustrative density plots show the gating strategy: I. Large cells, II. Singlets, III. Lineage negative, IV. MHC class II V. CD172a. Five myeloid cell populations were identified as shown by the annotated gates, pDC gated as, MHC-II^{low} CD172a$^{low/neg}$ CD4^{+} CADM1^{-} CD14^{-} CD163^{-} , a cDC1-like population gated as MHC-II^{high} CD172a$^{low/neg}$CD4^{-} CADM1^{high} CD14^{-} CD163^{-} , a putative cDC2 gated as MHC-II^{hi}, CD172a^{hi}, CADM1^{low}, a putative inflammatory CD14^{+} DC gated as, CD172a^{hi} ,CD163^{-}, CD14^{+} and putative MΘs as , CD172a^{hi} ,MHC II^{+}, CD163^{+}. **(B)** Flow cytometry histograms showing MHC class II, CD172a, CD4, CD14, CD163, CADM1 and CD152 (CD80/86) expression associated with each of the five defined myeloid cell populations. The histograms shown are illustrative for a single pig and are representative of 6 animals. **(C)** Plot demonstrating the relative proportion of each of the defined populations within the MHC II positive gate for six different pigs. Error bars and SD are shown for each individual population.

Figure 2. Expression of DC/MΘ/monocyte related genes by tonsil myeloid cell populations. The five identified populations were sorted, mRNA extracted and the expression of several DC/MΘ associated gene transcripts by the different populations assessed. mRNA expression levels of E2.2, IRF7, XCR1, IRF4, FLT3, ZBTB46, CSF1R, MAFB, SIRPa and Il-1b were evaluated by quantitative real-time RT-PCR (qRT-PCR). Gene expression was normalised to BACT (β-actin) and the relative expression of each gene was calculated with the $2^{-\Delta Ct}$ formula using the mean C_t values from duplicate samples. For each pig, data is expressed in arbitrary units (AU) obtained by assigning a value of 100 to the population giving the maximum level of expression and the remaining populations were compared to it for each gene. Each point on the graph represents the normalised $2^{-\Delta Ct}$ value from each cell population from each individual animal. This experiment was performed on a minimum of 3 animals in more than three independent experiments. Statistical analysis was performed by a one-way ANOVA and statistical significance is defined by ****$p<0.0001$, ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

Figure 3. Costimulatory molecule expression following 4-hour culture. The enriched tonsillar myeloid cells were stained, before and after culture, with the same antibody panel and gated as described above. Cells were also stained for fusion protein CD152 (CD80/86) expression to indicate cell maturation. Bar graphs showing the MFI values for CD80/86 and MHC class II corresponding to each of the defined myeloid populations. Values shown are from 8 individual pigs and error bars represent 1 SD. Values were compared using a two-way ANOVA and significance indicated by ****$p<0.0001$, *$p<0.05$.

Figure 4. Evaluation of Toll-like Receptors (TLRs) expression and cytokine responses to TLR ligands. The five populations were sorted and the mRNA was extracted to evaluate the expression of TLRs 1 to 10 by quantitative real-time PCR (qPCR). Each TLR gene expression level was normalised to β-actin and the relative expression of each gene was calculated with the $2^{-\Delta Ct}$ formula using the mean C_t values from duplicate samples. For each pig, data is expressed in arbitrary units (AU) obtained by assigning a value of 100 to the population giving the maximum level of expression and the remaining populations were compared to it for each gene. Each point on the graph represents the normalised $2^{-\Delta Ct}$ value from each individual animal. This experiment was performed on 3 or 4 animals in three independent experiments. Statistical analysis was performed by a one-way ANOVA and statistical significance is shown by ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

Figure 5. Evaluation of antigen processing and T cell stimulatory capacity (A). Differential endocytosis between cell populations was evaluated with OVA-DQ-FITC by culture of lineage depleted myeloid cells for 1.5 hours at 37°C (and 4°C) and APC fluorescence was assessed by flow cytometry. Histogram shows the mean percentage of cells taking up DQ from 8 pigs (each tested in triplicate), following subtraction of non-specific fluorescence (uptake at 4°C) for each cell population from 3 independent experiments. CD4 T cells were also assessed as a negative control. **(B)** Myeloid cells (APC) were sorted and PBMCs from allogeneic animals were stained with Violet CellTrace and mixed at a APC:T cell ratio of 1:10 before being culture for 5 days at 37°C. Proliferation of $CD3^+CD4^+$ (CD4 T cells), $CD3+CD4^-CD8\alpha^+$ (CD8 T cells) and $CD3+CD4^+CD8\alpha^+$ (memory T cells) was evaluated by flow cytometry. A value of 100 was assigned to the population with the highest proliferation value and all other populations were compared to this value (and repeated for each pig). Data is from 3 separate experiments and a minimum of four different animals for each cell type. A one-way ANOVA was performed and statistical significance is described by ****p<0.0001, ***p=0.0002, **p=0.0016 and *p=0.0108. **(C)** Isolated tonsil cells were depleted for lineage markers (CD3, CD8α, CD21 and IgM) and stimulated for 12 hours in the presence of TLR agonists CpG, Poly I:C or LPS. After incubation, the myeloid populations were defined using the same antibody panel as described above. IL-12 (top panel) and TNF-α (bottom panel) secretion was assessed by intracellular staining and flow cytometry. For each cell population, each point represents a single pig and the horizontal line represents the mean of at least 7 pigs tested in 3 independent experiments. The mean cytokine secretion of non-stimulated cells, was subtracted from each of the relevant data points. **(D)** Representative flow cytometry dot plots, showing IL-12 and TNFα secretion associated with cDC1 and $CD14^+$ cells respectively following CpG stimulation.

Figure 6. In situ localisation of the $CD14^+$ cells and plasmocytoid dendritic cells in porcine palatine tonsil. $CD14^+$ cells and pDCs were localised by confocal microscopy following ethanol fixation of tonsil slices. The areas assessed included the follicle (F), the interfollicular region (IFA), the crypt (C), and the epithelium (E). The tissue was stained using panel 1 antibodies; white arrow $CD14^+$ cells and yellow arrow pDCs Images are representative of at least two images from each section, from three different pigs. Objective used: (A) x63 oil immersion. Scale bars as shown.

Figure 7. In situ localisation of conventional dendritic cells and macrophages in porcine palatine tonsil. Two conventional dendritic cell subsets and macrophages were localised by confocal microscopy following ethanol fixation of tonsil slices. The areas assessed included

867 the follicle (F), the interfollicular region (IFA), the crypt (C), the connective tissue (CT) and
868 the epithelium (E). Tissue stained using panel two antibodies; blue arrow cDC1, white arrow
869 cDC2, yellow arrow macrophages. Images are representative of at least two images from
870 each section, from three different pigs. Objective used: x40 oil immersion. Scale bars as
871 shown.

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Figure 1.TIF

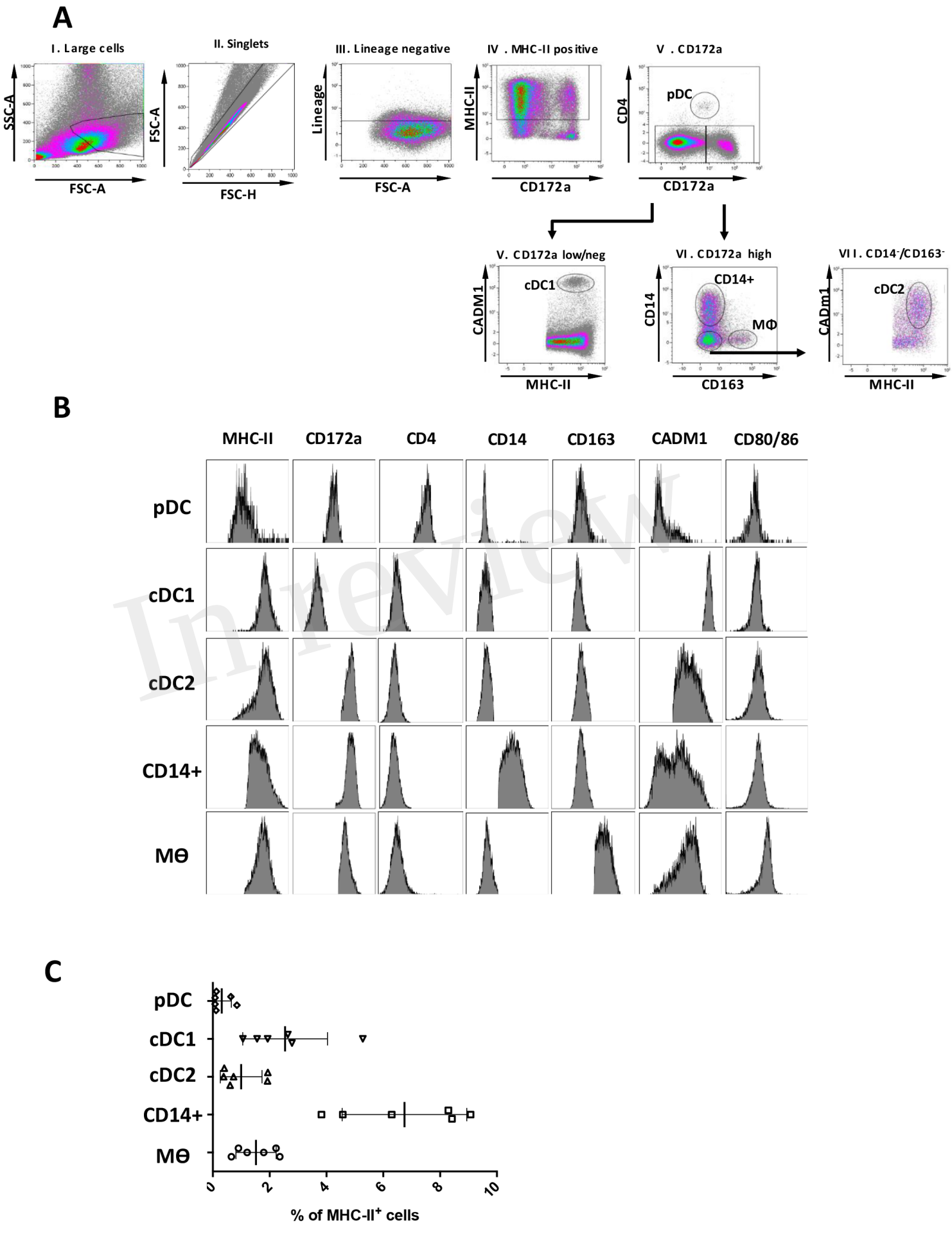

Figure 1.

Figure 2.TIF

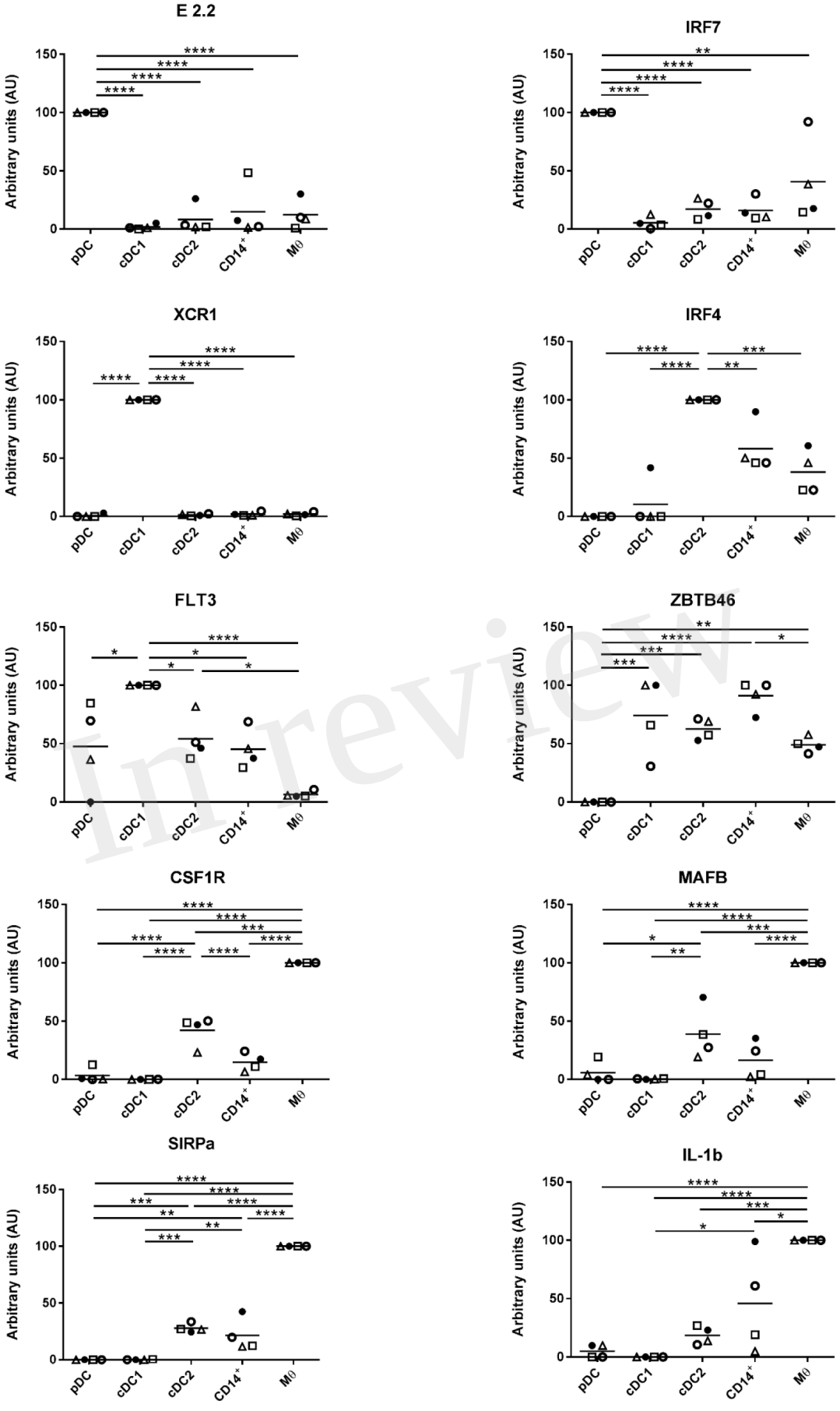

Figure 2

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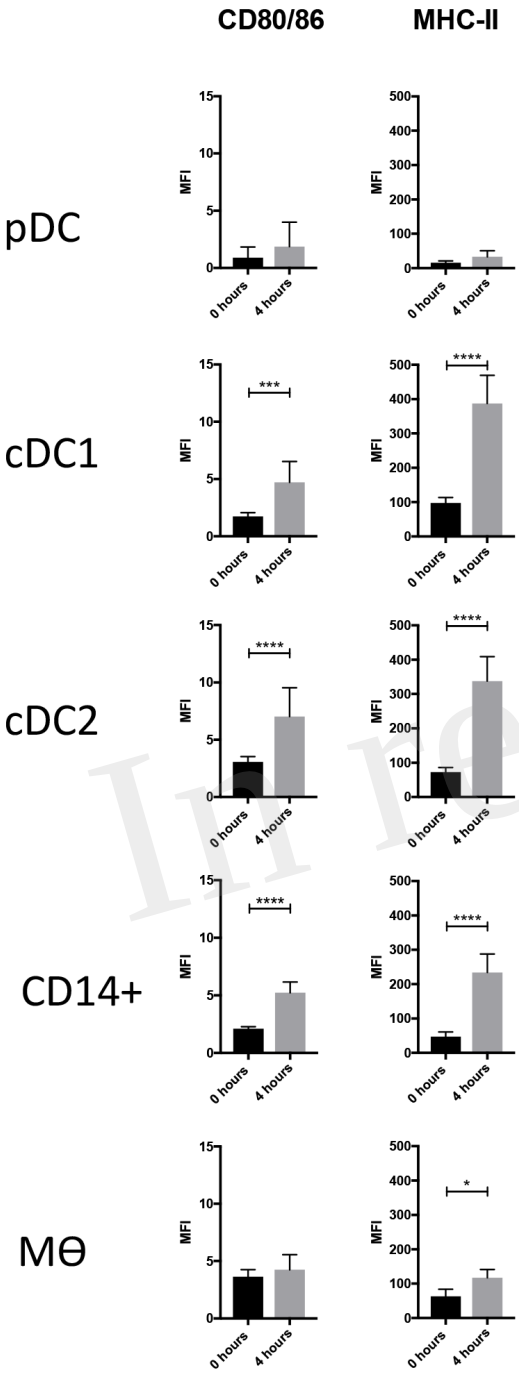

Figure 3.

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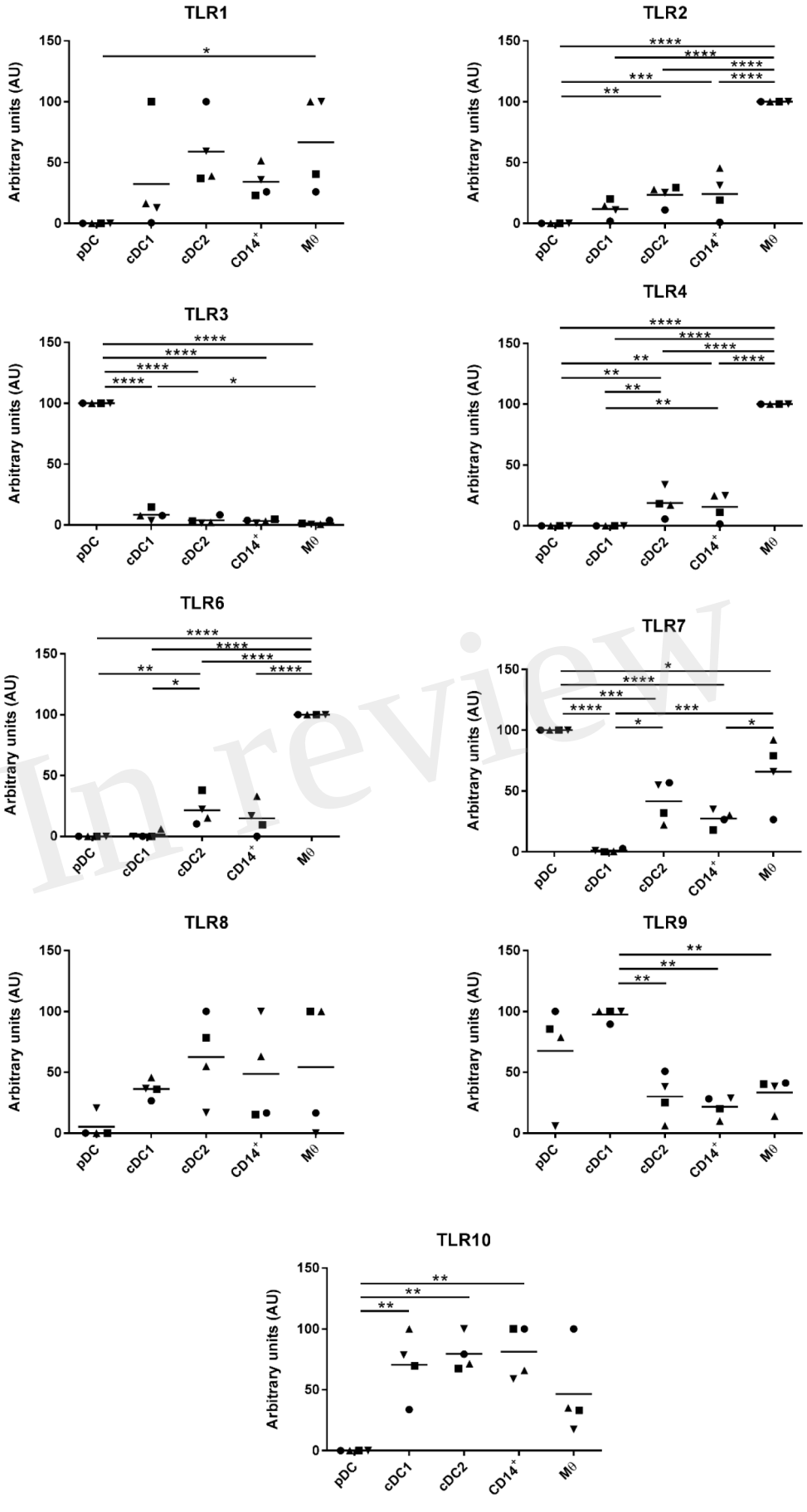

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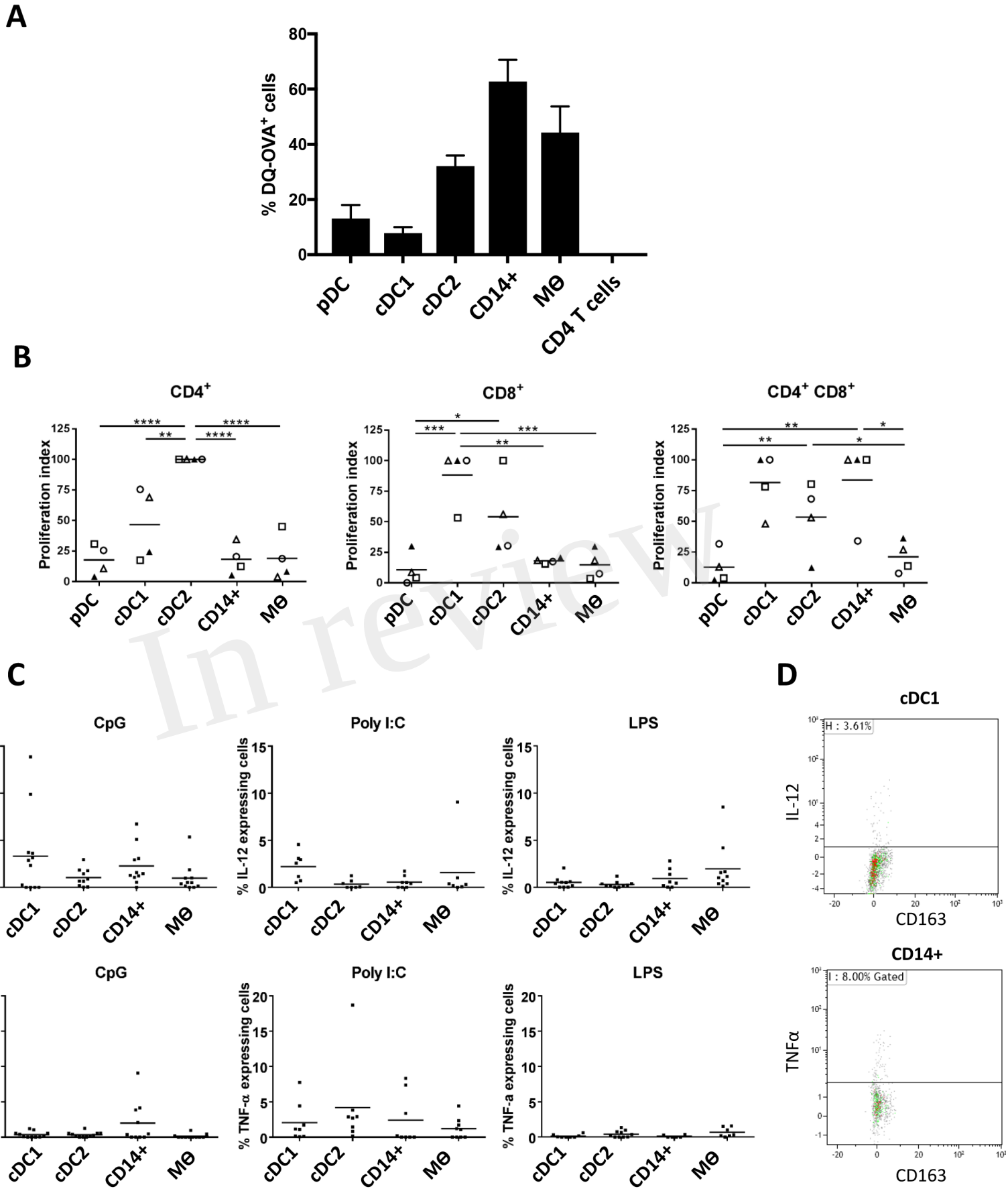

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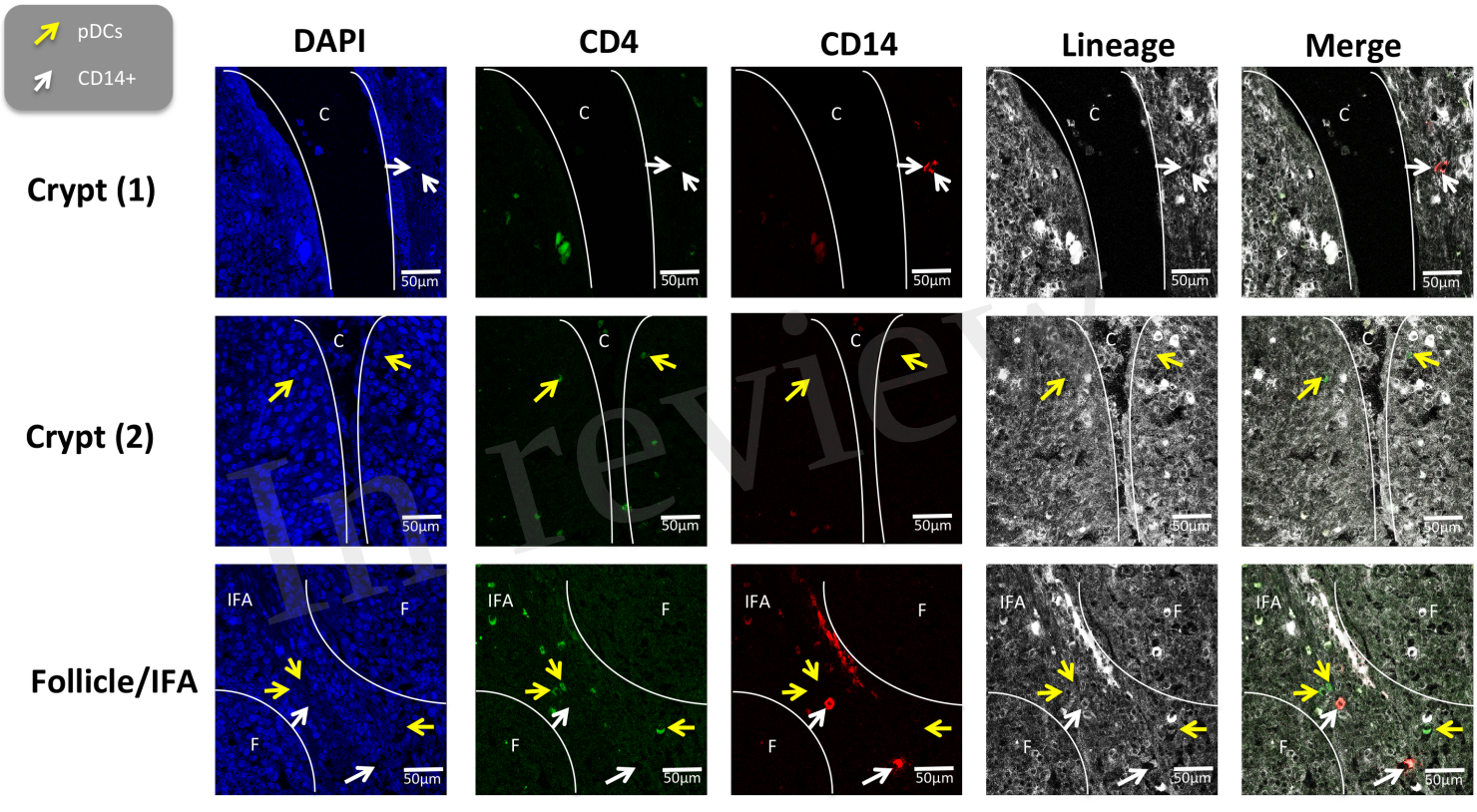

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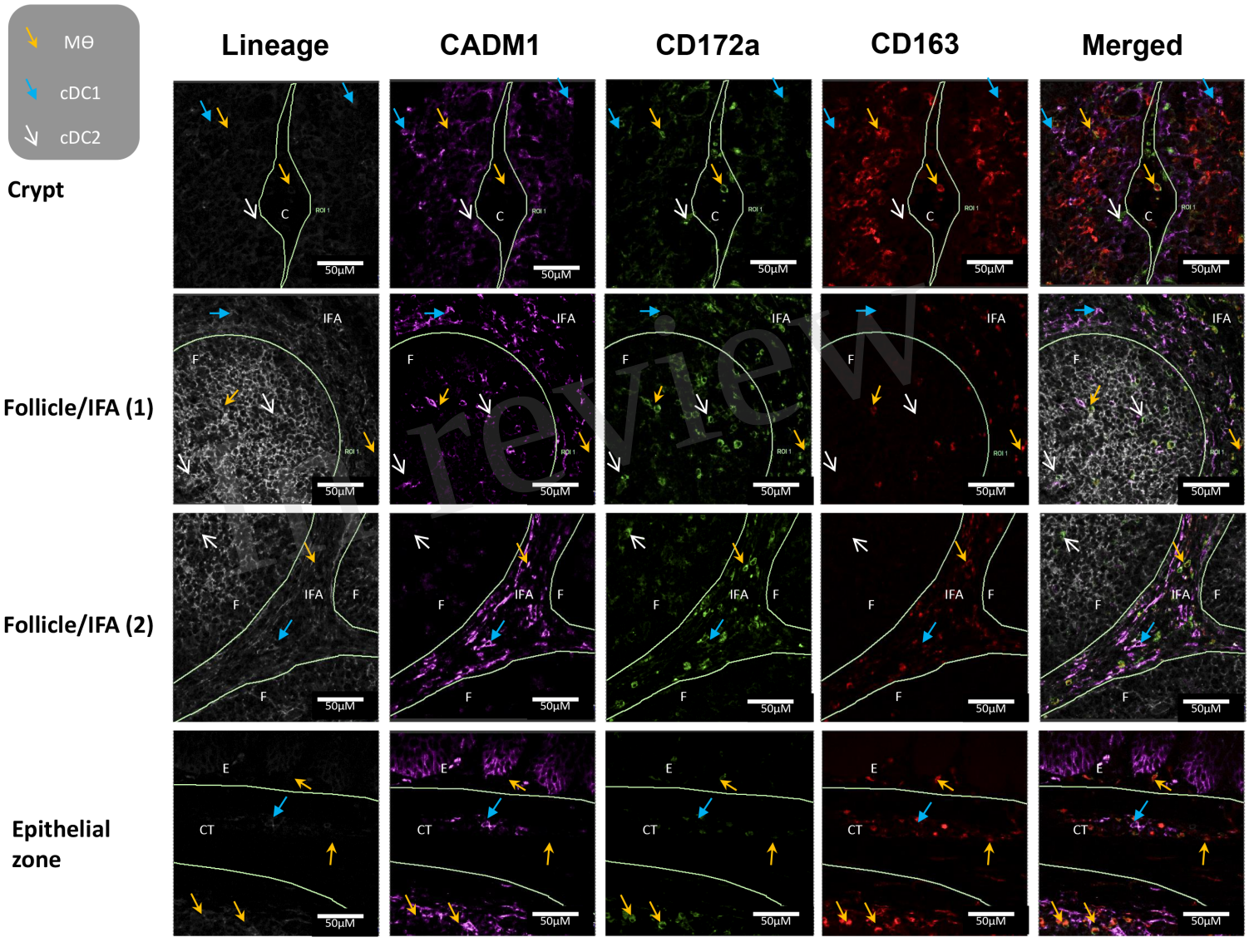
MΘ
cDC1
cDC2
Lineage
CADM1
CD172a
CD163
Merged
Crypt
Follicle/IFA (1)
Follicle/IFA (2)
Epithelial zone
C
IFA
F
E
CT
50μM