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Langerhans cells in porcine skin

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ABSTRACT

Langerhans cells (LCs) are resident dendritic cells (DCs) of skin and mucosal epithelium. The standard for identifying skin DCs as LCs is expression of langerin (CD207), a surface protein that mediates Birbeck granule (BG) formation upon internalization. Reports of BGs in porcine skin DC are contradictory, due to lack of langerin detection. Here, we present the sequence of porcine langerin/CD207, showing that the predicted porcine protein shares 75%/86% amino acid identity/similarity with human. Langerin mRNA was detected in porcine skin DCs by PCR and langerin protein was detected in both isolated skin DCs and skin sections by immunostaining. Approximately, 50–70% of skin DCs expressed langerin, demonstrating that the majority of porcine skin DCs are LCs. The full length sequence combined with the identification of antibodies reactive with porcine langerin, facilitates the study of LCs in swine, and advances the use of swine for studying skin diseases and infectious disease processes involving skin.

1. Introduction

Dendritic cells (DCs) are critical in both early innate and long-term adaptive immune responses (Banchereau and Steinman, 1998). Langerhans cells (LCs) are resident DCs of the epithelium, particularly the epidermis, characterized by the expression of the C-type lectin, langerin (CD207). Langerin is expressed on the cell surface and is internalized to form intracellular, racket-shaped Birbeck granules (BGs) in the cytoplasm (Valladeau et al., 2000), including the dendrites. After encountering antigen, LCs can migrate to regional lymph nodes where, like other DC subsets, they have the unique antigen presenting cell (APC) capacity to induce primary T-cell responses, thereby initiating an adaptive immune response. In mice, it is notable that langerin is also expressed by non-cutaneous, non-Langerhans cell derived dendritic cell subsets in lymphoid tissue and lung (Douillard et al., 2005; Sung et al., 2006).

The phenotype of human and mouse LCs has been extensively documented (Romani et al., 2006). LCs have also been described in domestic animals including pigs, cattle, sheep, dogs, cats and chickens (Holli and Lyne, 1972; Khalil et al., 1982; Marchal et al., 1995; Perez-Torres and Ustarroz-Cano, 2001; Romano and Balaguer, 1991). The description of porcine LCs is, however, based on the microscopic identification of BGs in epidermal DCs (Graber et al., 1994; Monteiro-Riviere, 1992; Romano and Balaguer, 1991) and LC-like cells derived from monocytes (Paillot et al., 2001). Conversely, we and others did not observe these granules in porcine skin and skin-derived DCs (Bautista et al., 2002; Gregg et al., 1995a). Since langerin is found exclusively in LCs in the skin, antibodies against this molecule have been used to identify human LCs by immunolabelling (Valladeau et al., 2000) and demonstration of expression of langerin has become the standard for designating skin dendritic cells as LCs.

We have previously characterized DCs isolated from porcine skin as coexpressing CD1 and CD172. These cells
also express high levels of MHC class II, CD80/86 and S100 antigen but lack expression of lymphoid specific proteins such as CD3, CD4, CD8 and CD21 (Bautista et al., 2002). However, in the absence of specific antibodies to porcine langerin, the further characterization of these porcine skin DCs has been limited relative to classifying Langerhans cells.

Pigs have important physiological and immunological similarities with humans. Therefore, they have been proposed as important models in human medicine and potential organ donors for xenotransplantation (Dunning et al., 1994; Sablinski et al., 1997; Sim et al., 1999). Furthermore, pigs are an important food source, necessitating high health standards through control and eradication of infectious diseases. As such, an understanding of the immunological properties of porcine LCs and other porcine APCs will be valuable in both human and veterinary medicine.

The objective of the present work was to identify langerin expressing skin dendritic cells in swine by multiple assays. We have sequenced the full length porcine langerin gene and here we present a comparative analysis of the predicted protein sequence relative to the langerin protein in human and other animal species. The predicted porcine CD207 protein shares high amino acid identity/similarity with human (75%/86%) and lesser amino acid identity/similarity to mouse (63%/77%) and rat (63%/76%) CD207. Further, we show langerin expression in porcine skin DCs by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), with mRNA levels more than 100-fold higher in putative LCs than in porcine monocyte-derived DCs (MoDCs). We then tested nine antibodies to porcine Langerhans cells (MoDCs). We have thus identified the tools necessary for further studies of porcine skin LCs (MoDCs).

### 2. Materials and methods

#### 2.1. Langerin (CD207) cloning, sequencing and structural and functional analysis

Cloning primers were designed using human (Genbank NM_015717)/bovine (Genbank XM_588243) cross reactive sequence using the Primer Express software package (Applied Biosystems, Foster City, CA) and are listed in Table 1. Duplicate 50 μl PCR reactions using these primers were performed using a cDNA pool made from eight porcine tissues and 2× PCR master mix (Abgene/Thermo, Waltham, MA). These products were amplified by 45 cycles of PCR on an iCycler thermocycler (Biorad, Hercules, CA). Primers and products under 100 bp were removed from amplified material with the Qiagen PCR purification kit (Qiagen, Valencia, CA). The presence of a single band of expected size was determined using the Agilent Bioanalyzer 2100 and a DNA 1000 Labchip kit (Agilent Technologies, Palo Alto, CA). These products were then amplified for sequencing using Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an iCycler thermocycler (Biorad). After purification by Performa spin columns (Edge Biosystems, Gaithersburg, MD), amplicons were spun dry, re-suspended in Hi-Di Formamide (Applied Biosystems), denatured at 95 degrees for 2 min and sequenced on a 3100 Genetic Analyzer 16 capillary array (Applied Biosystems) at the Environmental Microbial Safety Laboratory at the Animal and Natural Resources Institute, USDA, Beltsville, MD.

A consensus sequence for each gene target was assembled from four independent sequence reads, and then compared to the human reference sequences. Contiguous sequences were assembled using the CAP3 sequence assembly program (http://pbiol.univ-lyon1.fr/cap3.php). The predicted exon structure was determined by BLAST searching the deduced cDNA assembly against human and bovine genomic DNA (http://www.ncbi.nlm.nih.gov/blast/). The predicted protein structure was compared to other mammalian CD207 homologues by a translated BLAST search of the non-redundant protein database.

A phylogenetic tree comparing the predicted porcine CD207 protein with putative full-length amino acid sequences of known mammalian CD207 proteins was generated using Clustal X (v2.0.8 European Bioinformatics Institute). The alignment was compared by Bayesian Markov chain Monte Carlo algorithms employing a strict molecular clock model and a chain length of 10,000,000

#### Table 1 Primers used in the cloning of porcine CD207

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position in cDNA</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5'-CGACGCCAGAACGACCCTCTG-3'</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>F 5'-TGCCCATAGAAACGATAGAGA-3'</td>
<td>235–213</td>
<td>2</td>
</tr>
<tr>
<td>F 5'-CTTGTCGCCGCTCCTAC-3'</td>
<td>183–200</td>
<td>2</td>
</tr>
<tr>
<td>F 5'-GTGCTACGTCCTCTCTACACT-3'</td>
<td>433–412</td>
<td>3</td>
</tr>
<tr>
<td>F 5'-CCTGACGTCCGAGCACTAGAGA-3'</td>
<td>297–319</td>
<td>2</td>
</tr>
<tr>
<td>F 5'-ACGTTCCCGCGAGAATCT-3'</td>
<td>645–626</td>
<td>4</td>
</tr>
<tr>
<td>F 5'-ATGGACATGGGCTTTACGATATT-3'</td>
<td>593–618</td>
<td>4</td>
</tr>
<tr>
<td>F 5'-GGGACACATGGGCTTTACGATATT-3'</td>
<td>1000–977</td>
<td>6</td>
</tr>
<tr>
<td>F 5'-GCTTCTGGATCTCCAGGATACGC-3'</td>
<td>863–883</td>
<td>6</td>
</tr>
<tr>
<td>F 5'-CGGAGATGAGGAGGAACACACT-3'</td>
<td>NA</td>
<td>6</td>
</tr>
</tbody>
</table>

**PCR assay**

| F 5'-GGAATGTGCCCCCTGTTCA-3' | 944–962 | 6 |
| R 5'-CGTGGAACTTAAACTGCAAGCT-3' | 1030–1052 | 6 |
| Probe 5'-CGACCCTATTCCTCCATGACCGT-3' | 988–1013 | 6 |

Yorkshire pigs (female) aged 6–8 months were purchased from Animal Biotech Inc., Danboro, PA and allowed...
1 week to acclimatize and all animals were deamed healthy before the start of experiments. All procedures performed on these animals were approved by the Plum Island Animal Disease Center (PIADC) Institutional Animal Care and Use Committee.

Skin DCs were isolated following a published protocol (Bautista et al., 2002; Gregg et al., 1995b). Briefly, the skin of the entire back of a euthanized pig was washed, depilated with Nair (Carter Wallace Inc., New York, NY), surgically scrubbed, and disinfected. Skin layers (0.1–0.15 mm thick) were cut with a microtome blade and collected into Basal Eagle Medium (BEM, Invitrogen, Carlsbad, CA), supplemented with antibiotics (BEM-AB). Skin layers were washed twice in BEM-AB and then cultured (epidermis up) in RPMI-1640 containing 0.1 mM non-essential amino acids, 0.3 mg/ml L-glutamine, 8×10^{-5} M β2-mercaptoethanol, 1 mM sodium pyruvate, 4 mM dextrose, and 10% heat-inactivated fetal bovine serum (culture medium) supplemented with 10% heat-inactivated autologous porcine serum in 150 cm^2 culture petri dishes at 37 °C, 5% CO2, and 85% relative humidity overnight. Skin DCs that migrated from skin were recovered as previously described (Bautista et al., 2002).

Epidermal keratinocytes (EKS) were isolated from skin layers remaining after overnight skin DCs migration. The method for EK isolation was modified from a published protocol (Gray and Yardley, 1975). Briefly, small pieces of skin layers were digested in 0.05% trypsin-EDTA (Invitrogen) at 37 °C for 90 min with shaking. The cell suspension was withdrawn and more trypsin-EDTA added to the skin layers at 37 °C. After another 90 min, the reaction was stopped by adding 10% PBS and the cell suspension filtered to remove skin debris. The isolated cells were suspended in culture medium and viability assessed by trypan blue exclusion. Residual (CD1+) skin DCs in the EKS suspension was determined by flow cytometry.

Porcine alveolar macrophages (PAMs) were collected by lung lavage as previously described (Bautista et al., 2002). Briefly, lungs removed from a euthanized pig were filled with PBS containing antibiotics, gently massaged and the medium collected into a beaker. After centrifugation, contaminating erythrocytes were lysed in hypotonic buffer, followed by washes in PBS and the PAMs resuspended in culture medium.

For PBMC isolation, heparinized blood was diluted in an equal volume of PBS and layered over a separation gradient (Lymphoprep™, Life Technologies, Grand Is., NY) following the manufacturer's protocol. Negative selection with Dynal beads (Dynal Corp., Invitrogen, Carlsbad, CA) was used following the manufacturer's protocol to deplete PBMCs of T and B cells, and the remaining cells tested for langerin expression. Briefly, PBMCs were incubated with mouse antibodies to porcine CD3 and CD21 to eliminate T and B cells respectively and enrich for blood DCs. Bead-positive cells were then separated with a magnetic field. Isolated cells were used for flow cytometry and RNA isolation for RT-PCR.

2.3. Polymerase chain reaction

Cells for RNA extraction (5 × 10^5 skin DCs, PAMs or EKS) were preserved in Trizol (Invitrogen) and stored at −70 °C. As a negative control for langerin, RNA was also extracted from porcine monocyte-derived DCs differentiated from monocytes as previously described (Bautista et al., 2007). Total cellular RNA was isolated using the Invitrogen protocol for Trizol reagent and cDNA synthesized from approximately 1 μg of the extracted RNA as previously reported (Bautista et al., 2005).

For TaqMan realtime PCR, samples were analyzed in duplicates, with approximately 10 ng/well of cDNA used in a PCR mix of 25 μl containing optimal concentrations of primers, probes (Table 2) and the TaqMan universal PCR mastermix (Applied Biosystems, Foster City, CA), following the manufacturer’s protocol. Data was acquired in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The level of langerin mRNA expression for each sample was determined by the comparative cycle threshold (CT) method (user bulletin 2, Applied Biosystems), using ubiquitin gene as an endogenous control to normalize the data. Data for MoDCs was then used as a calibrator for the langerin expression in skin DC, EK and PAM samples applying the formula 2^-ΔΔCT (user bulletin 2, Applied Biosystems) to calculate the fold difference in mRNA. MoDCs were generated to yield DCs of high purity for preparation of polyA RNA as blood DCs are remarkably rare and purification above 50% very difficult.

2.4. Antibodies

All antibodies raised against human Langerhans cells and reactive with human langerin, clones 808E10.01, 310F7.02, 306G9.01, 817G7, and 122A11 (all mouse IgG1) and antibodies raised against recombinant human langerin and reactive with both mouse and human langerin, clones 929F3.01, 923B7, 926G4, 929F7 (all rat IgG2a) were provided by Dynetics (Lyon, France). Antibodies to porcine CD1 (clone 76–7–4, mouse IgG2a) and CD172 (formerly SWC3a) (clone 74–22–15, mouse IgG2b) were purchased from Southern Biotech (Birmingham, AL).

Table 2

<table>
<thead>
<tr>
<th>Primer or probe name*</th>
<th>Sequence (5’–3’)</th>
<th>Final concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-CD207-44F</td>
<td>GGAATGATGGCCCCCCTGTGA</td>
<td>900</td>
</tr>
<tr>
<td>p-CD207-152R</td>
<td>GCTTGGAACTAAGTGCAAGCT</td>
<td>900</td>
</tr>
<tr>
<td>p-CD207-88T</td>
<td>CGACCTTGTGGCCCCATGAAACCTGC</td>
<td>200</td>
</tr>
<tr>
<td>p-UBC-47F</td>
<td>GCGACACCCTGCTGACTACA</td>
<td>900</td>
</tr>
<tr>
<td>p-UBC-126R</td>
<td>AGATCTGATGCCACCCCTCTGA</td>
<td>900</td>
</tr>
<tr>
<td>p-UBC-79T</td>
<td>AGTCCACCTGCACCTGGTCCCTC</td>
<td>200</td>
</tr>
</tbody>
</table>

* p, porcine; F, forward primer; R, reverse primer; T, TaqMan TET-labeled probe. UBC, ubiquitin (house keeping gene).
2.5. Flow cytometry

The expression of the cell-specific antigens was analyzed by flow cytometry. Briefly, $1 \times 10^6$ cells were washed in chilled FACS buffer (PBS with 0.3% BSA and 0.1% sodium azide) prior to being stained for 30 min on ice with optimal dilutions of the specific antibodies (10 μg/ml for all the anti-langerin antibodies) or their respective isotype controls. For intracellular staining, cells were fixed and permeabilized with BD cytoperm/cytofix (BD Biosciences, San Jose, CA, USA) according to manufacturer’s protocol prior to incubation with specific antibodies. For double staining, cells were first stained for CD1, fixed, and permeabilized before staining for langerin. Either goat anti-mouse IgG-FITC (STAR117F, Serotec), goat anti-mouse IgG1-PE (STAR81PE, Serotec), goat anti-rat IgG-PE (AQ200H, Chemicon International, Temecula, CA, USA) were used as secondary antibodies for langerin staining. IgG1-PE (STAR81PE, Serotec) or goat anti-rat IgG-PE were further incubated with anti-rat IgG-Alexa Fluor 488 (Invitrogen) in blocking buffer at 37 °C for 60 min to detect langerin and CD1 respectively or the corresponding isotype controls. Note that the slides were washed between each step with BD perm/wash (BD Biosciences). Some slides were incubated with a nuclear stain (TOPRO-iodide, Invitrogen) for 5 min to reveal the architecture of the skin section. Slides were then mounted using a mounting medium for fluorescent microscopy (KPL, Gaithesburg, MD, USA), viewed on an OLYMPUS BX40 fluorescent microscope (Scientific Inc., Piscataway, NJ, USA) and images were captured with a DP–70 digital camera and analyzed using DP–BSW v2.2 software (Scientific Inc., Piscataway, NJ, USA).

2.8. Internalization of anti-langerin antibody

The internalization assay was performed as previously described (Valladeau et al., 1999). Briefly, skin DCs were washed in PBS containing 1% fetal calf serum (PBS-F) and stained with anti-langerin clone 122A11 on ice for 30 min. After washing twice with PBS-F, cells were further stained with biotin anti-mouse IgG (eBioscience) on ice for 30 min. As time zero, $5 \times 10^5$ cells were stained with streptavidin-PE (PharMingen) for 30 min. The rest of the cells were either incubated at 37 °C or on ice for 15 and 30 min. Some cells were fixed and incubated at 37 °C to assess the off-rate of the antibody at this temperature. At indicated timepoints, cells were washed in chilled FACS buffer and stained with streptavidin-PE as above. Finally, cells were analyzed by flow cytometry and antibody internalization measured as percentage decrease in mean fluorescence intensity relative to control samples kept on ice.

3. Results

3.1. CD207 cloning and predicted structural and functional analysis

The assembly of the porcine langerin gene (CD207) yielded a 1069 bp cDNA sequence containing the entire predicted coding region (Genbank EU442798). Nucleotides 1–24 contains part of the 5’ untranslated region. Nucleotides 1012–1069 contain part of the 3’ untranslated region. The predicted exon locations are as follows; exon 1bp 1–98, exon 2bp 99–217, exon 3bp 218–592, exon 4bp 593–744, exon 5bp 745–863, and exon 6bp 864–1096. The open reading frame of porcine CD207 is predicted to encode a 329 amino acid protein (Fig. 1). The predicted molecular weight is 37.5 kDa and if the protein is fully glycosylated, the molecular weight is predicted to be 40 kDa. Porcine CD207 is closest in size to the bovine (329) and human (328) proteins followed by the mouse (331), rat (332) and canine (200) proteins. The predicted canine protein is missing an internal stretch of 129 amino acids spanning the predicted membrane-proximal neck domain. The predicted porcine CD207 protein shares high amino acid identity/similarity with canine CD207 (76%/83%), human (75%, 86%) and bovine (73%/84%) CD207, and lesser amino acid identity/similarity to mouse (63%/77%) and rat (63%/76%) CD207 proteins.
Amino acids 23–29 of the predicted porcine CD207 molecule encode a proline rich potential signal transduction motif (WPREEPPP) that is conserved across species. In other proteins this motif associates with SH3 domain proteins and is involved in endocytic vesicle trafficking and cytoskeletal movement. Amino acids 46–68 encode the predicted transmembrane region. Amino acids 69–195 encode the membrane-proximal neck domain. This area displays a moderate level of conservation among species. According to the manufacturer, the rat anti-human monoclonal 929F3 used in this study was generated to an epitope in the neck domain. Two potential N-glycosylation sites occur in the membrane-proximal neck domain at amino acids 114 and 181. Amino acids 196–321 encode the C-type lectin domain. The tryptophan residue essential for human Bierbeck granule formation is conserved at position 263 (Verdijk et al., 2005). Amino acids 286–288 encode a conserved Glu-pro-asn (EPN) motif that is essential for mannose recognition (Stambach and Taylor, 2003). The C-type lectin domain contains conserved glutamic acid (262) and asparagine (289) residues and glutamic acid (286), asparagine (288, 308) and aspartic acid (309) residues that form 2 Ca2+ binding sites in the human CD207 protein (Stambach and Taylor, 2003).
evolutionary distance. One tree is shown in Fig. 2. Porcine and equine CD207 form a separate clade. Porcine CD207 is predicted to be closer to primate CD207 than rodent CD207, which forms a separate subclade with opossum CD207.

3.2. Transcription of langerin mRNA in porcine skin DCs

To demonstrate the transcription of langerin in porcine skin DCs, we designed a qRT-PCR assay to detect langerin specific mRNA. The level of langerin-specific mRNA in skin DCs was compared to that in PAMs and EKs, using MoDCs as the calibrator. Skin DCs \((n=3)\) expressed 105.3-fold more langerin mRNA than MoDCs. Though analysis of PAMs and EKs from three different animals showed 16.7- and 12.5-fold (respectively) more expression of langerin mRNA than MoDCs derived from the same three animals, these levels were significantly lower when compared to skin DCs.

3.3. Monoclonal antibody reactivity with porcine langerin

Of the nine monoclonal anti-human langerin antibodies tested for cross-reactivity with porcine langerin, three antibodies representing the difference in reactivity with intracellular verses extracellular langerin are shown in Fig. 3. Monoclonal antibodies 929F3 and 310F7.02 stained intracellular epitopes of langerin while clone 122A11 stained epitopes on the cell surface (Fig. 3). This dichotomy was reported by Saeland and colleagues (Valladeau et al., 1999) for another anti-langerin antibody, DCGM4, in the staining of human LCs. We previously reported DCGM4 does not cross-react with porcine skin DCs (Bautista et al., 2002). Analysis of the cellular localization of the epitopes recognized by the antibodies used here with the human langerin protein is available from the supplier, Dendritics (Lyon, France).

Two antibodies showed little or no reactivity with porcine langerin. Specifically, clone 808E10.01 showed moderate reactivity with a low mean fluorescence intensity (MFI) while clone 817G7 gave signal close to background level (Fig. 4). Seven clones reacted with 47–60% of DCs derived from porcine skin and four of these had high MFI (Fig. 4).

Porcine skin DCs are CD1 and CD172 double positive (Bautista et al., 2002). Therefore, to estimate the proportion of skin DCs that express langerin, we analyzed the CD1/CD172 double positive cells with anti-langerin and show that the percentage of skin DCs expressing langerin ranged from 32% to approximately 80% (Table 3). Indeed,
double staining of the skin DCs with anti-CD1 and anti-langerin clones 306G9.01 and 929F3.01 revealed reactivity with 50–73% of the CD1 positive cells in these two examples (Fig. 5A). Notably, these antibodies stained none or a negligible percentage of PAMs (Fig. 4A), blood DCs (Fig. 5B) and MoDCs (data not shown). Blood DCs were about 30% of the CD1 positive cells (reactive with both anti-CD1 and anti-CD172) after negative selection for CD3 and CD21, yet only 8% of the CD1 cells were detected with anti-langerin. It is worth noting that though blood DCs form the bulk of CD1/CD172 double positive cells, other mononuclear cells may also show this phenotype. Similarly, less than 8.0% of EKs isolated from the same skin biopsies as the DCs were stained (Fig. 4A). The small percent of EKs that stained with anti-langerin might represent residual skin DCs, as these were also CD1 positive (Fig. 5B).

3.4. Anti-langerin antibodies stain LCs in porcine skin by immunohistochemistry and immunofluorescent microscopy

Two anti-human langerin Mabs are recommended by the manufacturer for use in IHC, clones 306G9.01 and 310F7.02. We tested these antibodies for reactivity with porcine skin and show both antibodies reacted with LCs in the skin (Fig. 6). However, clone 310F7.02 apparently stained LCs in skin sections more intensely (Fig. 6A and B) compared to clone 306G9.01 (Fig. 6C and D). The langerin-positive cells were concentrated in the epidermis and isotype controls were negative (Fig. 6E).

Clone 929F3 is also recommended by the manufacturer for use in IHC on paraffin sections. We obtained a relatively high fluorescent intensity with this antibody by flow cytometry (Figs. 4B and 5A, right hand panel). We therefore used this antibody to stain isolated skin DCs and frozen
sections of porcine skin for immunofluorescent microscopy. Isolated skin DCs readily reacted with 929F3 antibody to human langerin (red, Fig. 7A) and also expressed CD1 (green, Fig. 7B), as further disclosed in Fig. 7C by the yellow color when the images were merged. Similarly, in situ, langerin expressing cells in skin (Fig. 7D) also expressed CD1 (Fig. 7E). Fig. 7F shows the double positive cells (yellow color resulting from the merge of images from Fig. 7D and E) and reveals LCs in situ with cytoplasmic projections (dendrites) characteristic of DCs. Cell nuclei were stained with TOPRO-iodide to outline the skin section and indicates that the langerin/CD1 coexpressing cells were found mainly in the epidermis (Fig. 7G). Anti-human langerin clone 923B7 also stained LCs in skin sections by IF (data not shown).

3.5. Anti-langerin antibody is internalized into porcine skin DC

Anti-langerin clone 122A11 binds an extracellular epitope of langerin (Fig. 3E) and can be endocytosed into BGs. An analysis of porcine skin DC interaction with this antibody revealed that anti-langerin antibody is rapidly endocytosed, with mean surface expression declining to approximately 50 and 30% after 15 and 30 min at 37 °C respectively when compared to cells incubated on ice for the same length of time (Fig. 8).

4. Discussion

We have previously isolated and characterized skin DCs of swine (Bautista et al., 2005, 2002). These cells are distinguished from other porcine mononuclear cells by coexpressing CD1, CD172 and S100 at high levels and no expression of CD14. Swine DCs derived from bone marrow express CD1, CD14 and CD172 (Carrasco et al., 2001) whereas monocyte-derived DCs from peripheral blood are CD172 positive, CD1 dull and CD14 negative (Bautista et al., 2007; Summerfield et al., 2003). All of these DC subsets are also characterized by high expression of MHC class II, CD80/86 and the ability to take up and process antigen and the induction of primary allogenic responses (Bautista et al., 2007; Summerfield et al., 2003). However, a distinguishing feature of LCs is the expression of langerin (CD207) concentrated in racket-shaped Birbeck granules within the cytoplasm (Valladeau et al., 2000).

Here, we characterize the porcine langerin gene, presenting the full length sequence and comparison with the CD207 sequence from other species presently in the database. Interestingly, where the porcine gene is most closely related to the equine, these two genes are more closely related to the simian species than are the rodent genes to simian. The predicted porcine CD207 protein shares (75%/86%) amino acid identity/similarity with human and lesser amino acid identity/similarity to mouse (63%/77%) and rat (63%/76%) CD207 proteins suggesting that antibodies to human langerin would be more likely to cross react with the porcine protein than antibodies specific for the murine CD207. In fact, binding data for the human protein using some of the anti-human CD207 antibodies tested in swine, predict specificity for the neck region of the protein, just external to the transmembrane region. This region is highly conserved in the simian, equine and porcine genes.

Before testing for protein, we confirmed the presence of langerin mRNA in skin DCs by PCR. These DCs had 105.3-fold more langerin-specific mRNA than MoDCs. However, there were detectable levels of langerin mRNA in both EKs and PAMs. EKs contained about 8.5% residual skin DCs as determined by flow cytometry. Given that PCR amplifies small quantities of RNA, the langerin detected in the EKs was confirmed by reverse transcriptase PCR, which showed 8.8%.

| Clone     | % LCs | n
|-----------|-------|---
| 808E10.01 | 32.5 ± 26.3 | 3
| 310F7.02  | 75.6 ± 16.0  | 3
| 306G9.01  | 66.9 ± 11.8  | 3
| 817G7     | 3.8 ± 0.7    | 3
| 929F1.01  | 74.7 ± 13.6  | 3
| 923B7     | 79.7 ± 9.2   | 3
| 926G4     | 78.0 ± 14.1  | 3
| 929F7     | 77.3 ± 19.4  | 3

a Langerin positive cells expressed as a percentage of CD1+ CD172+ double positive.
could be from the residual skin DCs. Similarly, PAMs had detectable levels of langerin mRNA. LCs are present in the lining of the respiratory tract and could be present in small numbers within the broncho-alveolar lavage cells along with the PAMs. In fact, Valladeau et al. (1999) detected significant levels of langerin in the lungs which they attribute to LCs lining the pulmonary epithelium.

Though LCs have been described in the pig based on the microscopic detection of BGs (Monteiro-Riviere, 1992; Romano and Balaguer, 1991), we could not confirm the presence of these granules in the porcine skin DCs described above (Bautista et al., 2002). Similarly, other researchers have failed to identify these granules in porcine epidermal DCs (Gregg et al., 1995a). This discrepancy could be addressed by identifying antibodies that react with porcine langerin. This C-type lectin is the principal protein component of the BGs and is found exclusively in LCs among skin DCs (Valladeau et al., 2000). Therefore, antibodies against langerin have been used to identify human LCs (Valladeau et al., 2000) and the present study shows porcine skin DC also express CD207/langerin.

We tested multiple anti-human langerin monoclonal antibodies for cross-reactivity with swine CD207/langerin and identified monoclonal antibodies that reacted with a significant proportion of porcine skin DCs. These antibodies showed very minimal reactivity to blood DCs, PAMs and EKs obtained from the same animals, suggesting that the reactivity was specific for porcine LCs. Based on the coexpression of langerin and CD1, we estimate that LCs make up at least 70% of DCs isolated from porcine skin. LCs are known to be the principal DCs of the skin and the langerin-negative skin DCs are probably of dermal origin, which are known to share other characteristics with LCs (Lenz et al., 1993).

We subsequently used these crossreactive anti-huCD207/langerin antibodies to identify LCs in situ in porcine skin sections by IHC and IF. Cells expressing both CD207 and CD1, with the characteristic dendrites of DCs, were found predominantly in the epidermis. This is in
agreement with the electron microscopic description of LCs in pig skin by Romano and Balaguer (1991). These investigators identified cells with characteristic BGs in the basal and suprabasal layers of the porcine epidermis. This is also consistent with data from human and other species in which LCs are typically found in the epidermis and stratified epithelia but can migrate to regional lymph nodes upon activation (Lukas et al., 1996).

Our previous report failed to identify BGs by electron microscopy, implying the absence of LCs (Bautista et al., 2002). Various factors could account for this including the failure to visualize BGs in certain studies due to the age of the donor animals or the handling or processing of samples for electron microscopy. Indeed, LCs are rare in the skin of young pigs (Monteiro-Riviere, 1992) and LCs may lose the characteristic BGs in the maturation process in vitro (Romani et al., 1989). A more definitive way to identify LCs in porcine is by the reactivity of these antibodies against langerin. In fact, analysis of langerin expression by DC of the skin by immunohistochemistry presented in this report (Fig. 6) shows very similar staining as we previously reported for CD1, CD172 and Class II MHC in skin sections when considering either the epidermal location of cells expressing these proteins or the number (Bautista et al., 2002). Further, detection of both CD1 and langerin in frozen skin sections shows all cells reacting with either antibody, react with both (Fig. 7). Taken together, these data strongly indicate the vast majority of dendritic cells (CD1/CD172 expressing cells) in the epidermis are expressing langerin and are bonafide Langerhans cells.

Pigs have been proposed as important models in human medicine and potential organ donors for xeno-
transplantation because of physiological and immunological similarities with humans (Dunning et al., 1994; Sablinski et al., 1997; Sim et al., 1999). A better understanding of porcine immune responses compared to humans is required in order to fully exploit the potential of pigs for modeling human disease. Knowledge of the role of LCs in porcine immunity is critical to achieving this understanding. Grabbe et al. (1994) showed that inbred miniature pigs possess skin APCs which function in the same way as human and murine LCs. In addition, other studies have shown that porcine skin DCs are important in delayed hypersensitivity reactions (Grabbe et al., 1994; McFarlin and Balfour, 1973) and are involved in allergic contact dermatitis (Vana and Meingassner, 2000). Our data show that porcine skin DCs transcribe CD207/langerin mRNA and express langerin protein, which is reactive with antibodies raised against human langerin and that the majority of DCs in porcine skin epithelium are LCs.

**Fig. 8.** Anti-langerin antibody clone 122A11 is internalized into porcine skin DC at 37°C. Skin DCs were stained with anti-langerin clone 122A11, followed by biotin anti-mouse IgG, incubated at 37°C for indicated time periods and antibody binding revealed with streptavidin-PE. Surface antibody intensity at 37°C (●) was expressed as a percentage of that on cells incubated on ice (○) for an equal time. Fixed cells (●) were incubated at 37°C served as controls for the off-rate the antibody.

**Fig. 7.** Immunofluorescent staining of porcine LCs in cytopsins of isolated skin DCs and in situ in skin cryosections: Cytopsins of skin DCs and cryosections of porcine skin were fixed before staining with optimal concentrations of specific antibodies or isotype control for langerin (clone 929F3) and CD1. Images were captured from same sections for langerin+ cells (red) and CD1+ cells (green) and the images fused to reveal doubly stained cells (yellow). Langerin+ cells (A for cytopsins and D for in situ) were also CD1+ (B for cytopsins and E for in situ). C and F show a composition of the respective preceding figures. G shows TOPRO-stained nuclei of all cells to reveal the architecture of the skin. d = dermis; e = epidermis. Similar results were obtained from two animals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
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References


