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Expression of toll-like receptor 2 (TLR2) in porcine leukocyte subsets and tissues

Belén ÁLVAREZ¹, Concepción REVILLA¹, Nieves DOMÉNECH², Carlos PÉREZ¹,
Paloma MARTÍNEZ¹, Fernando ALONSO¹, Angel EZQUERRA¹,
Javier DOMÍGUEZ^{1*}

¹ Dpto. Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA),
28040 Madrid, Spain

² Unidad de Investigación, CHU Juan Canalejo, 15006 La Coruña, Spain

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Abstract – Toll-like receptors (TLR) are a group of pattern recognition molecules that play a crucial role in innate immunity. TLR2 recognises a variety of microbial components leading to the development of inflammatory and immune responses. To characterise the expression and functional properties of porcine TLR2 (pTLR2), we have raised a panel of monoclonal antibodies (mAb) against this molecule. Mouse 3T3 cell transfectants expressing pTLR2 were used for immunisation of mice. The specificity of these antibodies was confirmed by their reactivity with CHO cells transfected with pTLR2 but not with pTLR4 or with non-transfected cells. Using one of these mAbs, named 1H11, pTLR2 was found on cells of the innate immune system, including monocytes, macrophages, and granulocytes, but not on peripheral blood lymphocytes. Staining of tissue sections showed that pTLR2 is also expressed on epithelial cells lining the tracheobronchial and intestinal tracts, bile ducts in the liver and renal tubules, and on the basal layer of the epidermis. This distribution is consistent with a surveillance function at entry sites, allowing for early detection of microbial invasion.

toll-like receptor / monoclonal antibody / monocyte / epithelial cell / pig

1. INTRODUCTION

Cells of the innate immune system express a range of receptors referred to as pattern-recognition receptors (PRRs) that serve to sense microbial infection leading to development of inflammatory and immune responses. These receptors recognise conserved molecular structures, called pathogen-associated molecular patterns (PAMPs), which are shared by large groups of micro-organisms [21].

Toll-like receptors (TLRs) constitute an ancient family of PRRs characterised by an extracellular domain containing multiple leucine-rich repeats (LRRs) and a cytoplasmic domain homologous to that of the interleukin 1

(IL-1) receptor, known as the TIR domain. Up to date 13 TLRs have been identified in mammals, although their expression varies among species [5].

Stimulation of macrophages or mast cells through their TLRs leads to synthesis and secretion of pro-inflammatory cytokines and other mediators, thereby initiating an inflammatory response. Additionally TLR activation of dendritic cells induces the initiation of an adaptive immune response through the upregulation of co-stimulatory molecules [20, 31].

TLR2 recognises a variety of microbial components including lipoproteins and lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from

* Corresponding author: juncal@inia.es

mycobacteria, and zymosan from yeasts. It is believed that TLR2 associates in heterodimers with TLR1 and TLR6 [31]. Whereas the response to diacylated bacterial lipoproteins requires TLR2 and TLR6, the response to triacylated bacterial lipoproteins depends on TLR2 and TLR1 [32, 33]. TLR2 ligands induce maturation of DCs with up-regulation of MHC class II, CD80 and CD86 [18, 25] and secretion of IL-12 from monocytes and DC [6, 34]. Upon ligation TLR2 is rapidly internalised into endosomes. Targeting of antigen to TLR2 may therefore be a useful vaccination strategy for enhancing CD4⁺ T cell responses by delivering epitopes into the MHC class II pathway [29].

To characterise the expression and functional properties of porcine TLR2, we raised a panel of monoclonal antibodies (mAb) against this molecule. Using one of these mAbs, named 1H11, pTLR2 was found on cells of the innate immune system, including monocytes, macrophages, and granulocytes, but not on peripheral blood lymphocytes. Staining of tissue sections showed that TLR2 is also expressed on epithelial cells lining the tracheobronchial and intestinal tracts, bile ducts in the liver, and renal tubules, and on the basal layer of the epidermis, a distribution consistent with its surveillance function of bacterial invasion.

2. MATERIALS AND METHODS

2.1. Tissues and cells

Tissue samples for immunohistochemical analyses were collected from three healthy, conventionally reared, 3-month-old Large-White pigs that had been euthanised with azaperone and ketamine.

Blood samples were obtained from six 14- to 20-month-old Large-White outbreed pigs. Peripheral blood mononuclear cells (PBMC) were isolated on Percoll (Pharmacia, Uppsala, Sweden) discontinuous gradients after blood sedimentation in Dextran (Pharmacia), as has been previously described [17]. Granulocytes were recovered from the lower Percoll phase after lysis of residual erythrocytes by hypotonic treatment.

Blood monocytes were magnetically isolated using anti-CD172a mAb BA1C11 [2] and the VarioMACS cell sorting technique (Miltenyi Biotec, Bergisch-Gladbach, Germany). Dendritic cells (DC) were derived from monocytes by culturing

them in the presence of rpGM-CSF and rpIL-4, as described elsewhere [10]. On day 5, rpTNF- α was added to induce their maturation. Alveolar macrophages were collected by bronchoalveolar lavage as described by Carrascosa et al. [9].

Mouse embryo NIH/3T3 cells were obtained from the American type culture collection (ATCC, Manassas, VA) and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (BioWhittaker, Verviers, Belgium) supplemented with 30 μ g/mL gentamicin, 2 mM L-glutamine and 10% fetal bovine serum (FBS) (BioWhittaker), at 37 °C in a humidified 5% CO₂ atmosphere.

The reported experiments have been executed in full compliance with guidelines by the ethical committee of the Institute.

2.2. Cloning of porcine TLR2 cDNA

Messenger RNA was extracted from porcine alveolar macrophages using μ MACS mRNA isolation kit (Miltenyi Biotec). One microgram of poly A⁺ RNA was used to synthesise cDNA and a series of 5'- and 3'-rapid amplification of cDNA ends (RACE) PCR reactions were performed to determine the full-length TLR2 cDNA sequence using the SMARTTM RACE Amplification Kit (BD Biosciences Clontech, CA, USA). TLR2-specific 5' and 3'RACE primers (Tab. I) were designed based on a porcine TLR2 partial coding sequence (CDS) (GenBank accession number AY289531). The PCR products from partial TLR2 cDNAs were cloned into pcDNA3.1/V5-His TOPO vector using a TA cloning kit (Invitrogen, San Diego, CA, USA). The plasmid DNAs were purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and sequenced with universal oligonucleotides T7 and BGH.

While we were checking the sequences of our clones, it was published in the databases the first complete CDS of porcine TLR2 (GenBank accession number AB085935). Based on our results and on the new sequence, the primers ST2F and ST2R (Tab. I) were designed for PCR amplification of full-length coding cDNA of pig TLR2. These primers flank the TLR2 translation start and stop codons. cDNA from alveolar macrophages was amplified using BD AdvantageTM 2 PCR Enzyme System as recommended by the manufacturer (BD Biosciences Clontech). Amplification was performed by 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and finally 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. A

Table I. Primer sequences used in this study.

Primer	Sequence	Size (bp)	
R4F	TGG TGT CCG TCG TGT GCT GTG CCC TCT TCC TG	—	3'-RACE PCR
R5R	AGC AGC AGG AAG AGG GCA CAG CAC ACG ACG GAC	—	5'-RACE PCR
ST2F	GGA CCA TGC CAT GTG CTT TGT GGA CAG CAT GGG	2 402	Full-length TLR2
ST2R	AGA CCA GCA TCG GAC CAA GAC TGG CCT TTC		
T2ATGF	GGA CCA TGC CAT GTG CTT TGT GG	2 361	TLR2-GFP
T2CDSGFPR	GGG ACT TGA TCG CAG CTC TCA AAT TT		

band of 2 300-2 500 bp was purified after agarose gel electrophoresis of PCR-amplified products, and cloned into the pcDNA3.1/V5-His TOPO (Invitrogen). Different clones were sequenced to confirm the fidelity and orientation of inserts. The sequence obtained only differs from those reported by Muneta et al. [26] (accession number AB085935) and Tohno et al. [35] (accession number AB072190) by 3 and 13 nucleotides, respectively, resulting in 2 amino acid differences with the former sequence (3 R→C and 616 Q→L) and 4 with the latter (126 A→T, 338 T→A, 451 G→S, and 633 L→R).

2.3. Construction of eukaryotic expression vector for porcine TLR2

Based on the complete sequence of porcine TLR2, primers T2ATGF and T2CDSGFPR (Tab. I) were designed to clone the full-length ORF as a fusion protein to GFP by PCR. A 2361-bp product was cloned into pcDNA3.1/CT-GFP-TOPO (Invitrogen) according to the manufacturer's protocol. Several clones of this construct (pTLR2-GFP) were sequenced to confirm the orientation and the integrity of the inserts.

2.4. Transient expression of TLR2-GFP construct and analysis by flow cytometry

NIH/3T3 or CHO cells were transfected with plasmid pTLR2-GFP encoding full-length TLR2-GFP fusion protein by using the LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the day prior to transfection, cells were seeded into 6-well plates (3×10^5 cells/w) and grown in DMEM supplemented with 30 µg/mL gentamicin, 2 mM L-glutamine, and 10% FBS. Immediately before transfection, cells were rinsed with medium without serum nor antibiotic (transfection medium, TM). For each well, 1 µg of plasmid DNA plus 6 µL of PLUS reagent were mixed, diluted with 500 µL of TM and incubated for 15 min at room temperature. Then 4 µL of Lipofectamine were mixed with

500 µL of TM and added to the former mixture. After 15 min incubation, the DNA/LipofectAMINE PLUS complexes were added to the cells. Cells were incubated for 3 h at 37 °C in 5% CO₂, and then 1 mL of fresh growth medium was added. Cells were harvested at 24 h post-transfection and directly analysed on a FACScalibur flow cytometer. Cells transfected with a plasmid encoding the full-length pTLR4-GFP fusion protein [1] or non-transfected cells were used as negative control.

2.5. Monoclonal antibody production

To raise mAb against porcine TLR2 10-week-old BALB/c mice were i.v. immunised with pTLR2-GFP-transfected NIH/3T3 mouse cells (2×10^6 cells/0.1 mL of sterile PBS), and boosted on days 15 and 30 with the same amount of cells. Serum from immunised mice was collected 7–10 days after each boost, and the presence of specific Abs was tested in flow cytometry using pTLR2-GFP-transfected CHO cells; as a negative control, non-transfected CHO cells were used. Selected mice were boosted i.v. with 2×10^6 cells in 0.1 mL sterile PBS four days before fusion of spleen lymphocytes with the SP2/0 murine plasmacytoma, using polyethylene glycol 4000 (Merck, West Point, PA, USA) according to current protocols [23].

Class and subclass of mAbs were determined by immunodot with a mouse monoclonal antibody isotyping test kit from Serotec (Oxford, UK). MAb 1H11 is isotype IgG₁κ.

MAbs to porcine CD3 (BB23-8E, IgG_{2b}), CD4 (74-12-4, IgG_{2b}), and CD8α (76-2-11, IgG_{2a}) were kindly provided by M. Pescovitz (Indiana University, USA); mAb to CD16 (G7, IgG₁) was a gift from Y.B. Kim (Finch University of Health Sciences, IL, USA); mAbs to porcine CD172a (BA1C11, IgG₁) and CD45RA (3C3/9, IgG₁) were produced in our laboratory; mAbs to CD8β (PG164A, IgG_{2a}) and TCRγδ (PGBL22A, IgG₁) were purchased from VMRD (Pullman, WA, USA)

and mAb to CD21 (B-Ly4; IgG₁) was obtained from BD Pharmingen (CA, USA).

For multi-colour immunofluorescence assays, mAbs were purified by affinity chromatography on Protein A-Sepharose CL4B (Pharmacia), and labelled with either biotin (Sigma, Saint Louis, MO, USA), Alexa Fluor 488 or Alexa Fluor 633 (Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol.

2.6. Flow cytometry

For single-colour staining, cells (5×10^5) were incubated with 75 μ L hybridoma supernatant for 30 min at 4 °C. After two washes in PBS containing 0.1% BSA and 0.01% sodium azide (washing buffer), cells were incubated with FITC or phycoerythrin (PE)-conjugated rabbit F(ab')₂ anti-mouse Ig (Dako, Glostrup, Denmark). Then, they were washed and fixed in 0.1% formaldehyde prior to analysis on a FACScalibur cytometer (Becton Dickinson, USA). Irrelevant isotype-matched mAbs were used as negative controls.

For two-colour staining, after incubation with FITC-conjugated rabbit F(ab')₂ anti-mouse Ig, cells were washed and free binding sites blocked with 5% normal mouse serum for 15 min. Then, biotinylated mAbs were added and detected with streptavidin-PE (BD Pharmingen). Subsequently, cells were washed and fixed in 0.1% formaldehyde prior to analysis on the cytometer. Irrelevant isotype-matched mAbs, unlabelled or labelled with biotin were used as negative controls.

For three-colour staining, cells were incubated with 1H11 hybridoma supernatant and Alexa 488-labelled anti-CD4 mAb for 30 min at 4 °C. After washing, cells were incubated for 30 min at 4 °C with PE-conjugated goat F(ab')₂ anti-mouse IgG1 (Southern Biotech, AL, USA). Cells were washed and blocked with 5% normal mouse serum, and then Alexa 633-labelled anti-CD172a mAb was added. Cells were washed twice and fixed in 0.1% formaldehyde prior to analysis in the cytometer. Irrelevant isotype-matched mAbs, unlabelled or labelled with the different fluorochromes, were used as negative controls.

2.7. Immunoprecipitation analysis

Non-transfected or pTLR2-GFP-transfected CHO cells (3×10^6) were washed twice with PBS and solubilised in 0.3 mL of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 10 μ g/mL aprotinin and 1 mM

phenylmethylsulfonylfluoride) for 1 h at 4 °C. After centrifugation at $12\,000 \times g$ for 30 min, the supernatants were precleared with 25 μ L of a 25% (v/v) suspension of protein G-Sepharose (Pharmacia) in lysis buffer, and then subjected to immunoprecipitation with mAb 1H11 or an irrelevant mAb. Hybridoma supernatant (300 μ L) was added to 0.1 mL of lysate and incubated for 2 h at room temperature. Then, 40 μ L of 25% (v/v) suspension of protein G-Sepharose were added and incubated for 1 h with gentle mixing. Beads were washed three times with lysis buffer and boiled in electrophoresis sample buffer (0.062 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.7 M 2-mercaptoethanol, 0.001% bromophenol blue). Bound proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and analysed by Western blotting with an anti-GFP mAb conjugated to horseradish peroxidase (HRP) (Miltenyi Biotec). Peroxidase activity was visualised with the ECL detection assay following the recommendations of the manufacturer (Amersham, Uppsala, Sweden).

2.8. Immunohistochemical analyses

Part of the tissue samples were snap frozen in isopentane/liquid nitrogen and stored at -80 °C. Frozen sections were cut at 6 μ m thick, mounted on poly-L-lysine coated glass slides, and air-dried overnight at room temperature. Slides were then fixed for 10 min in acetone and stained according to previously described protocols [13].

Another part of the tissues were fixed in neutral buffered 10% formalin for 12 h, and embedded in paraffin wax. Serial 4 μ m sections were cut, placed on silane coated slides, and allowed to dry at room temperature. Slides were dewaxed in xylene and rehydrated through graded alcohols to distilled water. Slides were then subjected to heat-mediated antigen retrieval treatment in 10 mM Tris, 1 mM EDTA pH 9.0, and stained as previously described [13].

2.9. Effect of anti-TLR2 antibody in TNF- α production

To induce TNF- α production through TLR2, PBMC were cultured in 12-well tissue culture plates at concentrations of 5×10^6 cells/well with Pam₃CysSer(Lys)₄ (hereafter P₃CSK₄) (100 ng/mL) or Malp-2 (1.3 ng/mL) (both from Alexis Biochemicals, Lausen, Switzerland) for 24 h at 37 °C. Concentrations of TNF- α in supernatants were measured by ELISA (BioSource, CA, USA). Blocking of the PBMC activation was attempted

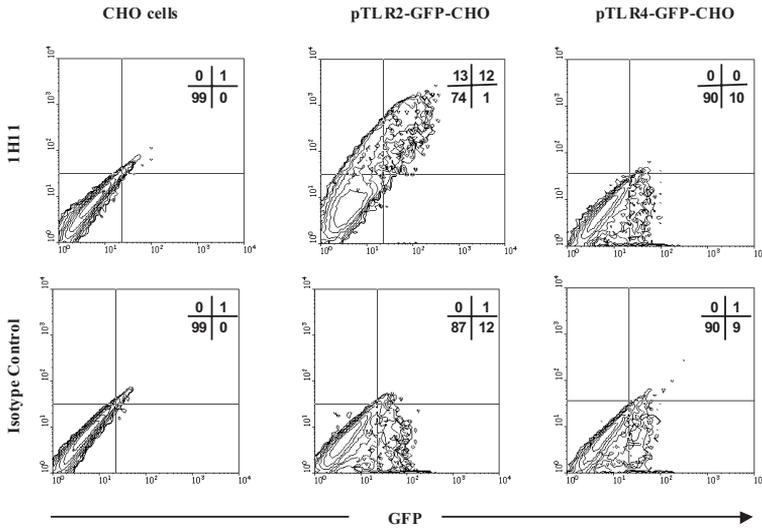


Figure 1. MAb 1H11 specifically recognises porcine TLR2. CHO cells transfected with the pTLR2-GFP or pTLR4-GFP constructs were stained with mAb 1H11 or an irrelevant isotype-matched antibody followed by PE-rabbit F(ab')₂ anti-mouse Igs and analysed by flow cytometry. A control staining of non-transfected CHO cells is also shown.

by incubating cells with mAb 1H11 (50 µg/mL) or an isotype-matched control antibody, for 30 min at 37 °C prior to the addition of the stimuli.

Additionally, the agonistic activity of mAb 1H11 on TNF-α production was tested by incubating PBMC with this antibody or an isotype-matched control antibody, cross-linked or not with rabbit anti-mouse Ig (Dako, Glostrup, Denmark), for 24 h.

3. RESULTS

3.1. MAb 1H11 specifically recognises porcine TLR2 on transfected cells

To generate anti-porcine TLR2 mAbs, TLR2 cDNA was amplified and cloned into pcDNA3.1/CT-GFP-TOPO to obtain a GFP-tagged version of the porcine TLR2, that was then used to transfect mouse NIH/3T3 cells. These cells were used for mice immunisation. The specificity of mAbs obtained was checked by flow cytometry. MAb 1H11 reacted with CHO cells transfected with the pTLR2-GFP construct, as shown by the co-expression of GFP fluorescence and 1H11 labelling, but not with CHO cells transfected with a pTLR4-GFP construct or with non-transfected cells (Fig. 1). Moreover, when lysates from pTLR2-GFP-transfected CHO cells were immunoprecipitated with mAb 1H11 and subsequently analysed by Western blotting with an HRP-conjugated anti-GFP mAb, a specific band of 115 kDa, the expected size for the TLR2-GFP fusion protein, was clearly detected (Fig. 2).

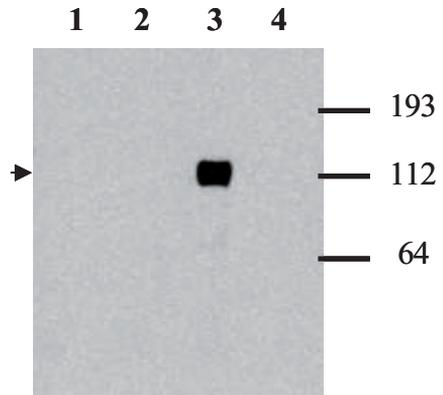


Figure 2. Immunoprecipitation of pTLR2 with mAb 1H11. Cells lysates (3 × 10⁶ cells) were subjected to immunoprecipitation with mAb 1H11 (1 and 3) or an irrelevant mAb (2 and 4). Bound proteins were resolved by 7.5% SDS-PAGE under reducing conditions and analyzed by Western blotting with an HRP-conjugated anti-GFP mAb. Lane 1 and 2: non-transfected CHO cells, lane 3 and 4: CHO cells transfected with pTLR2-GFP. Arrowhead show specific band corresponding to the full TLR2 fused to GFP (115 kDa). Numbers on the right indicate the position of MW markers.

These results confirm the specificity of this antibody for porcine TLR2.

3.2. Distribution of TLR2 in leukocyte subsets

The reactivity of mAb 1H11 was next analysed by flow cytometry on different blood cell

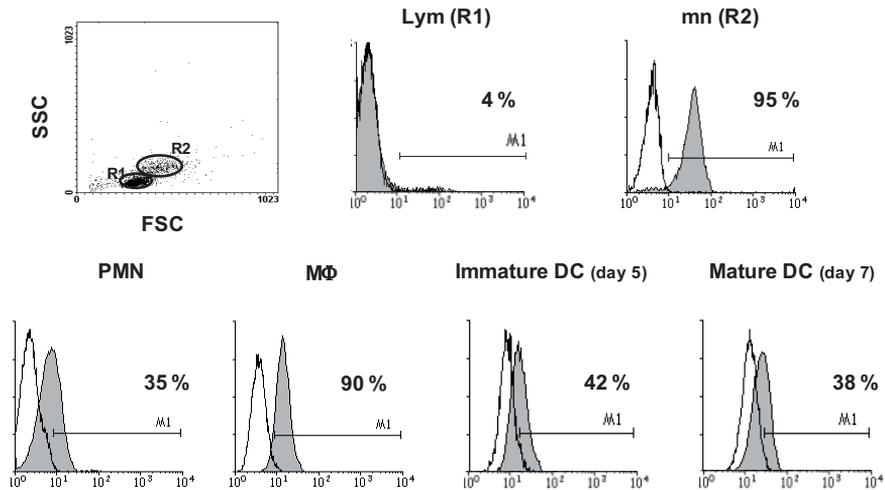


Figure 3. Porcine TLR2 is expressed on cells of the innate immune system. Staining of lymphocytes (Lym), monocytes (mn), granulocytes (PMN), alveolar macrophages ($M\Phi$), immature DC (day 5) and mature DC (day 7). Cells were stained with mAb 1H11 followed by FITC-rabbit F(ab')₂ anti mouse Ig. An irrelevant isotype matched mAb was used as negative control (open histogram). Lymphocytes (R1) and monocytes (R2) were gated according to their forward (FSC) and side scatter (SSC) profiles. Results are representative of three independent experiments.

populations. TLR2 was detected at high levels on a subset of peripheral blood mononuclear cells, which, accordingly to their FSC and SSC characteristics, corresponded to monocytes. It was also detected, although at lower levels, on the majority of granulocytes. Lymphocytes were negative. Alveolar macrophages also stained positive but with lower intensity than monocytes. Differentiation of monocytes into DCs in the presence of GM-CSF and IL-4, resulted in down-modulation of TLR2 expression (Fig. 3).

The distribution of porcine TLR2 on PBMC was further examined by two-colour flow cytometry (Fig. 4). Porcine TLR2 antigen was expressed on most of CD172a⁺, CD16^{hi} monocytes. No significant expression was detected on the surface of T cells or their subsets as defined by the expression of CD3, CD4⁺, CD8 α ⁺, CD8 β ⁺, or $\gamma\delta$ TCR markers. NK and B cells, identified as CD16^{lo} and CD21⁺ cells respectively, were also negative.

Within the TLR2⁺CD172a⁺ cells, a small population expressing low levels of CD172a was identified. Since plasmacytoid dendritic cells (pDC) have been shown to express

low levels of CD172a, we carried out three-colour flow cytometric analyses using a CD4/CD172a/TLR2 combination, to investigate whether these cells corresponded to this lineage. As shown in Figure 5, these cells did not express CD4 and therefore did not fit the phenotype described for pDC [30].

3.3. TLR2 is expressed at portals of pathogen entry

The distribution of TLR2 in non-lymphoid tissues was analysed by immunohistochemistry on frozen sections (Fig. 6). In the lung, epithelial cells lining tracheobronchial airways were strongly stained, whereas alveolar macrophages were barely detectable. In the small intestine, epithelial cells of villi showed a weak staining that appeared to be predominantly intracellular, whereas goblet cells were negative. The lamina propria was mainly negative. TLR2 expression was also observed in the epithelial cells of renal tubules and in bile ducts within the liver but not in hepatocytes. In the skin, mAb 1H11 strongly labelled the basal layer of the epidermis; positive cells were also occasionally seen in dermis. A similar

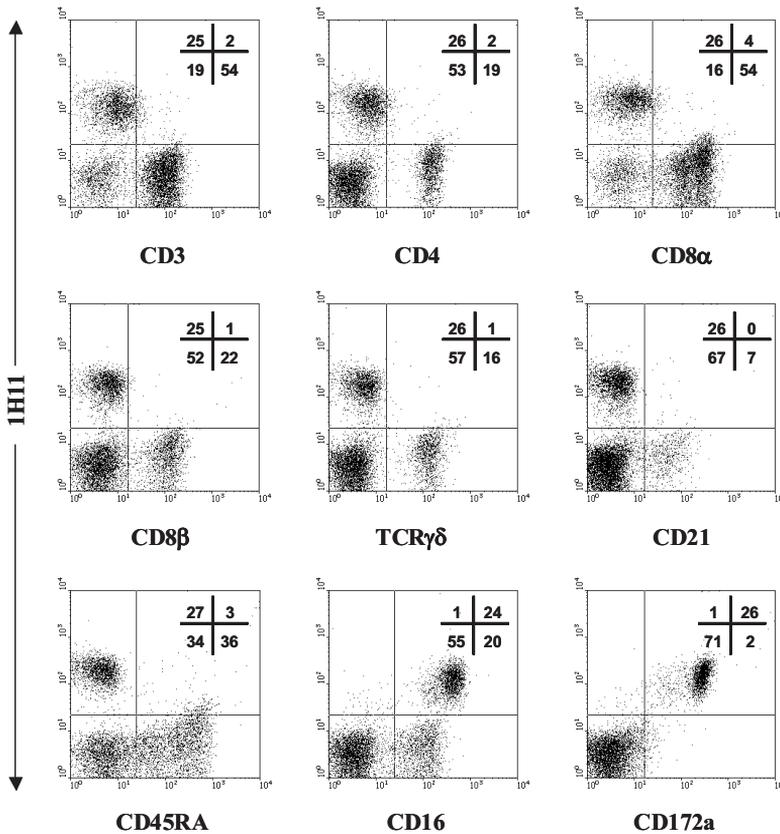


Figure 4. Two-colour flow cytometric analysis of TLR2 expression in PBMC subsets. PBMC were double-stained with anti-CD3, anti-CD4, anti-CD8 α , anti-CD8 β , anti-TCR $\gamma\delta$, anti-CD21, anti-CD45RA, anti-CD16 or anti-CD172a mAbs, and rabbit F(ab')₂ anti-mouse Ig FITC (x-axis), followed by biotin-conjugated 1H11 and streptavidin-PE (y-axis). Isotype-matched irrelevant mAbs, unlabelled or biotin-labelled were used as negative controls. Thirty thousand events were acquired. The data shown are from a representative experiment out of four performed with cells from different donors.

staining pattern was observed on formalin-fixed paraffin-embedded skin sections that have been subjected to heat-mediated antigen retrieval treatment (Fig. 6F).

3.4. Effect of mAb 1H11 on TNF- α production by PBMC in response to lipopeptides

Lipopeptides are known to activate monocytes/macrophages through a TLR2-dependent pathway leading to the secretion of significant levels of TNF- α . To investigate whether mAb 1H11 was able to interfere TLR2-mediated cell activation, we treated PBMC with this mAb before the addition

of P₃CSK₄, a ligand for TLR1/TLR2 heterodimer, or Malp-2, that binds to TLR2/TLR6 heterodimer, and measured the production of TNF- α . As shown (Fig. 7), mAb 1H11 did not block the production of TNF- α triggered by P₃CSK₄ or Malp-2. This mAb also failed to induce TNF- α production, even after cross-linking with an anti-mouse Ig antibody (data not shown).

4. DISCUSSION

Invasion of host tissues by microbial pathogens causes the activation of first-line defense mechanisms. TLRs are a family of

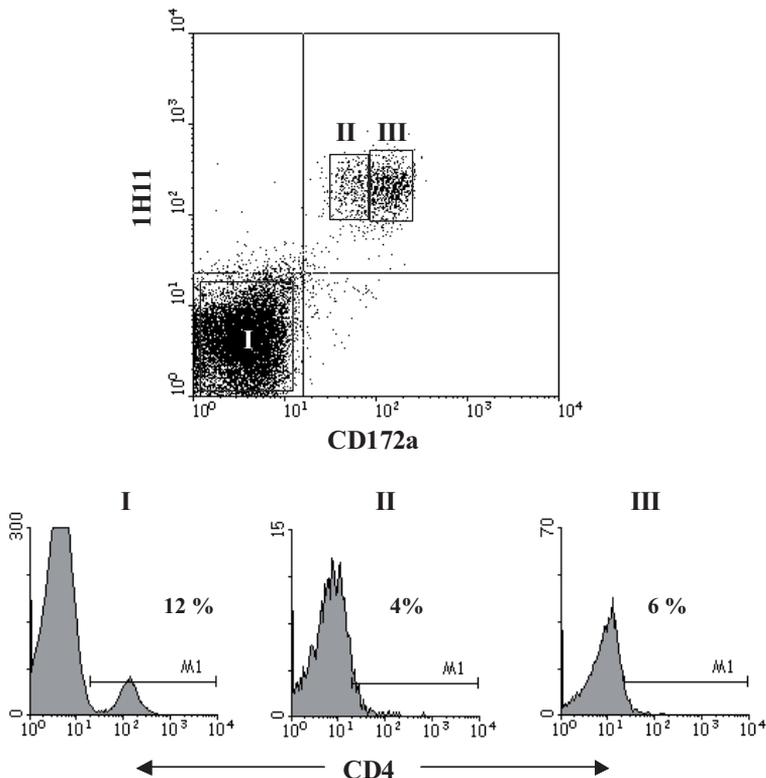


Figure 5. CD4 expression in CD172a⁻ 1H11⁻ (I), CD172a^{lo} 1H11⁺ (II), and CD172a^{hi} 1H11⁺ (III) cells assessed by three-colour immunofluorescence. Cells were stained, as described in Materials and Methods, with anti-CD4 labelled with Alexa 488 and mAb 1H11 followed by PE-conjugated goat F(ab')₂ anti-mouse IgG1, and then with Alexa 633-labelled anti-CD172a. Isotype-matched irrelevant mAbs, unlabelled, labelled with Alexa 488 or Alexa 633 were used as negative controls. One hundred thousand events were acquired. Data from one representative out of four independent experiments are shown.

pattern-recognition receptors that detect conserved molecular products of micro-organisms and are involved in the activation and regulation of inflammatory and immune responses. Of the TLRs so far identified, TLR2 plays a major role against infections by Gram-positive bacteria, mycobacteria, and yeasts, binding a wide variety of microbial products such as peptidoglycan, lipoteichoic acid, lipoproteins, lipoarabinomannan, and zymosan. Upon activation by PAMPs, TLR2 predominantly signals to NF-κB, which induces the transcription of an array of genes encoding cytokines and other molecules involved in the initiation of host innate and adaptive immune responses [31].

In this report we describe the characterisation of a mAb to porcine TLR2 and analyse the pattern of expression of this receptor in different leukocyte subsets and tissues to investigate how it can contribute to anti-microbial immune responses. The TLR2 specificity of this mAb was determined by its reactivity with CHO transfectants expressing the pTLR2 but not the pTLR4 nor with non-transfected cells. To our knowledge, this is the first report describing the development and characterisation of mAbs against porcine TLR2.

In general, the distribution of TLR2 on porcine leukocyte populations is similar to that reported in humans, being predominantly expressed on myelomonocytic cells such as

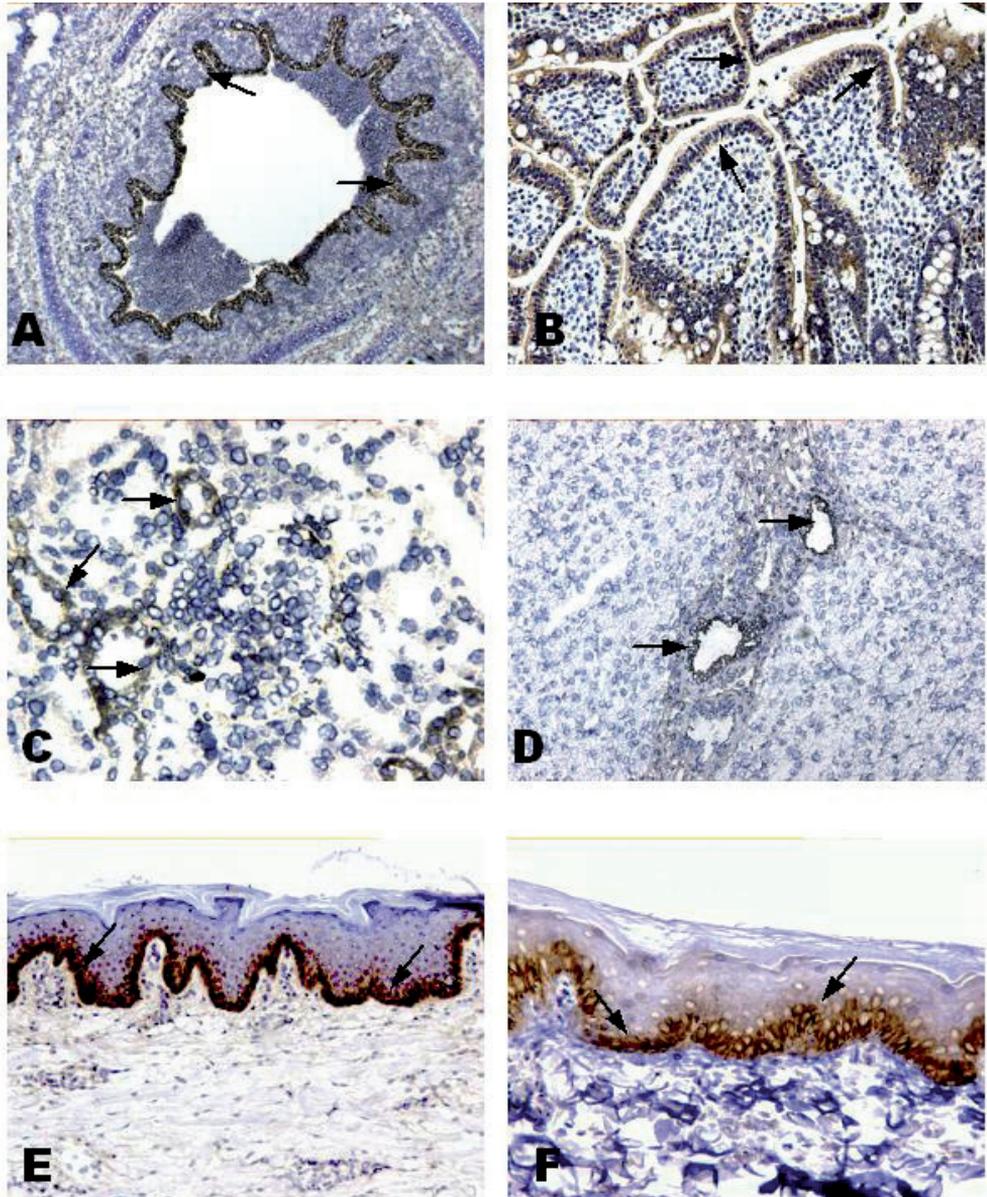


Figure 6. Immunohistochemical staining of frozen sections (A-E) and formalin-fixed, paraffin-embedded sections (F) of porcine tissues with anti-TLR2 mAb 1H11 using the immunoperoxidase method. (A) Lung: Bronchial epithelial cells stain strongly positive. (B) Jejunum: Positive staining of enterocytes. (C) Kidney: TLR2 expression in the epithelium of some renal tubules. (D) Liver: Positive staining of bile ducts. (E, F) Skin: Strong expression of TLR2 in cells of the basal layer of the epidermis. Representative areas of positive staining are indicated by arrows. Original magnifications: (A) $\times 40$, (B, D, and E) $\times 100$, (C and F) $\times 200$. Results are representative of three independent experiments with different donors.

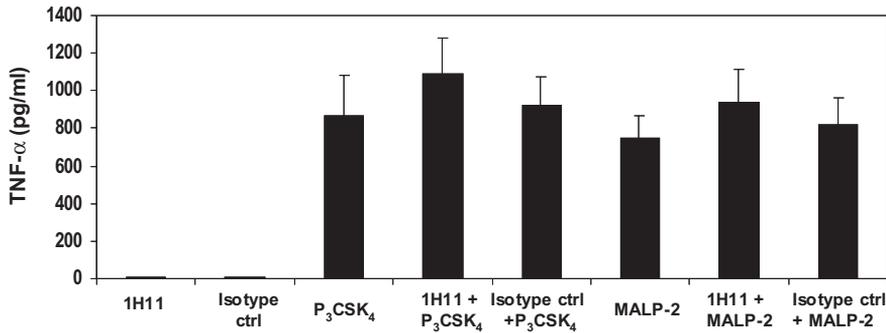


Figure 7. Effect of mAb 1H11 on TNF- α secretion by PBMC in response to P₃CSK₄ and Malp-2. Cells were preincubated with anti-TLR2 mAb 1H11 (50 μ g/mL) or an isotype-matched control antibody (50 μ g/mL) for 30 min and then stimulated with P₃CSK₄ (100 ng/mL) or Malp-2 (1.3 ng/mL) for 24 h. TNF- α level in culture supernatants was determined by ELISA. Data are expressed as the mean \pm SEM of triplicates of one representative out of three experiments performed.

monocytes, macrophages, and neutrophils [15, 27, 28, 38]. Like in humans, swine neutrophils express relatively low levels of cell surface TLR2, whereas monocytes express it at high levels [28]. Likewise, differentiation of monocytes into immature DCs by the addition of GM-CSF and IL-4 results in the progressive loss of this receptor [29, 38].

Although TLR2 protein has been detected intracellularly in non-stimulated human T cells [24], none or very few T cells express TLR2 on the cell surface when they have not been activated [3, 11, 24]. We have not detected expression of TLR2 on the surface of porcine blood T, B, or NK cells. Nevertheless, Tohno et al. [36] have reported a strong expression of TLR2 in a considerable proportion of T lymphocytes in porcine Peyer patches and mesenteric lymph nodes (MLNs). They also detected a weak expression on some CD21⁺ B cells. This apparent discrepancy may be due to differences in the affinity of the antibodies used (a polyclonal antiserum in their study), differences in the state of activation of cells, or to the different lymphoid compartments analysed (peripheral blood vs lymphoid organs). Indeed, these authors found a stronger TLR2 expression in gut-associated lymphoid tissues (GALT) than in other lymphoid tissues, such as spleen or thymus.

The skin and mucosal surfaces are the most important routes of entry of micro-organisms into the host. In addition to cells of the innate

immune system, we have found expression of TLR2 on epithelial cells of the skin, renal tubules, and respiratory and intestinal tracts. These cells provide the first line of defense against invading pathogens. Besides functioning as a mechanical barrier, they produce, in response to microbial products, a variety of cytokines, chemokines and antimicrobial peptides that trigger the activation of tissue-resident innate cells and the recruitment of leukocytes to the sites of infection [19, 20, 22]. Constitutive expression of TLR2 has also been reported in epithelial cells of the proximal and distal renal tubules in mouse and in human tracheobronchial epithelial cells and alveolar epithelial type II cells [12, 14, 19, 39]. In the human skin, TLR2 is expressed throughout the epidermis, but shows an increased expression in basal keratinocytes [4], like we have seen in pigs.

Our results show that in skin and bronchial epithelial cells TLR2 expression was relatively strong, whereas enterocytes appeared weakly stained. Previous studies by Tohno et al. [36, 37] reported a low expression of TLR2 mRNA by real-time quantitative PCR in the porcine gut, but failed to detect TLR2 at the protein level except in the M cells which cover the mucosa-associated lymphoid tissue. Nevertheless, a low expression of TLR2 has been reported in human intestinal epithelial cells (IECs) *in vitro* and *in vivo* [7, 8, 16]. This low expression may be a way of minimising

luminal recognition of molecular patterns of commensal flora in the healthy intestine [8]. In IECs, TLR2 and TLR4 have been shown to traffic from apical to cytoplasmic compartments in response to specific bacterial ligands, and might play important roles in conveying bacterial products to the underlying immune and inflammatory cell populations of the lamina propria [8].

Targeting Ag to TLR2 has been proposed as a useful strategy to enhance vaccine immunogenicity [29]. Signalling through TLR2 leads to maturation of APCs (DCs) resulting in the up-regulation of MHC class II and costimulatory molecules (CD80, CD86) [18]. It also induces local inflammation with the consequent increase in the number of these APCs at the inoculation site. Besides, TLR2 is an endocytic receptor that delivers ligands into the MHC class II presentation pathway. Although mAb 1H11 failed to induce the production of TNF- α by monocytes, that we analysed as a measure of activation, it may still be useful for the development of Ab-based vaccines by improving the uptake of antigen by APCs. Further studies will determine the ability of this mAb to deliver antigens into MHC class I and MHC class II pathways for T cell priming.

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