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Pig Bone Marrow-Derived Macrophages Resemble Human Macrophages in Their Response to Bacterial Lipopolysaccharide

Ronan Kapetanovic,* Lynsey Fairbairn,* Dario Beraldi,* David P. Sester,[†] Alan L. Archibald,* Christopher K. Tuggle,[‡] and David A. Hume*

Mouse bone marrow-derived macrophages (BMDM) grown in M-CSF (CSF-1) have been used widely in studies of macrophage biology and the response to TLR agonists. We investigated whether similar cells could be derived from the domestic pig using human rCSF-1 and whether porcine macrophages might represent a better model of human macrophage biology. Cultivation of pig bone marrow cells for 5–7 d in presence of human rCSF-1 generated a pure population of BMDM that expressed the usual macrophage markers (CD14, CD16, and CD172a), were potent phagocytic cells, and produced TNF in response to LPS. Pig BMDM could be generated from bone marrow cells that had been stored frozen and thawed so that multiple experiments can be performed on samples from a single animal. Gene expression in pig BMDM from outbred animals responding to LPS was profiled using Affymetrix microarrays. The temporal cascade of inducible and repressible genes more closely resembled the known responses of human than mouse macrophages, sharing with humans the regulation of genes involved in tryptophan metabolism (IDO, KYN), lymphoattractant chemokines (CCL20, CXCL9, CXCL11, CXCL13), and the vitamin D3-converting enzyme, Cyp27B1. Conversely, in common with published studies of human macrophages, pig BMDM did not strongly induce genes involved in arginine metabolism, nor did they produce NO. These results establish pig BMDM as an alternative tractable model for the study of macrophage transcriptional control. *The Journal of Immunology*, 2012, 188: 3382–3394.

Cells of the mononuclear phagocyte system, including monocytes, bone marrow progenitor, and tissue macrophages and dendritic cells (1–3), are the first line of defense against potential pathogens and have numerous trophic roles in development and homeostasis (4). These cells comprise 10–15% of the total cells in most organs of the body, and are especially concentrated adjacent to mucosal surfaces (5). Their differentiation and maturation are controlled by hemopoietic growth factors, notably CSF-1 and IL-34, which share a receptor encoded by the c-fms protooncogene (6, 7).

Upon recognition of a potential pathogen, resident macrophages within tissues initiate an early inflammation response that leads to recruitment of neutrophils and inflammatory monocytes (8). Recognition is mediated through pattern recognition receptors that bind to pathogen-associated molecules. The archetypal pattern recognition receptor is TLR4, which, with the coreceptor MD-2, recognizes LPS, a component of the cell wall of Gram-negative organisms (9). TLR4/MD2 ligation induces the recruitment of multiple adaptors interacting though their Toll/IL-1R domain. One pathway, requiring the adaptors MyD88 and TIRAP/Mal leads to translocation of NF-κB and transcription of proinflammatory cytokines. A second, initiated following endocytosis, and involving TRAM, activates IFN regulatory factor (IRF) 3 and leads to type 1 IFN induction (9). The regulatory cascade initiated following TLR4 ligation involves regulation of thousands of transcripts, with sequential induction and repression of numerous transcriptional regulators generating a complex network (10–13). Appropriate regulation of this cascade determines the outcome of infection, because many of the induced genes are required for defense against the pathogen, but also causes much of the pathology. Feedback control by numerous negative regulators is needed to ensure that the response is limited and appropriate (14, 15).

Many studies of LPS signaling have been carried out using murine bone marrow-derived macrophages (BMDM): cells grown from bone marrow in the presence of M-CSF (CSF-1) (16). CSF-1 is required for the maintenance of macromolecule synthesis and ultimately for survival of macrophages. It regulates the expression of the TLRs in mouse macrophages, and alters the response to LPS (17). Conversely, one of the earliest detected responses to TLRs is downregulation of the CSF-1 receptor and growth arrest (18), and a subset of genes is induced solely as a consequence of the removal of the CSF-1 signal (19).

Most of our knowledge of TLR signaling derives from studies of the mouse, taking advantage of knockout technology to define the roles of specific signaling molecules. However, CSF-1 signals

rather differently in humans than in mice (20); numerous differences have been observed in the responses of the two species to LPS and the sets of innate immune effectors (21). The immunity-related GTPase family provides one example, as follows: mice possess 23 different members, whereas only one can be found in humans (22). Another important example is the regulation of inducible NO synthetase (NO synthase 2 [NOS2]). By contrast to mice, human macrophages in vitro do not upregulate arginine metabolism to produce NO in response to LPS, but instead metabolize tryptophan via IDO (23).

We recently reviewed the differences between mouse and human inflammatory biology in the context of consideration of the domestic pig as an alternative model (21). The comparison is somewhat compromised by the use of distinct populations of cells. Most human studies employ monocyte-derived macrophages grown in CSF-1 (24). Mouse monocytes are not readily accessed in substantial numbers and may differ from humans in terms of their maturation state/subpopulations (25) and in their response to inflammatory stimulus like LPS (26). Conversely, access to human bone marrow is also not straightforward, and human BMDM have not been reported. We therefore decided to bridge the mouse and human systems by studying BMDM from pigs. Pigs are economically important in their own right, and are susceptible to many viral and bacterial pathogens that can also infect humans, or that cause similar pathologies to human infections (27, 28). So, there is the reciprocal interest in whether humans or mice provide an adequate model for pig innate immunity. The tractability of the pig as an experimental model is enhanced greatly by the impending completion of a high quality genomic sequence (29). Previous work demonstrated that pig bone marrow cultured in L929-conditioned medium (a source of mouse CSF-1) and horse serum generated cells with the morphological and functional characteristics of macrophages (30). In the current study, we have established a system for generating BMDM from the pig using human rCSF-1 (rhCSF-1) and FCS, the same reagents used in mouse and human studies. We used this system to determine the time course of regulation of gene expression in response to LPS and the variation among individual animals. The results indicate that pig BMDM resemble human monocyte-derived macrophages in their response to LPS and could provide a better predictive model for testing candidate therapeutic approaches in sepsis and other infectious pathologies.

Materials and Methods

Animals

Male Large White or F_1 cross Large White-Landrace pigs of 8–12 wk of age were used, as indicated. All the pigs spent at least 2 wk in the same facility before experimentation. All animal care and experimentation was conducted in accordance with guidelines of Roslin Institute and the University of Edinburgh and under Home Office Project License PPL 60/4259.

Cell isolation and culture

Pigs were sedated with a mixture of ketamine (6 mg/kg) and azaperone (1 mg/kg), left undisturbed for a minimum of 15 min, and then killed by captive bolt. Five posterior ribs from each side of the animal were removed. The outer surface of the bone was cleaned with alcohol, both extremities were cut, and, using a 20-ml syringe with an 18-g needle, bone marrow was flushed from both ends with RPMI 1640 (Sigma-Aldrich) containing 5 mM EDTA to prevent clotting. Cells were spun, suspended in red cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA [pH 8.0]) for 2 min, spun again, and washed with PBS, followed by RPMI 1640. Bone marrow cells were finally suspended in a freezing medium (90% heat-inactivated FCS-10% DMSO) and frozen overnight in a "Mr. Frosty" isopropanol box at $-80°C$ (Nalgene), allowing a controlled decrease of temperature. The next day, cells were transferred to a $-150°C$ freezer for long-term storage. To recover cells from the freezer, they were thawed rapidly in a 37°C waterbath, then slowly diluted by dropwise addition of complete medium

over 2–3 min to avoid the shock of sudden dilution of DMSO. After being washed to remove DMSO, cells were cultured in complete medium-RPMI 1640, 10% heat-inactivated FCS (PAA Laboratories), penicillin/streptomycin (15140; Invitrogen, Paisley, U.K.), and GlutaMAX-I supplement (35050-61; Invitrogen). BMDM were obtained by culturing bone marrow cells for 5–7 d in presence of rhCSF-1 (10^4 U/ml; a gift of Chiron, Emeryville, CA) on 100-mm^2 sterile petri dishes, essentially as described previously for the mouse (18). The resulting macrophages were detached by vigorous squirting with medium using a syringe and 18-g needle, washed, counted, and seeded in tissue culture plates at 10^6 cells/ml in CSF-1–containing medium. The cells were treated where appropriate with LPS from *Salmonella enterica* serotype minnesota Re 595 (L9764; Sigma-Aldrich) at a final concentration of 100 ng/ml.

ELISA

TNF was measured in culture supernatants by ELISA, as specified by the manufacturer (DuoSet; R&D Systems, Minneapolis, MN). Nitrite (the product of rapid NO oxidation in culture) concentration was measured by Griess assay [1% sulfanilamide, 0.1% N-(1-naphtyl) ethylenediamine diHCl, 2.1% phosphoric acid], using sodium nitrite standards (Flucka Analytical). Samples were diluted 1/2 with RPMI 1640, and an equal volume of Griess reagent was added. Absorbance was measured at 540 nm.

Flow cytometry

Cells were incubated 15 min with high-blocking solution (PBS, 0.1% sodium azide, 2% FCS, 0.1% BSA), and then washed with low-blocking solution (PBS, 0.1% sodium azide, 0.2% FCS, 0.1% BSA). Cells were stained with either a mouse anti-pig CD14 Ab (clone MCA1218, 1:50; AbD Serotec), a mouse anti-pig CD16 Ab (clone MCA1218, 1:200; AbD Serotec), a mouse anti-pig CD163 Ab (clone MCA1218, 1:200; AbD Serotec), a mouse anti-pig CD172a Ab (clone MCA1218, 1:400; AbD Serotec), or an IgG2b or IgG1 isotype control (MCA691 and MCA928PE; AbD Serotec; same concentration as primary Ab) in Low Block. The cells were then washed and resuspended in 500 μl Low Block. Data were acquired on 50,000 cells using a CyAn ADP Analyzer (Beckman Coulter, High Wycombe, U.K.) and analyzed with Summit software (v4.3).

Phagocytosis assay

Cells were plated either on acid-washed glass coverslips (fluorescence microscopy) or 24-well plates (flow cytometry) and cultured overnight. Phagocytosis was initiated by centrifugation ($600 \times g$, 1 min) of FITC-conjugated zymosan bioparticles (Molecular Probes) at a particle:cell ratio of ~10:1, followed by incubation at $37°C/5\%$ CO_2.

For fluorescence microscopy, coverslips were washed with ice-cold PBS/0.1% sodium azide 5 min for a total of six times, fixed with 4% paraformaldehyde/PBS for 10 min, followed by an additional four washes with PBS. Cells were then permeabilized with 1% Triton X-100 for 5 min, followed by four 5-min washes with PBS. Cells were blocked with PBS/1% BSA for 1 h, and then stained for 30 min with a 1/100 dilution of Alexa Fluor 546-conjugated phalloidin (Molecular Probes) in PBS/0.2% BSA. Coverslips underwent three additional 5-min washes with PBS, a rapid wash in milliQ water, followed by mounting using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Peterborough, U.K.).

MTT assay of viable cells

Mouse and pig BMDM (2×10^4 cells) were seeded in 100 μl complete medium, in a 96-well plate with or without rhCSF-1 (10^4 U/ml). Every 24 h, 10 μl 5 mg/ml MTT (Sigma-Aldrich) was added in the culture for 2.5 h at 37°C. Medium was then homogenized by adding 100 μl solubilization buffer (0.1 M HCl-10% Triton isopropanol) and left overnight at 37°C. OD was read the next day at 540 nm.

Microarray-RNA preparation

To investigate the macrophage response to LPS, BMDM extracted from three pigs (one female, two males, F_1 cross Landrace × Large White; see above) were cultured 1 wk in medium (RPMI 1640, 10% FCS, penicillin/streptomycin, GlutaMAX-I) in presence of rhCSF-1 (10^4 U/ml). Cells were then harvested and plated in 6-well plates at a concentration of 10^6 cells/ml and left to rest overnight. The next day, BMDM were stimulated with LPS (100 ng/ml). RNA for gene expression analysis was collected at time points 0, 2, 7, and 24 h post-LPS stimulation. Each time point included BMDM from the same three pigs, and each cell culture was replicated. Therefore, a total of 24 microarrays was hybridized (four time points, three pigs, two cell culture replicates). For further analyses, the two replicates of each cell culture were averaged. We detected probes differentially

expressed between time point 0 (unstimulated macrophages) and each of the other time points by fitting a linear model through each probe set using the Bioconductor package LIMMA. The design matrix for the linear models included a coefficient for each of the three pigs and a coefficient for the comparisons of interest, as follows: 0 versus 2 h, 0 versus 7 h, and 0 versus 24 h. RNA was extracted from BMDM using the Qiagen RNeasy kit, as specified by the manufacturer (Qiagen, Crawley, U.K.). RNA concentration was measured using ND-1000 Nanodrop (Thermo Scientific). The quality was controlled by running the samples on the RNA 6000 LabChip kit (Agilent Technologies, Waldbronn, Germany) with the Agilent 2100 bioanalyzer in which samples are assigned an integrity classification from 10 (intact RNA) to 1 (highly degraded) by the 2100 bioanalyzer expert software. RNA used for the microarray had a concentration between 30 and 539 ng/μl (mean = 257, SD = 126). RNA integrity number ranged from 9.70 to 10 (mean = 9.96, SD = 0.02). Total RNA was prepared for hybridization using the Affymetrix 3' IVT Express kit (Affymetrix, Santa Clara, CA), following the manufacturer's instructions, and hybridized to the Affymetrix GeneChip Porcine Genome Array including 24,123 probe sets by ARK-Genomics (http://www.ark-genomics.org). Probe set expressions were normalized using the RMA algorithm (31), as implemented in the Bioconductor package affy (32, 33). The annotation of the genes was based upon an update of the Affymetrix porcine annotation, in part upon cross-mapping of Affymetrix human annotation, as described (34); the updated annotation is available at http://www.anexdb.org.

Normalized array data were uploaded to the software Biolayout Express (3D) (http://www.biolayout.org/) (35), and a graph was created using parameters of R mean 0.95, Markov clustering algorithm of 2.2, and a minimum number of 6 nodes per cluster (36).

RT-PCR

cDNA was synthesized from 1 μg RNA using Superscript III (Invitrogen, Carlsbad, CA), and mRNA expression was quantitated using the SYBR Green quantitative PCR system (Invitrogen). All oligonucleotides were designed using Primer3 (37) and synthesized by Invitrogen (Paisley, U.K.). Primers were designed with an optimal amplicon size between 80 and 150 bp. Primer pairs were designed so that one of the primers overlapped an exon junction to prevent possible amplification of any remaining genomic DNA. Primers are designed with an annealing temperature of 60°C and optimized using a pool of cDNA consisting of the samples to be tested. The dissociation curve analysis for each primer pair ensured that only a single amplification product was produced and efficiency was between 95 and 105%. Primers were used at 500 nM, as follows: hypoxanthine phosphoribosyltransferase (forward, 5'-ACACTGGCAAAACAATGCAA-3'; reverse, 5'-ACACTTCGAGGGGTCCTTTT-3'), CCL20 (forward, 5'-GGTGCTGCTGCTCTACCTCT-3'; reverse, 5'-GCTGTGTGAAGCCC-ATGATA-3'), IDO1 (forward, 5'-GGTTTCGCTATTGGTGGAAA-3'; reverse, 5'-CTTTTGCAAAGCATCCAGGT-3'), STAT4 (forward, 5'-GA-AAGCCACCTTGGAGGAAT-3'; reverse, 5'-ACAACCGGCCTTTGTT-GTAG-3'), and NOS2 (forward, 5'-CCACCAGACGAGCTTCTACC-3'; reverse, 5'-TCCTTTGTTACCGCTTCCAC-3').

Promoter sequence alignment

All sequences have been extracted from the Ensembl database, and alignment was performed using the software MacVector. The software Alibaba2.1 was used to predict the most probable transcription factor binding sites by constructing matrices on the fly from TRANSFAC 4.0 sites (http://www.gene-regulation.com).

Results

A large number of cells can be obtained from a single pig and give rise to macrophage-like cells in response to human CSF-1

In mice, bone marrow cells can be easily isolated from the femur of the animal and differentiated into macrophages in response to rhCSF-1 (38). Typically, cells from several mice are required for a single experiment. However, mouse cells have been frozen and recovered, expediting the study of knockout lines held by other researchers (39). Clearly, many more cells can be generated from a pig, so to avoid unnecessary animal use, we optimized a freezing protocol. Bone marrow cells were isolated by flushing 10 posterior ribs of each pig. Approximately 10^9 cells were obtained routinely from a single animal and cryopreserved in a freezing me-

dium comprising 90% FCS and 10% DMSO at a temperature of −155°C. Following thawing, the percentage of viable cells was ~80%. The early published study of pig BMDM (30) used femoral marrow from piglets as the starting population, but this is impractical in older animals because of the adiposity of long bone marrow. They added 30% L929 conditioned medium and 15% horse serum and grew the cells in Teflon bags. The maximal cell yield occurred after 10 d. Ribs provide a convenient alternative source of marrow. Thawed cells cultured in the presence of rhCSF-1 for 5–7 d on bacteriological plastic, the identical conditions used for mouse BMDM, acquired a macrophage-like morphology based upon an increase in size granularity and adherence to the bacteriological plastic substratum (Fig. 1A, 1B). Thus, we had a simple method for producing macrophages from the pig that could be used to recover such cells for multiple investigations sequentially from the same animal. The system is as close as possible to that used in published studies of gene expression in mouse BMDM.

Pig BMDM display appropriate marker expression for macrophage-like cells

Human blood monocytes can be subdivided into subpopulations based upon the expression of CD14 and CD16 (40). We and others have shown that pig monocytes can also be separated into subpopulations that differ in their expression of CD16 and the scavenger receptor CD163 (21). As shown in Fig. 1, all pig BMDM obtained following cultivation in rhCSF-1 expressed CD14, the coreceptor for LPS. CD16, Fc receptor 3, was also expressed at high levels on all cells, as was SCW3a and CD172 (SIRP α). The pig BMDM had almost undetectable expression of CD163 and in that respect resembled more mature pig (21) and human (41) monocyte subsets.

Each of the positive markers was induced with time in culture of bone marrow cells in CSF-1. Based upon the forward and side scatter, the apparent granularity was retained, and cells became significantly larger with time (Fig. 1C–E). CD14 was expressed on a subset of the initial heterogeneous bone marrow cell population, but increased substantially in both intensity and frequency with time. CD163, also known as scavenger receptor cysteine-rich type 1 protein M130, was weakly expressed on some bone marrow cells. CD16 and CD172a expression appeared rapidly on the cells in the presence of CSF-1 (Fig. 1F–Q). Hence, we have a system in which to study the differentiation of macrophage in the pig. With the very large number of cells that can be obtained readily, this system may lend itself to purification of progenitors and detailed mechanistic studies.

Pig BMDM are potent phagocytic cells

The phagocytic activity of the pig BMDM was confirmed using FITC-zymosan bioparticles (yeast cell wall component) (Supplemental Fig. 3). The majority of pig BMDM rapidly ingested multiple particles. Similar results were observed with latex microspheres (data not shown). In quantitative flow cytometric assay, pig BMDM had comparable phagocytic ability to the murine macrophage cell line RAW-264.7. By contrast, a nonphagocyte control, pig fibroblasts, had no internalized particles.

Pig BMDM respond to LPS, but do not make NO

The main purpose for the generation of pig BMDM was to examine the response to LPS under similar conditions to those used in mouse and human studies, to assess the evolutionary divergence in the transcriptional regulatory cascade. Prior to a detailed examination of the LPS response, we examined whether the cells were able to produce the archetypal inflammatory cytokine, TNF-α. Secretion
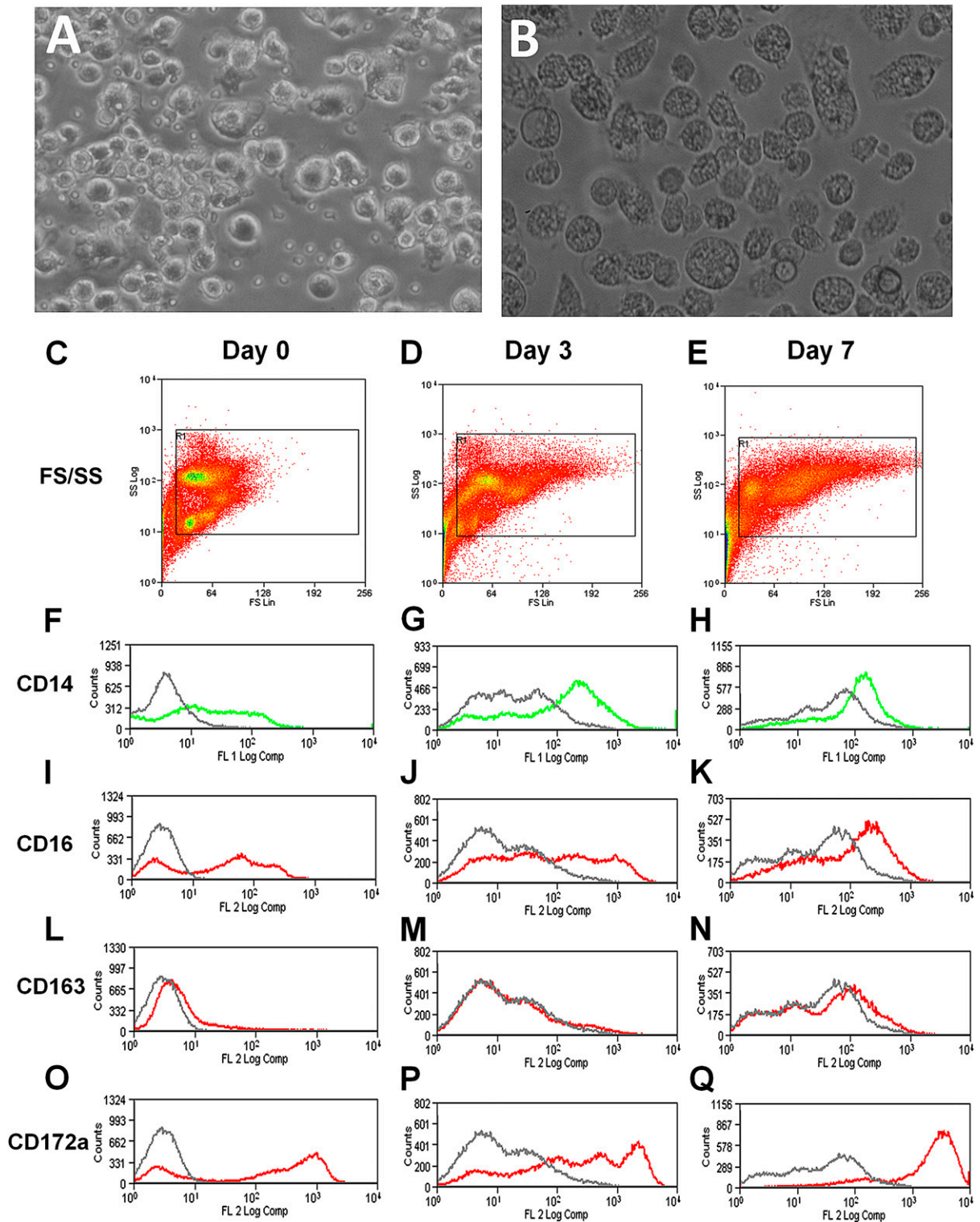
FIGURE 1. Characterization of pig BMDM. Bone marrow cells were isolated from pig ribs and cultured on bacteriological plastic in rhCSF-1. On day 0 (**A**), the cells were a mixture of small mononuclear cells and granular cells. After 7 d in rhCSF-1, the population was largely adherent and highly vacuolated (**B**). Original magnification ×200. (**C–E**) The FACS profiles of granularity (side scatter y-axis) and cell size (forward light scatter x-axis) are shown increasing as the cells mature in macrophages. At days 0, 3, and 7 of the maturation, expression of CD14 (**F–H**) was measured along with either CD163, CD16, or CD172a (respectively, **I–K**, **L–N**, and **O–Q**). The color line represents the mean fluorescence intensity of the receptor targeted, whereas the gray line represents the mean fluorescence intensity of the isotype control. Figures are representative of a minimum of three different experiments.

of TNF-α involves a multistep induction process including post-translation cleavage of the protein from the cell surface by TNF-α–converting enzyme (42, 43). As shown in Fig. 2A, TNF-α secretion was induced in BMDM by LPS, reaching a peak after 10 h

of stimulation and then slowly decreasing. No TNF-α was detected in the supernatant from unstimulated cells.

As noted in the introduction, mouse macrophages respond to LPS with induction of arginine metabolism, inducible NO syn-

FIGURE 2. Pig BMDM produce TNF-α, but not NO, in response to LPS. (**A**) BMDM were incubated with LPS for the time indicated, and TNF-α protein was measured in the supernatant by ELISA. (**B**) NO production was measured in supernatant from pig and mouse BMDM stimulated with LPS for 24 h. In each panel, the results represent the mean ± SEM of a minimum of five independent experiments.
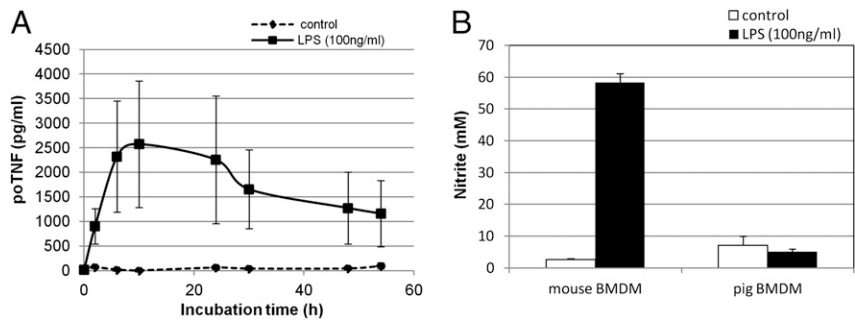
thase, and production of NO, whereas human macrophages do not (44, 45). One difficulty in comparisons of mouse and human has been that comparisons are made between monocytes-macrophages from different locations or differentiation states. Fig. 2B compares mouse BMDM and pig BMDM, grown under identical conditions in rhCSF-1. As expected, the LPS-stimulated mouse macrophages produced large amounts of NO. By contrast, under the same conditions, there was no detectable NO released from stimulated pig BMDM. Benga et al. (46) have reported that pig monocytes also do not produce NO in response to LPS.

By contrast to mouse cells, human macrophages respond to LPS with induction of IDO (47). In a separate study, we confirmed two other genes, the chemokine CCL20 (also known as MIP-3-α) and the transcription factor STAT4, as being upregulated in human monocyte-derived macrophages (K. Schroder, K.M. Irvine, M.S. Taylor, N.J. Bokil, K.-A. Le Cao, K.-A. Masterman, L.I. Labzin, C.A. Semple, R. Kapetanovic, L. Fairbairn, et al., submitted for publication) (24), but not in mouse in response to LPS (http://www.BioGPS.org) (10). The lack of induction of these genes in mouse BMDM responding to LPS is also evident from genome-scale 5′RACE (CAGE) expression profile in the FANTOM project (2) and in expression profiling of the RAW264 macrophage cell line (48). We therefore investigated the expression of these four genes (IDO1, NOS2, CCL20, and STAT4) in porcine BMDM after LPS stimulation using quantitative RT-PCR (qRT-PCR) (Fig. 3). IDO1 expression was massively upregulated after 6 h and slowly

decreased by 24 h. The levels of CCL20 and STAT4 mRNA were also substantially elevated after LPS stimulation, continuing to increase up to 24 h. We did not detect any expression of NOS2 mRNA in pig BMDM, consistent with the absence of NO production.

*Expression of genes after LPS stimulation of pig BMDM, using a microarray approach*

Having established the viability of the pig BMDM model, and confirmed that the pattern of gene induction resembled published studies of human monocyte-derived macrophages, at least for a limited set of index genes, we investigated the gene expression profiles in the primary pig BMDM population when stimulated with LPS. BMDM were produced from three individual (one female, two males) F_1 cross Landrace × Large White pigs. Using Affymetrix porcine microarrays, we have analyzed changes in gene expression at the transcript level using RNA from across a time course of activation (0, 2, 7, and 24 h) in response to LPS in two experimental replicates. To verify the response of the BMDM to LPS treatment in the same cells used for expression profiling, TNF-α production was measured by ELISA in the supernatant. In all three time courses, TNF-α mRNA reached a peak of expression at 2 h after stimulation, whereas the TNF-α protein production was maximal at 7 h, reaching a concentration close to 3000 pg/ml for 10^6 cells/ml (Fig. 4A). As expected from studies in the mouse, LPS stimulation of pig BMDM induced the expression of a very large number of genes, 3343 probe sets corresponding to 2159 genes (Fig. 4B), peaking at 7 h with 70.3% of the total upregulated genes (2340 probe sets corresponding to 1518 upregulated genes), including numerous known LPS-responsive cytokines and chemokines. As observed in the mouse BMDM response (10), many of the gene inductions were transient and there was a sequential cascade. Thirty percent of the probe sets were upregulated only at 7 h, 25.7% were upregulated only at 24 h, and 26.4% stay upregulated between 7 and 24 h. In keeping with the view that negative regulation and feedback control is a critical determinant of the duration and magnitude of LPS responses (49), LPS stimulation of pig BMDM triggered even more downregulation of genes than upregulation (a total of 5721 downregulated probe sets, corresponding to 4258 genes) (Fig. 4C). At 2 h, 14.3% of the probe sets (609 genes) were already downregulated, including the top downregulated Hairy/Enhancer of Split 1, a transcriptional repressor/activator, the Growth-Arrest and DNA damage-inducible gene α (GADD45A), and the oncogene MYC (fold of 0.13, 0.14, and 0.18, respectively, relative to 0 h). The 25 most up- and downregulated genes, with their fold increase, have been provided (Supplemental Fig. 1).

Fig. 4D shows the histograms of the number of probes significantly affected by the LPS treatment at each of the three time points. The 2-h time point produced a relatively small number of probes affected by LPS (as shown also in Fig. 4C), but the ma-
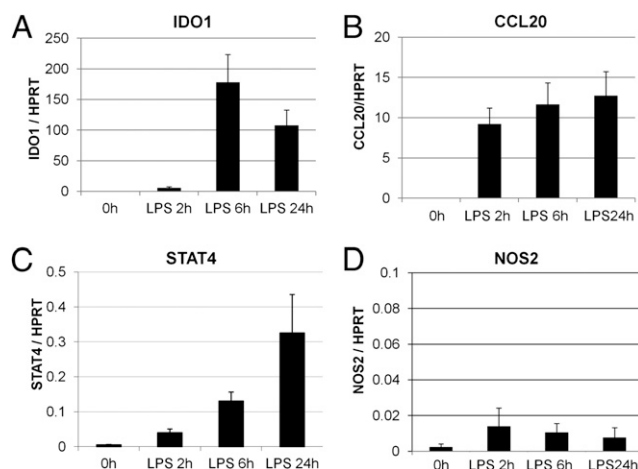
FIGURE 3. Pig BMDM expression of IDO1, CCL20, STAT4, and NOS2. Pig BMDM were stimulated with LPS, as described in *Materials and Methods*, for 2, 6, and 24 h. RNA expression of IDO1 (**A**), CCL20 (**B**), STAT4 (**C**), and NOS2 (**D**) was measured by qRT-PCR. Results are expressed as fold increase, normalized against the value for hypoxanthine phosphoribosyltransferase. Results represent the mean ± SEM of five independent experiments.
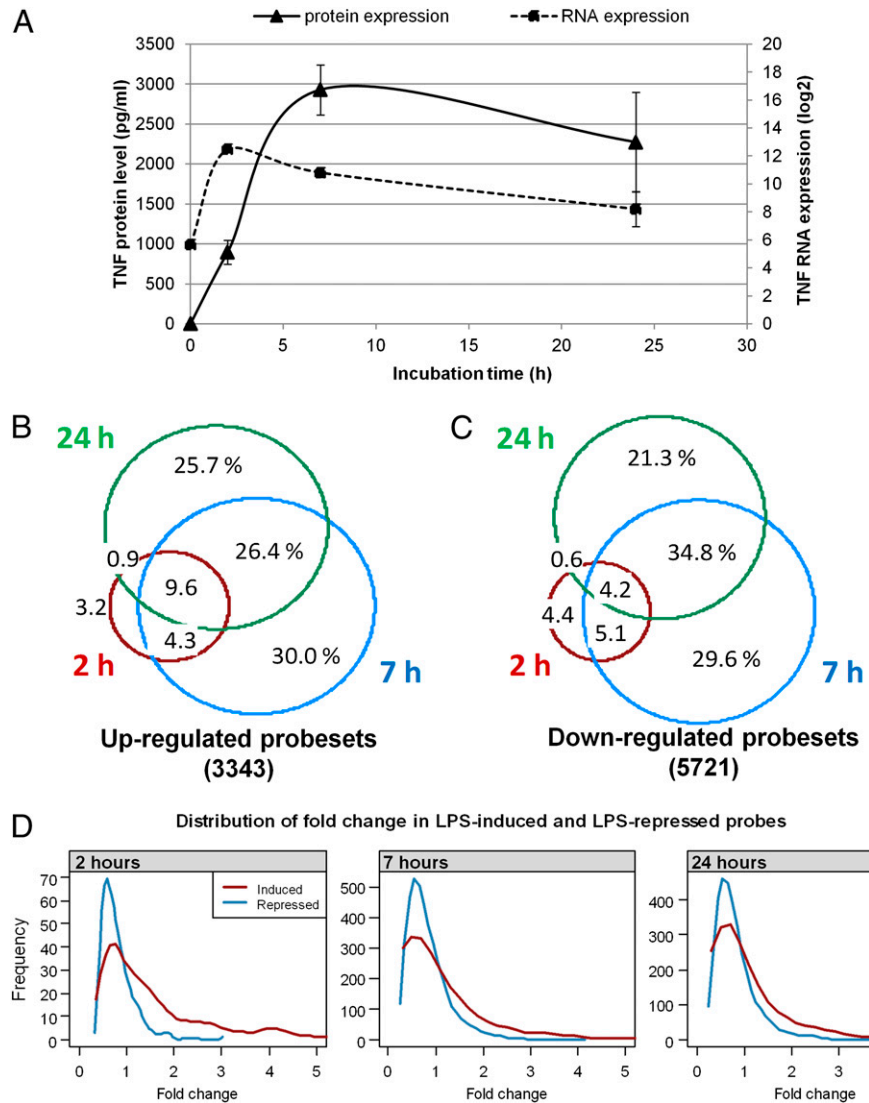
FIGURE 4. The mRNA expression profile of pig BMDM stimulated with LPS. BMDM from three individual Large White-Landrace F_1 cross pigs were stimulated with LPS for 0, 2, 7, and 24 h. (**A**) The expression of TNF-α mRNA and protein measures by qRT-PCR and ELISA, respectively, in the cells used for expression profiling. (**B** and **C**) The relative numbers of probe sets up- and downregulated at a significance threshold of $p < 0.01$ at each of the time points. (**D**) The frequency distribution of fold change (in base 2) for the LPS-induced and LPS-repressed probe sets at each time point. Note that at the 2-h time point, there is a long tail of very highly regulated genes.

jority of these genes were very highly regulated, and most genes induced later in the regulatory cascade were induced, or repressed, to a lesser extent. Among the 24,124 probe sets of the microarray, a remarkable 15,450 were expressed at detectable levels, and, of these, 11,819 showed a significantly different level of expression between 0 and 2 h, 0 and 7 h, or 0 and 24 h. The data from the microarray are published on the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov; GSE30956), and are also available on http://www.macrophages.com. As previously noted in studies of the mouse (10, 12), gene induction follows a distinct temporal profile with unique classes of genes induced maximally at each time point. Numerous cytokine genes were upregulated shortly (2 h) after LPS stimulation, including IL-1, TNF, IL-8, and IL-10. This cluster did not include so-called immediate early response genes such as c-fos and EGR1, which have been identified as LPS-inducible genes in studies by one group (12), but not in data from our laboratory (10) (biogps.gnf.org), in which LPS actually represses c-fos. This difference arises from differences in the experimental procedures; in our studies, we include CSF-1, which is a considerably more effective inducer of early response genes like c-fos than is LPS. Xie et al. (50) and Myers et al. (51) have noted previously that LPS causes rapid growth arrest in mouse BMDM. The latter authors noted a transient increase, followed by

repression of the proliferation-associated c-myc oncogene. In this respect, the pig system clearly resembles the mouse (see above). Similarly, and by contrast to the large induction of c-fos seen in CSF-1–starved BMDM (12), Myers et al. (51) saw only a minor increase in expression of c-fos mRNA above the CSF-1–induced constitutive expression, followed by repression. We feel that retention of CSF-1 is a more physiological model, because CSF-1 is present constitutively in tissues and circulation (4). What is clear from the findings is that immediately early gene induction, growth repression, and inflammatory cytokine induction can be dissociated.

Supplemental Fig. 2 summarizes other known classes of genes that are regulated in response to LPS. Upregulated genes include many components of the TLR and other signaling pathways, including multiple transcription factors as NF-κB1, the IRF family (IRF1, 2, 3, 5, 7, 8, and 9), CEPBP-β and δ, and PU-1, and coregulatory factors such as JMJD3, a demethylase 6B (52). As discussed by Wells et al. (14), we found that a major component of the inducible genes comprises feedback repressors, including inhibitory transcription factors (NF-κBI) and corepressors (NCOR), members of the suppressor of cytokine signaling family, dual specificity phosphatase 1 (DUSP1/MKP1), ATF3, tristetraprolin, also known as ZFP36, and several inhibitory cytokines (IL-1RA also known as IL-1RN, TGF-β, and IL-10).

The software package Biolayout Express(3D) has previously been used to analyze large data sets that included time course of LPS stimulation in BMDM and thioglycolate-elicited peritoneal macrophages (53, 54). The software allowed us to visualize the entire data set as a network graph, and to identify sets of genes that are robustly coexpressed across the three different animals examined. The 24,124 (pig) probe sets were clustered using a correlation of $R = 0.95$ and Markov clustering algorithm of 2.2, generating 95 distinct clusters (Fig. 5A). The two largest clusters are one group of downregulated genes and one group of upregulated genes (Fig. 5B). The first cluster contains 226 different probe sets downregulated, and cluster 2 contains a group of 209 probe sets, all upregulated after LPS stimulation. A group of three clusters shows a rapid upregulation after 2 h of LPS stimulation and then a slow decrease in their expression (Fig. 5C, Table I).

This list includes the set of genes shown in the mouse system to be induced at the level of transcription elongation, among which the archetype is TNF-α (55). Many of these early response genes initiate downstream signals, including IFN-β; IRF3, the IFN regulator; IFNAR2, the receptor for IFN; and Jagged 1, a molecule that is known to be a Notch ligand and a potential Th2-promoting factor (56). Interestingly, GADD45B is also rapidly upregulated at 2 h. GADD45B has been shown to inhibit the apoptosis signal of TNF-α by interfering with the JNK cascade (57). Upregulation of GADD45B may then allow the macrophages to release TNF-α without themselves undergoing apoptosis. A second cluster of genes was not linked to the others and is composed of genes quickly downregulated at 2 and 7 h, followed by a return to normal expression at 24 h (Fig. 5D, Table II). Multiple genes of this group have DNA-binding function, including CGC-binding
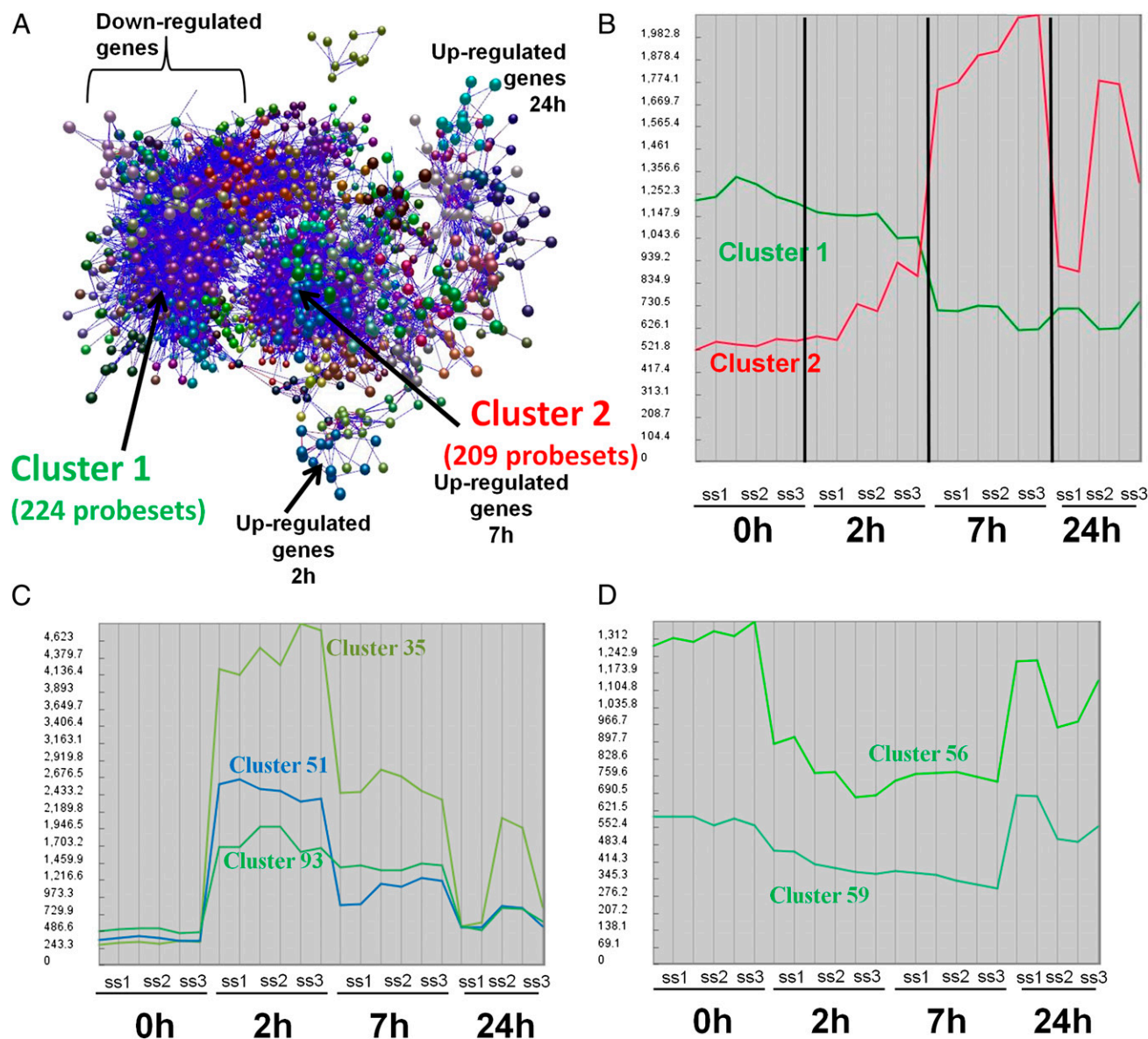
FIGURE 5. Network analysis of the response of pig BMDM to LPS using Biolayout(3D). (**A**) A network graphical representation of the pig macrophage gene expression data in which genes with similar expression profiles are clustered together within the same region using the analytical tool Biolayout(3D). This tool allows the visualization of the average expression profile of genes within a cluster across the data set. (**B**) Cluster 1 containing 226 probe sets was repressed in all three animals (ss1, ss2, ss3) by 7 h; cluster 2 containing 209 probe sets was induced in all three animals, peaking at 7 h, but was more rapidly repressed at 24 h in animal ss1. (**C**) Three clusters that show peak induction at 2 h. (**D**) Clusters in which the genes are transiently downregulated.

Table I. Gene list of the three clusters (35, 51, and 93) showing an upregulation peak at 2 h (linked to Fig. 5C) in pig BMDM responding to LPS

Gene	Full Name	Cluster
CFLAR	CASP8- and FADD-like apoptosis regulator	35
DPH3	DPH3 homolog	35
GADD45B	Growth arrest and DNA damage-inducible gene	35
GTPBP10	GTP-binding protein 10	35
IRF1	IFN regulatory factor 1	35
JUNB	Oncogene JUN-B	35
PLTP	Phospholipid transfer protein	35
SOCS3	Suppressor of cytokine signaling 3	35
BTG2	B cell translocation gene 2	51
DEPDC7	DEP domain-containing protein 7	51
FAM148B	Nuclear localized factor 2	51
IER5	Immediate-early response gene 5	51
JAG1	JAGGED 1	51
RND3	RHO family GTPase 3	51
TNF	TNF	51
IFNAR2	IFN α, β, and ω receptor 2	93
RASSF2	RAS association domain family protein 2	93
SLC11A2	Solute carrier family 11, member 2	93
TNFAIP2	TNF-α–induced protein 2	93
UBTD2	Ubiquitin domain-containing protein 2	93
VHL	VHL gene	93

protein 1, coiled-coil-helix-coiled-coil helix domain-containing 8, or CCCTC-binding factor. This cluster also includes signaling molecules, like MAPK 3 kinase 4 and the PI3K.

Using the expression data to identify unannotated genes highly upregulated after LPS stimulation

Because a stable draft genome sequence for the pig is not yet available for annotation, the array elements on the Porcine GeneChip were annotated using RNA level comparisons to RefSeq entries (34). The chip had a large number of unannotated probe sets; the Affymetrix GeneChip information leaves 18,313 probe sets unannotated. Using the ANEXdb (http://www.anexdb.org/), we managed to lower this number down to 5,395 probe sets out of 24,123 (22.3%). But still, among these unannotated probe sets, many were very highly regulated by LPS. To recover this information, we blasted the probe set sequences of the 10 most in-

ducible genes against the human and pig genome. The revised annotation revealed that CXCL11 (IFN-inducible T cell α chemoattractant) was strongly upregulated at 7 h (388.5-fold), as well as CD274 (programmed cell death ligand 1, 45-fold), alveolar macrophage-derived chemotactic factor-II (42-fold), guanylate-binding protein 2 (32-fold), DEAD box polypeptide 60 (22-fold), and family with sequence similarity 49, member A (9.7-fold). The data set will be further annotated after the completion of the pig genome annotation effort.

Highlighting the similarity of pig and human macrophage LPS response

CSF-1 primes mouse macrophages to respond to LPS with increased TNF-α production, but CSF-1 pretreatment had no effect on TNF-α in human monocyte-derived macrophages (58). As in human macrophages, the presence or absence of CSF-1 for the 24 h prior to LPS stimulation did not significantly alter the TNF-α production level in pig BMDM (Fig. 6A). As a control, we stimulated mouse BMDM, and recapitulated the reported priming effect on TNF-α production (Fig. 6B). The lack of effects of exogenous CSF-1 on TNF-α production by pig BMDM could be explained by constitutive production of CSF-1 by pig BMDM. To test this hypothesis, we cultured pig BMDM and mouse BMDM for 4 d in the presence or absence of rhCSF-1 and measured cell viability using the MTT assay. Mouse cells do not produce CSF-1 constitutively, and viability decreased rapidly following CSF-1 removal. Unlike mouse BMDM, pig BMDM did not undergo cell death in the absence of rhCSF-1, although cellular metabolic activity was increased when cultured with rhCSF-1 (Fig. 6C). We examined the expression of the three probe sets annotated for CSF1 on the microarray and their specific targets on the National Center for Biotechnology Information database. CSF1.1 (Ssc.19697.1.A1_at) targets the CSF1 gene on the wrong strand and was not detected. Weakly expressed probe set CSF1.2 (Ssc.19697.1.S1_at) targets exons 4, 5, and 6, which encode the transmembrane part of the protein. Finally, the strongly expressed probe set CSF1.3 (Ssc.6369.1.A1_at) targets the 3′ region of the CSF1 gene. This region is contained in both membrane and secreted forms. Hence, the difference of expression between CSF1.2 and CSF1.3 suggests that pig BMDM produce a large amount of mRNA encoding secreted CSF-1 constitutively (Fig. 6D).

Table II. Gene list of the two clusters (56 and 59) showing a rapid downregulation at 2 h in BMDM stimulated with LPS, followed by a return to normal value at 24 h (linked to Fig. 5D)

| Gene	Full Name	Cluster
AASDHPPT	α-aminoadipate semialdehyde dehydrogenase-phosphopantetheinyl transferase	56
ADNP	Activity-dependent neuroprotecto homeobox	56
CGGBP1	CGG-binding protein 1	56
CHCHD8	Coiled-coil-helix-coiled-coil-helix domain-containing protein 8	56
CTCF	CCCTC-binding factor	56
MID1IP1	MID1-interacting protein 1	56
NAT12	N-acetyltransferase 12	56
NDUFS4	NADH-ubiquinone oxidoreductase Fe-S protein 4	56
RAB14	RAS-associated protein RAB14	56
SMAD2	SMA- and MAD-related protein 2	56
AOF2	Amine oxidase (flavin-containing) domain 2	59
C16orf53	PTIP-associated 1 protein	59
DCTN2	Dynactin 2	59
DVL2	Dishevelled 2	59
GFOD2	Glucose-fructose oxidoreductase domain containing 2	59
HHEX	Hematopoietically expressed homeobox	59
MAP3K4	MAPK kinase kinase 4	59
PHF17	PHD finger protein 17	59
PIK3CG	PI3K, catalytic, γ	59
WHSC2	WHS candidate 2 gene (NELFA)	59
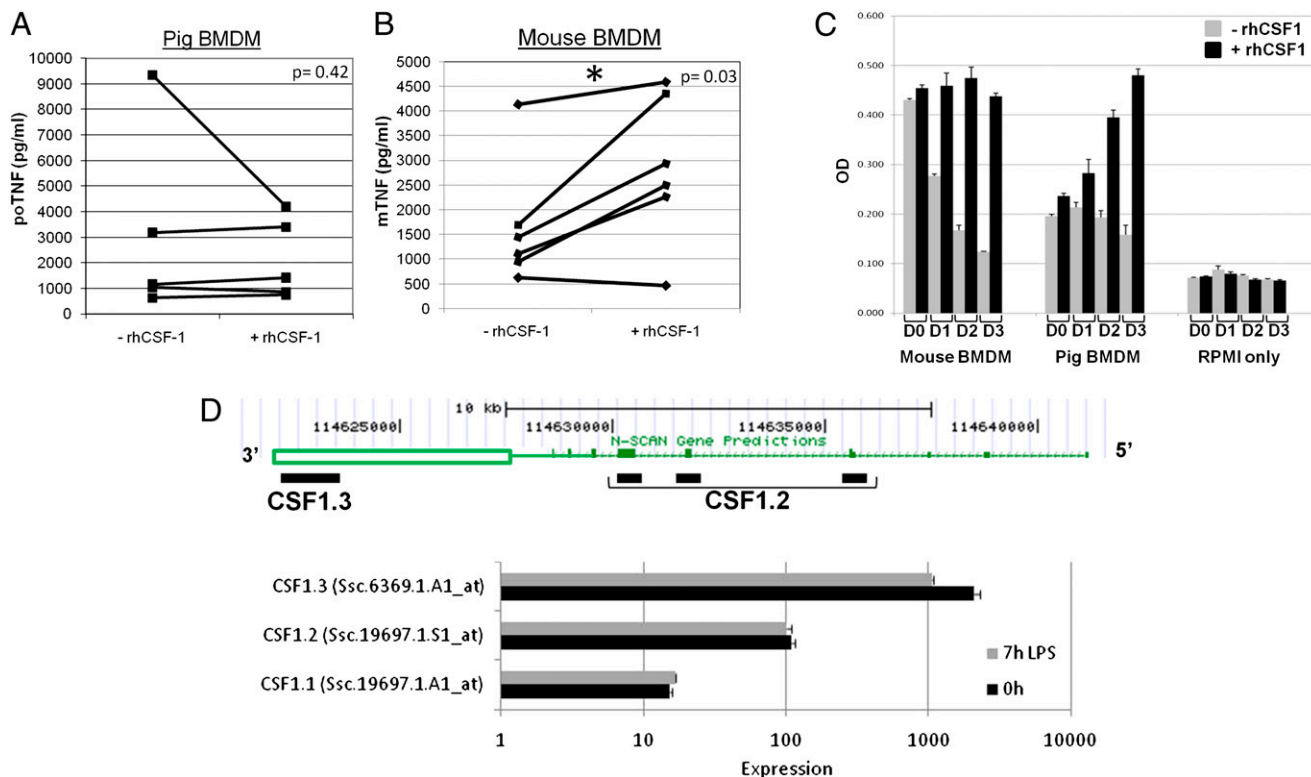
FIGURE 6.   Species-specific effect of CSF-1 on cell viability and responsiveness to LPS. Pig BMDM (**A**) or mouse BMDM (**B**) were incubated for 24 h with or without rhCSF-1, and then stimulated with 100 ng/ml LPS. TNF-α protein was measured in the supernatant after an additional 24-h incubation. The lines join pig or mouse BMDM from the same individual animal. Note that the priming effect of CSF-1 was significant in mouse (*), but not in pig BMDM . (**C**) Mouse or pig BMDM at 2×10^5 cells/ml in RPMI 1640–10% FCS were incubated in 96-well plates for 4 d with or without 10^4 U/ml rHCSF-1 prior to assay of viable cells based upon MTT reduction (see *Materials and Methods*). Medium-only background is shown at *right*. Note the decline in MTT reduction in mouse BMDM in the absence of added CSF-1. (**D**) The expression of CSF-1 mRNA by pig BMDM. Expression of the three probe sets for CSF1 was recovered from the array data above. Probe set CSF1.1 targets the opposite strand of CSF1 gene, whereas CSF1.3 targets the 3′UTR common to all forms of CSF-1.

The data already presented indicate that pig BMDM resemble human macrophages in that they induced IDO, STAT4, and CCL20 in response to LPS, whereas these genes were unaffected in mouse macrophages. Conversely, NOS2 was not inducible, nor was there any production of NO. The metabolism of arginine in mouse macrophages also involves induction of an arginine transporter (SLC7A2 or CAT2), GTP cyclohydrolase (GCH1), and arginase (ARG1 and ARG2). Of these, GCH1 and ARG2 were weakly induced in pig BMDM at 2 h, respectively, with a fold of 2.29 ($p = 0.0006$) and 2.72 ($p = 0.015$), whereas the limiting arginine transporter CAT2 was not detectable at any time.

To identify additional genes that distinguish pig from mouse BMDM, we compiled a list of the 500 most induced probe sets (comprising 174 gene names) in the pig BMDM at 7 h, and then examined the regulation of these genes in mouse macrophages from earlier studies now displayed on BioGPS (http://www.biogps.org) and confirmed the lack of induction in other data sets from the mouse (10, 59). From among this list, we identified 80 genes that were detected in the mouse with multiple independent probe sets (or CAGE Tags), but were not significantly regulated by LPS at any time point. We compared the list of discordantly regulated genes with the most similar human data set in the public domain (GSE5099 and GSE8608) (24). Of those 80 genes, we found that 30 were highly induced in activated human monocyte-derived macrophages (Fig. 7). Some of them are related to the IDO and vitamin D3 pathway (KYNU and CYP27B1), active pathways in humans, but not in mice. The heparin-binding epidermal growth factor-like growth factor is also upregulated in humans and pigs,

but not in mice. Heparin-binding epidermal growth factor-like growth factor is also known as the diphtheria toxin receptor, and, interestingly, humans and pigs are sensitive to the toxin, whereas mice are more resistant (60).

The genomic DNA sequence of the domestic pig is more similar to the human than to the mouse; the exonic sequences are most conserved across species, followed by 5′ untranslated region (UTR), 3′UTR, intergenic, and intronic regions (61). To begin to explain the concordance of pig and human inducible gene expression, we examined the sequence conservation of promoter regions of divergently expressed genes across the three species. One example, Cyp27B1, a gene upregulated in pigs and humans, but not in mice, is shown in Fig. 8A. The protein product of this gene is conserved 81% in mice and 87% in pigs (http://www. ensembl.org), but the promoter region is considerably more divergent between humans and mice. The divergence is highlighted in Pustell DNA matrix alignment of the promoter region of humans versus mice (Fig. 8B), compared with humans versus pigs (Fig. 8C). In the latter case, there is a gap caused by a repeat insertion in the pig, but otherwise, there is very substantial conservation extending almost 2 kb upstream. The conservation of this 2-kb region is also seen in dogs and cattle, whereas rats, like mice, are completely divergent (ecrbrowser.dcode.org). A detailed ClustalW alignment shows subtle variation even in the proximal promoter conserved across all three species. At −70 bp (relative to the start codon ATG), the TATA box sequence is identical in pig and human, but varies from the consensus in mouse. At −120 bp, a consensus C/EBP binding site is found in all three species, but
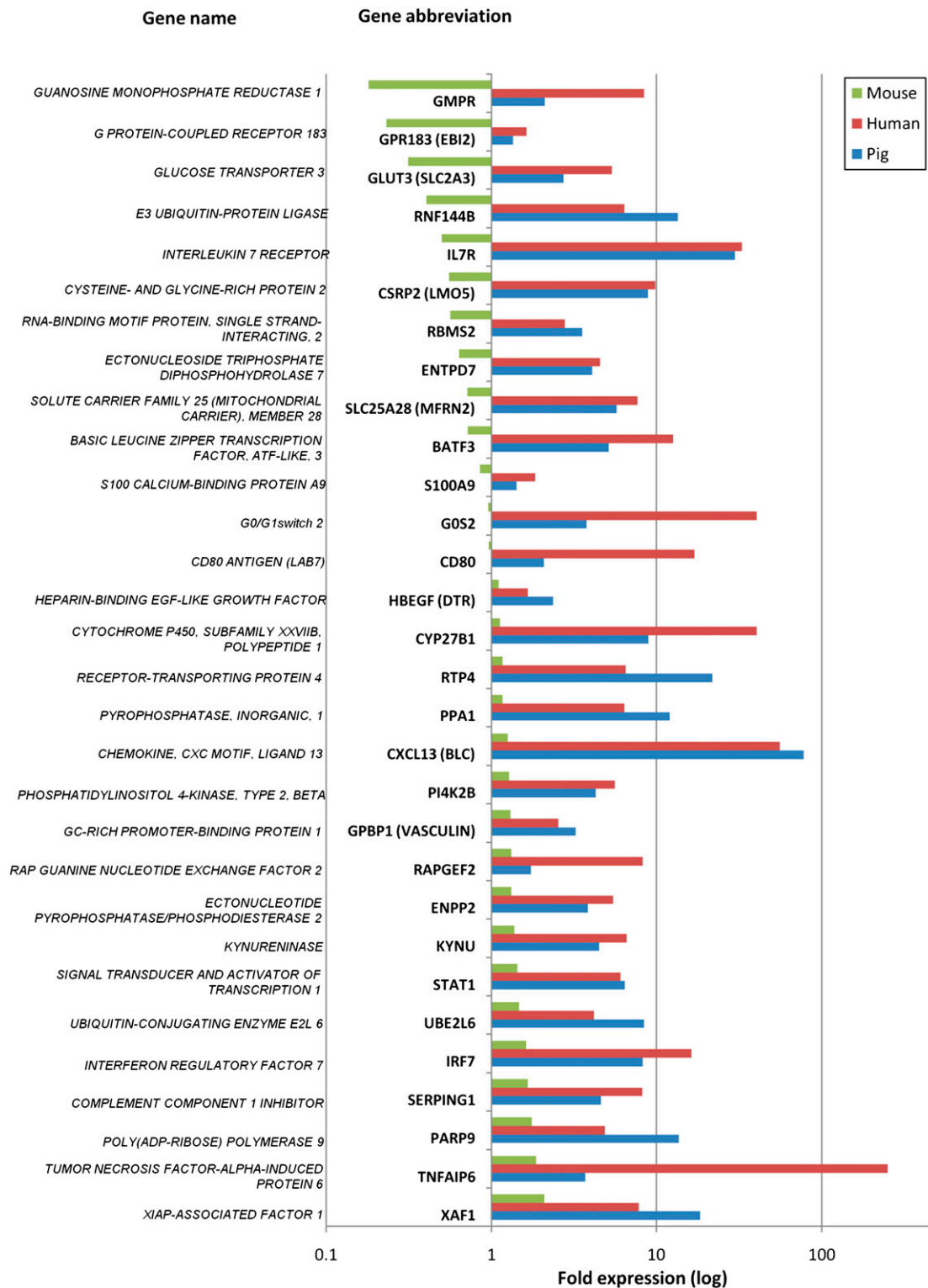
FIGURE 7. A set of genes induced by LPS in both pig and human macrophages, but not in mouse. The set of inducible genes for pig BMDM is derived from the microarray data in Fig. 5, and shows the fold induction at 7 h. The expression for human monocyte-derived macrophages + LPS is derived from published data sets (GSE5099 and GSE8608) and shows the maximal fold induction. Data for the mouse are derived from an analysis using Agilent custom arrays (K. Schroder et al., submitted for publication). None of the genes shown was induced by LPS in either mouse BMDM or thioglycolate-elicited peritoneal macrophages in a separate study using Affymetrix arrays (http://www.biogps.org), nor when expression was detected using genome-scale 5′RACE or CAGE (2).

a NF-κB site at −425 in the human is conserved in pig, but not in mouse. We have carried out similar analyses of the divergently regulated IDO1, CCL20, NOS2, STAT4, and IL-7R genes, in each case highlighting the extensive similarities of the promoters of

these genes in pigs and humans (data not shown). It is especially notable that the upstream enhancer region that mediates induction of NOS2 in mouse macrophages, and which is poorly conserved in humans (45), is absent in pigs (where the pig and human promoter
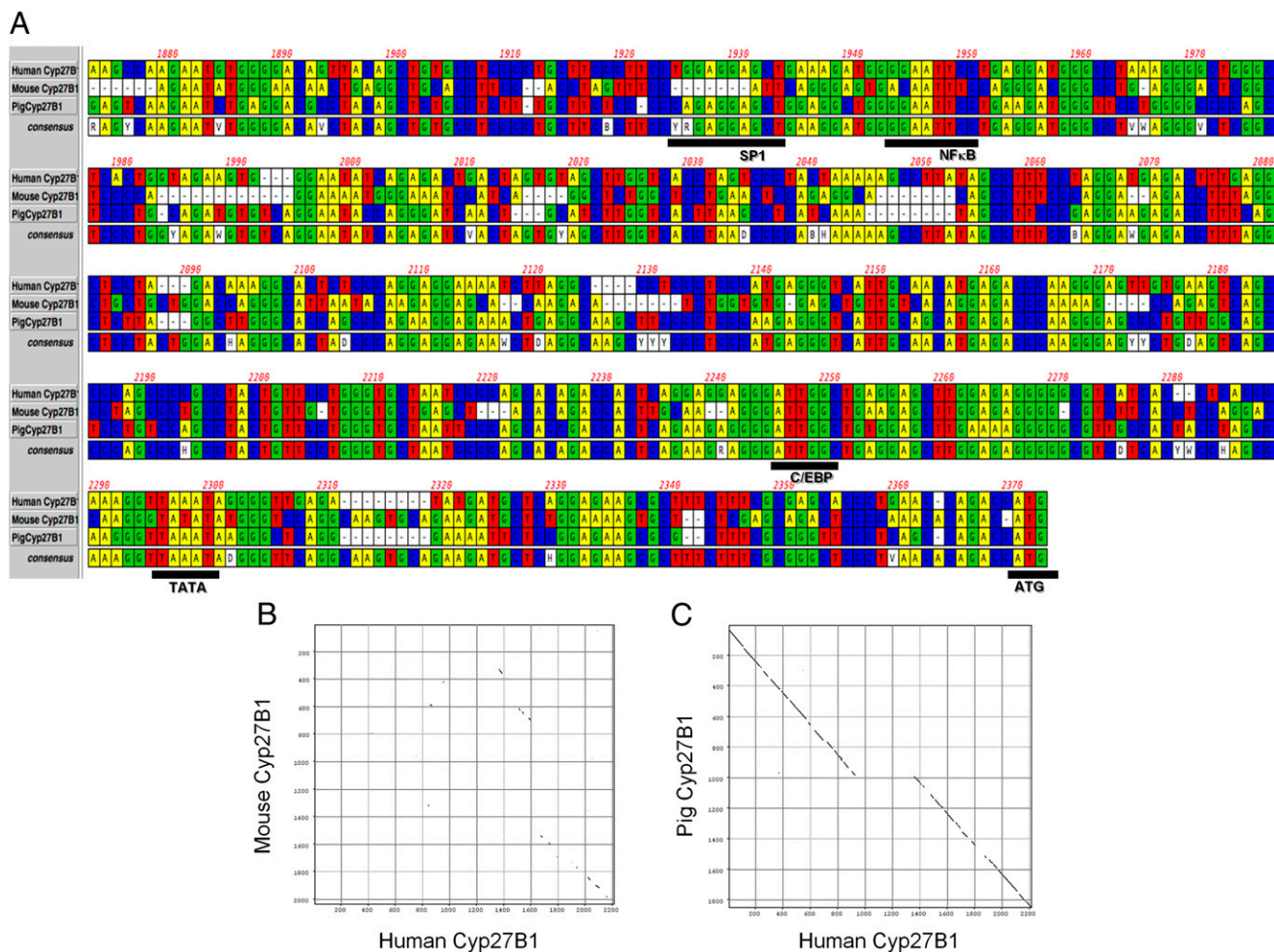
FIGURE 8. Divergence of the promoter sequence of the Cyp27B1 gene between humans, pigs, and mice. The 500-bp sequence upstream of the ATG of the human and mouse cyp27B1 genes was extracted from ENSEMBL, or for the pig from the pre-ENSEMBL build. A Clustal W alignment of the three sequences is shown in (**A**). A Pustell DNA matrix alignment of 2 kb upstream of the ATG between human and mouse promoter sequences (**B**) and between humans and pigs (**C**) is shown below. Note the divergent sequence of the mouse around the TATA box and initiator region, and the upstream NF-κB motif. The discontinuity in alignment of pigs and humans in (**C**) is due to a repeat insertion in human. All analysis used the MacVector package.

regions are conserved). Furthermore, in the case of IL-7R, the mouse promoter lacks the TATA box, which is conserved in pigs and humans. TATA boxes are commonly associated with highly regulated mammalian promoters (59).

Discussion

Marim et al. (62) reported that marrow cells from the mouse could be cryopreserved for an extended period and still generate macrophages. We have shown in this study that cells from pig marrow could also be cryopreserved, thawed, and grown on bacteriological plastic dishes to generate macrophages in response to rhCSF-1. The pig BMDM were actively phagocytic, uniformly positive for CD14, CD16, and CD172a, and responded to LPS with secretion of the proinflammatory cytokine, TNF-α. As discussed recently (26), mice have been studied extensively as a model for understanding human diseases, including sepsis, but they differ radically from humans in their resilience in the face of endotoxemia. Quite apart from the differences in sensitivity to LPS, mouse and human macrophages differ in the set of genes that are induced upon activation. These differences are not subtle differences in the magnitude of induction; they are all or nothing. A striking example is the production of NO in response to LPS. Human macrophages, even when primed with IFN-γ, do not induce NOS2, nor do they regulate arginase or the arginine transporter (CAT2, Slc7a2)

(GSE5099) (24), found previously to be induced in mouse macrophages (63). In most respects, the pig BMDM studied in this work resembled human monocyte-derived macrophages more closely than mouse BMDM. All three species induce the enzyme GCH1, which generates the cofactor tetrahydrobiopterin (BH4), and the by-product neopterin, which is a common marker of inflammation (64). Like human macrophages, and in keeping with earlier findings on other pig macrophage populations, pig BMDM did not produce NO, nor did they induce arginine-metabolizing genes or CAT2 in response to LPS (44, 45). In addition, the response of pig macrophages was unaffected by preincubation with CSF-1 in common with human, and quite distinct from mouse macrophages (58).

The microarray analysis revealed that approximately one-third of the most regulated genes in pig BMDM were not found to be induced in previous studies of mouse BMDM or other primary macrophage populations (http://www.biogps.org). Of these, two-thirds were previously found to be induced in activated human monocyte-derived macrophages (GSE5099 and GSE8608). In addition to CCL20, which binds specifically to CCR6, and has been implicated in homing of T cells and APCs (65), pigs shared with humans the induction of CXCL13 and CXCL9, which are also chemotactic for lymphocytes (66, 67). The most upregulated gene at 7 h in the pig was CXCL11, an IFN-inducible T cell α che-

moattractant (68). Induction of this set of chemokines suggests that the pig and human responses to LPS could have a distinctive engagement with the acquired immune system, a suggestion that could also explain the shared expression of the IL-7R, because IL-7 is associated with lymphoid development. In mice, IL-7R is not induced by LPS, but it is expressed constitutively on inflammatory macrophages in the peritoneum (http://www.biogps.org). In addition to IDO1, pig BMDM also share with human macrophages the induction of other tryptophan pathway enzymes, kynurenine hydoxylase, kynureninase, and tryptophan-tRNA synthetase. This pathway is commonly referred to as IFN-γ inducible (69), but is clearly responsive to LPS alone in the pig BMDM. The pathway generates a wide range of bioactive metabolites, including hydroxykynurenine and various pteridines, quinolinic acid, and 3-hydroxy-anthranilate (69). Another well-known human-specific inducible gene shared with pigs is CYP27B1, the enzyme that generates active vitamin D. In humans, vitamin D has been implicated in the control of antibacterial defense (70), and the vitamin D receptor (VDR) is expressed constitutively by human monocytes and macrophages, but not by mouse (http://www.biogps.org). Others have reported that the VDR is expressed in pig bone marrow (71). VDR is not annotated on our microarray, but we have confirmed robust constitutive expression of VDR mRNA in the pig BMDM by qRT-PCR (data not shown).

The segregation of the LPS-regulated genes into clusters of genes with similar patterns of expression using Biolayout generated 95 distinct clusters. The distinctions arise because each gene has its own distinct temporal profile of regulation, and some of them also varied between the three animals and the biological replicates. Genes that are subject to the same regulatory inputs and/or which contribute to the same pathway tend to be stringently coregulated in such a network. The genes that are induced rapidly by LPS, such as TNF-α (cluster 51), include many of the so-called primary response genes identified by Hargreaves et al. (55) that are bound by preassembled RNApol II in the resting state and induced via signal-dependent transcript elongation. A subset of these declines rapidly from an initial peak, through mRNA degradation mediated by the inducible RNA-binding protein tristetraprolin (72). Genes that are induced maximally at 7 h, the secondary response genes, include targets of inducible transcription factors, of which there are at least 50 (73), including some that are shared by pigs and humans, but not mice (STAT4, STAT1, IRF2, IRF7, GPBP1, BATF3, VDR [through induction of its ligand], and NCOA1, the nuclear hormone coactivator).

In summary, the generation of pig BMDM provides a model system for studying macrophage functional genomics that more closely resembles human biology than traditional mouse models. The domestic pig also offers a potential intersection of genomics and genetics. For example, Benga et al. (46) have reported significant variation in the response of individuals within and between pig breeds to microbial challenge. The use of BMDM in future studies will permit the assessment of macrophage autonomous variation in populations of animals from diverse sources, separated from the influence of husbandry, nutrition, and other variables. This in turn may contribute to understanding the genetic control of disease susceptibility in humans.

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Disclosures

The authors have no financial conflicts of interest.

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