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Manufacturing porcine islets: culture at 22°C has no advantage above culture at 37°C:

A gene expression evaluation

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Abstract

Background—The manufacturing process of islets includes a culture step which was originally introduced to ease the logistics of procedures in preparing the graft and transplant recipient. It has been suggested that culture at room temperature has an advantage over culture at 37°C, in part by reducing immunogenicity via preferential elimination of contaminating cells (such as passenger leukocytes) within islets. We investigated this using islets isolated from pancreata of adult pigs.

Methods—Porcine islets were isolated from three donors and cultured at 37°C for 1 day, and then under three different conditions: 37°C for 6 days (condition A); 22°C for 6 days (condition B); or 22°C for 5 days followed by 37°C for 1 day (condition C). Recovery was assessed by DNA measurement, viability by oxygen consumption rate normalized for DNA (OCR/DNA), and gene expression by RT-PCR for a series of 9 lymphocyte markers, 11 lymphokines and chemokines, and 14 apoptotic and stress markers.

Results—Post-culture islet recoveries were similar for the three culture conditions. Average OCR/DNA values were 129–159 nmol/min.mgDNA before culture, and 259–291, 204–212, and 207–228 nmol/min.mgDNA, respectively, for culture under conditions A, B, and C, respectively. Irrespective of culture condition, examined gene expression in all three series of lymphocyte markers, lymphokines and chemokines, and apoptotic and stress markers manifested a statistically significant decrease upon culture for 7 days. This decrease was most dramatic for condition A: in particular most of lymphocyte markers showed a >10-fold reduction and also 6 markers in the

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lymphokine and chemokine series: these reductions are consistent with the elimination of immune cells present within islets during culture. The reduction was less for apoptotic and stress markers. For culture under condition B the reduction in gene expression was less, and culture under condition C resulted in gene expression levels similar to those under condition A: this indicates that 24 hours at 37°C is sufficient to re-equilibrate gene expression levels from those in islets cultured at 22°C to those in islets cultured at 37°C. Results were consistent among the preparations from the three donors.

Conclusions—Culture of porcine islets at 37°C provides benefits over culture at 22°C with respect to OCR/DNA outcomes and reduced expression of genes encoding lymphocyte markers, lymphokines and chemokines, and markers for apoptosis and stress.

Keywords

apoptotic markers; chemokines; cytokines; lymphocyte markers; porcine islets; islet culture; temperature

Introduction

Clinical islet transplantation using islets from cadaveric donors is an accepted procedure in many countries. In the US phase 3 clinical trials are being undertaken towards approval of a Biological License Application. Xenotransplants using islets from porcine pancreata present a promising alternative to using human islets which are only available in limited amounts, since pigs essentially provide an “unlimited” donor source.

The manufacturing process of islets includes a culture step at 37°C after isolation and purification [1], which was originally introduced to reduce contaminating cells such as passenger leukocytes [2]. The current protocol for allogeneic transplants in clinical trials includes culture for the first 24 hours at 37°C and then at a lower temperature of 22°C for the remaining 24–48 hours prior to transplant to reduce metabolic rate and hypoxia [3]. Already in 1987 reduced immunogenicity of human islets due to culture for seven days at 24°C was inferred from the lower rejection rate of such islet preparations upon transplantation in diabetic mice [4]. Also, a two-day culture at 4°C was associated with a higher islet yield than was a two-day culture at 24°C or 37°C, as well as longer survival and normoglycemia upon transplantation in diabetic nude mice [5].

Culture at different temperatures is a relevant aspect in islet manufacturing, as there are various claims regarding the optimal culture temperature with respect to viability and recovery. Culture at 22–24°C is reported to decrease the level of necrotic cell death in islets, and to increase islet recovery [6,7]. In particular, low temperature protects islets under suboptimal culture conditions, e.g., inadequate oxygenation when islets are cultured at higher surface densities to reduce the number of flasks needed without providing oxygen and nutrient needed [8]. It could also prevent hypoxia by decreasing the metabolic rate [9]. Low temperature culture is believed to reduce the immunogenicity of islets, but there are also reports that endothelial cells, known to be present in islets and a target for rejection, are more highly depleted in cultures at 37°C [10,11]. Islets cultured at 24°C possess a better morphological structure than islets cultured at 37°C, and experiments in rats have shown that the antigenicity and proinflammatory cytokines levels are reduced [12]. Purified human islet preparations remain functionally and morphologically intact after culture at 24°C for seven days [4]. However, islets cultured at 24°C exhibit increased apoptosis [13]. Low-temperature culture may cause the degranulation of islets or result in impaired insulin production. Therefore, it has been suggested to apply a combination of these two temperatures to most efficiently maintain human islets in culture for transplantation [14].

Post-isolation culture of islets has the potential to facilitate elimination of undesirable immunoreactive cell populations, and to prepare cells for survival and function in a human environment. In our most recent preclinical xenotransplant studies, porcine islets are cultured at 37°C for 6 to 9 days prior to transplant into nonhuman primates. This period of culture may, in itself, reduce the immunogenicity of the islets [10,11,15]. Here, we examined various temperature conditions during culture of adult porcine islets to further evaluate the effects on immunogenicity and quality, using physiological indicators of viability and molecular gene expression markers to assess immunological, inflammatory, and stress responses.

Materials and Methods

Pancreas donors, islet isolation

Three adult female wild-type Landrace retired breeder pigs, aged 3–6 years and weighing about 250 kg, served as pancreas donors. The animals were sacrificed, pancreata were immediately procured, and islets were isolated using a modified Ricordi method [16]. After purification, islets were incubated in ME199 culture medium (Mediatech, Manassas, VA, USA) with 10% heat-inactivated porcine serum (Gibco, Aukland, NZ), L-glutamine (Mediatech) and heparin (10 U/ml, APP Pharmaceuticals) on gas-permeable culture devices (G-Rex100, Wilson-Wolf Manufacturing, New Brighton, MN, USA) at 1,000 islet equivalents (IE)/cm² at 37°C for 1 day, and then under three different conditions: 37°C for 6 days (condition A); 22°C for 6 days (condition B); or 22°C for 5 days followed by 37°C for 1 day (condition C) (Fig. 1).

Quality assessments

Islet (cellular) recovery was assessed by DNA measurement, and viability by oxygen consumption rate normalized for DNA (OCR/DNA). Recovery was calculated by DNA concentration at the end of culture divided by DNA concentration put into culture, and expressed in percentage. The calculation for DNA recovery relates to IE recovery (10.4ng DNA/IE) [17]. For islets that were cultured at 22°C, the samples for OCR were allowed to equilibrate to 37°C for 30 minutes prior to assessment. OCR and DNA assays were conducted as described previously [18,19]. The coefficient of variation of the 3 chambers was generally less than 20%.

Rationale for selected genes in the series of lymphocyte markers, lymphokines and chemokines, and stress and apoptotic markers

The selection of markers (Table 1) was based on published literature providing evidence of their involvement in graft loss or survival of allo- and xeno-islet transplantation. Markers for inflammation, angiogenic revascularization, and hypoxia-induced apoptosis and inflammation were investigated [20–22], as well as histoprotective genes and indicators for a stress response [23,24]. We also selected markers activated in the death signaling pathway [22,23,25–27]. It should be noted that β -cell death is not caused by a simple mechanism of apoptosis, and therefore cytotoxic molecules are involved in the evolution of allograft rejection and islet graft destruction were included [28]. We also identified markers that have been shown to change relative to culture at 24°C or 37°C [12].

Gene expression analysis

Cultured islet samples containing 2,500 IE in lysis buffer were homogenized using the QIAshredder (Qiagen, Valencia, CA) and RNA was extracted using the RNeasy minikit (Qiagen) following the manufacturer's instructions. RNA concentrations were determined by NanoDrop 1000 spectrophotometry (Thermo Fisher Scientific, Wilmington, DE) and

quality was verified with the Agilent 2100 expert Eukaryote Total RNA Nanokit (Agilent Technologies, Waldboronn, Germany). To confirm the RNA quality, electropherograms were evaluated to generate an RNA Integrity Number (RIN) number. Purified RNA had RIN values of 10, except for two samples with RIN numbers of 7 that were isolated from pigs immediately after isolation and purification, but prior to culture.

After genomic DNA was eliminated using the gDNA Wipeout buffer (Qiagen), 2µg of total RNA was converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen). Perfecta SYBR Green FastMix, with low rox (Quanta BioSciences, Foster City, CA) was used for qRT-PCR with 100nM gene-specific primer sets and 6ng of sample. To design primers, available porcine gene and mRNA sequences were obtained from Genbank and a consensus sequence was generated for each gene by ClustalX alignment. The consensus was imported into Primer 3 v4.0 (<http://frodo.wi.mit.edu/primer3/>) for oligo selection. Candidate primers were blasted again to the NCBI nonredundant database to confirm that the primers were specific. Primers which gave a single peak on the dissociation curve and no amplification in the non-template control were chosen. Specific primer sequences are listed in Table 1. Quantitative RT-PCR was performed on an ABI 7500 machine with the following parameters; 95°C for 1 minute, then 50 cycles at 95°C for 4 seconds and 60°C for 45 seconds, followed by a dissociation step. Each sample was run in triplicate using the plate as a replicate. The internal control housekeeping gene was cyclophilin. Negative control was RNase-free water. Data were recorded as the mean Ct value normalized to the cyclophilin mean (ΔCt). $\Delta\Delta Ct$ (ΔCt target sample – ΔCt control sample) and relative fold changes ($2^{-\Delta\Delta Ct}$) were computed using the $\Delta\Delta Ct$ values of the samples. Genes with a mean Ct values 38 or no value were given Ct values of 38 for computational purposes and are indicated with “LD” in Table 2.

Statistical analysis

Arithmetic mean values were evaluated using the parametric student t-test and analysis of variance with respect to individual pig source and culture condition as independent variables. The level of significance was set at $P < 0.05$.

Results

Quality assessment

The effect of culture temperature on cell viability, as determined by recovery of DNA, is presented in Fig. 2. For the islet preparations from the three pigs, the DNA recovery after 37°C culture (condition A) ranged between 73 and 90%, for 22°C (condition B) between 70 and 104%, and at 22°C for 5–6 days followed by 1 day at 37°C (condition C) between 76% and 110%. The differences in recovery among the three culture conditions were not statistically significant.

Oxygen consumption increased significantly over time under all culture conditions ($P < 0.001$, Fig. 3). The average OCR/DNA values before culture ranged between 129 and 159 nmol/min•mg DNA. After 6–7 days in culture, the average values for condition A were highest and ranged between 259 and 291 nmol/min•mg. Average values for condition B were significantly lower ($P < 0.001$) and ranged between 204 and 212 nmol/min•mg, and those for condition C ranged between 207 and 228 nmol/min•mg (no statistically significant difference compared to A or B).

Gene expression

The Ct values for the housekeeping control gene cyclophilin consistently ranged between 18 and 20 with a standard deviation between 0.10 and 0.50. This consistent outcome of the

control value justified the analysis of ΔCt values, including also the comparison of ΔCt values, for the individual marker genes in samples from individual pigs after the three culture conditions.

A semi-quantitative score of marker gene expression with respect to the cyclophilin standard is presented in Table 2 for each individual pig donor. Almost all genes showed detectable expression, at levels lower than the abundant CyC, i.e., with Ct values above that of the CyC housekeeping gene. Hence the range of Ct values between that of the cyclophilin housekeeping gene (18–20) and undetectable (LD in Table 2, Ct >38) was a quantifiable range for statistical comparison. The variation between the three donor pigs in levels for an individual marker expression was reasonably low. We therefore combined the data of all three donor pigs in the subsequent evaluations.

In general the expression levels of apoptotic and stress markers were higher than that of lymphokines and chemokines, and the expression of lymphocyte markers was lowest. The outcomes were consistent with the quality of isolated and purified islets, in which the contamination with leukocytes is generally low, and expression of apoptotic and stress markers might already have been upregulated during the period between porcine pancreas procurement and initiation of the culture, which typically requires approximately 6 hours.

Variation in gene expression during culture

Incubation of purified islets in culture media for 7 days under all three experimental conditions significantly altered the global patterns of gene expression ($P < 0.001$). The change was most dramatic at 37°C (condition A), which associated with significantly reduced gene expression levels compared to day 0 in all series, including lymphocyte markers, lymphokines and chemokines, and apoptosis and stress markers (analysis of variance, $P < 0.001$, Fig. 4). Greater than 10-fold reductions were consistently noted in the series of lymphocyte markers, including CD4, CD3e, Perf, GB, SLA-DQB and SLA-DRB (Fig. 4); and in the series of lymphokines and chemokines for IL1b, IL8, MCP-1, Mig, Mip2, and SOCS3 (Fig. 4). These reductions are consistent with elimination of immune cells during culture. In the series of apoptotic and stress markers, only Hsp70 was reduced as much as 10-fold (Fig. 4).

Extended islet incubation at 22°C (condition B) was associated with reduced expression of markers for lymphocytes and antigen-presenting cells that was similar to the effect of 37°C culture (Fig. 4). Statistical analysis showed significance for the differences between day 0 and day 7 samples for the series of lymphocyte markers and for the lymphokines and chemokines series ($P < 0.001$). However, expression of lymphokines and chemokines was less affected, with only IL6 and MCP1 showing >10-fold reductions. Apoptosis and stress marker expression levels were similar to those observed at 37°C culture, except for TXNIP, which was increased more than 5-fold (Fig. 4). Overall, there was no significant difference in the series of apoptotic and stress markers between day 0 and day 7 (Fig. 4).

Incubation of islets at 22°C for 5 days then increasing the temperature to 37°C for 1 day (condition C) resulted in gene expression patterns similar to 7 day culture at 37°C (condition A), both in terms of $\Delta\Delta\text{Ct}$ values and relative differences (Fig. 4). A minor exception was noted for iNOS, which was unchanged at low temperature but just exceeding the 2-fold threshold at 37°C culture (Fig. 4).

Difference in gene expression between three culture conditions

Incubation of islets at 22°C resulted in increased levels of gene expression relative to incubation at 37°C, and the magnitude of the difference was related to the time at 22°C. As shown in Fig. 5, eleven genes are more highly expressed (minimum expression is 2-fold

different) at 22°C compared to 37°C, and two genes show reduced expression. In condition C, in which islets at 22°C were moved to 37°C for the final 24 h, one gene, IL6, was more highly expressed and none were suppressed (Fig. 5). Statistical analysis comparing $\Delta\Delta C_t$ values for cumulative gene expression in lymphocyte markers, lymphokines and chemokines, and apoptosis and stress markers showed an order of condition B > condition C > condition A. A more than 10-fold higher expression was consistently observed for IL6, Mig and Mip2 in the series of lymphokines and chemokines (Fig. 5). Interpretation of IL8 expression ratios was confounded by its lack of expression at 37°C. The data indicated that 24 hour at 37°C was sufficient to re-equilibrate gene expression levels from 22°C to the level observed in islets cultured at 37°C for 7 days.

The patterns were consistent for all three pigs in the series for lymphocyte markers for CD4, CD3e, Perf; in the series of lymphokines and chemokines for IL15, IL8, IL6, Mig, and Mip2; and in the series of apoptotic and stress markers for SOD2, c-jun, TXNIP, and FasL.

Discussion

Islet isolation from the pancreas is a stressful procedure that induces an increase in expression of proinflammatory genes and a poor prognosis following transplantation [2]. Hence, post-harvest culture was introduced to reduce inflammatory markers, reduce or eliminate immunogenicity mediated by passenger leukocytes, and improve transplant survival in diabetic recipients [2]. Here, we confirm that adult porcine islet culture after isolation elicits a substantial reduction in expression of lymphocyte markers, expression of lymphokines and chemokines, and some markers for apoptosis and stress. Panels of gene expression markers were designed to provide information about treatment effects on key physiological processes involving inflammation, immunogenicity, stress, and apoptosis, that are central to islet function and maintenance in recipients. Data quality was established through the demonstration that quantitative RT-PCR data was obtained in the linear range of the assay (C_t values between 18 and 38) in nearly all cases, and good reproducibility among donor pigs. Therefore we concluded that the evaluation of gene expression panels related to key processes is a good approach for molecular characterization of islet preparations. This conclusion is presented with the remark that it is based on data of three donor pigs, but seems justified considering the low interanimal variability in outcome.

The literature claim for human islets that culture for two days at 22–24°C results in a higher recovery than culture at 37°C [5] is not confirmed in the present evaluation showing similar recovery for the three culture conditions. It is worth noting that the present results are for a 7-day culture period and recovery is based on DNA determination and not on manual counting, because manual counting and DNA determination can reveal large differences. Also, the distribution regarding islet diameter appears to change dependent on the temperature of culture [5]; at lower temperature there is less core cell damage and hence a better survival of large-sized islets [6,7]. It needs to be established whether this difference in survival during culture for islets of different size affects the outcome in manual counting followed by size correction to transfer data in Islet Equivalents. It should also be emphasized that cultures in the present study were done in flasks using gas-permeable silicone rubber membranes enabling optimal oxygen supply, so that necrosis observed in other studies might not occur.

The OCR/DNA value has been introduced as a marker for fractional viability [18,19]: the increase during culture is a well-known phenomenon that is ascribed to the disappearance of dead cells. Remarkably, OCR/DNA values after a 7-day culture are higher for culture at 37°C than at lower culture, resulting in better viability of islets after culture. This outcome is relevant for the transplant setting, because the OCR/DNA value is a predictor for graft

outcome. This has been demonstrated in a number of transplant settings including human and rat islets transplanted into diabetic mice [30,31] and recently for adult porcine islets transplanted into diabetic monkeys. Noteworthy, for all three culture conditions the threshold value acceptable for the release for transplantation in nonhuman primates (175 nmol/min•mgDNA [32]) was widely passed.

The reduction in gene expression after culture occurred for almost all lymphocyte markers, which is in agreement with the loss of 'passenger' leukocytes during culture [3]. The strong reduction in SLA is worth mentioning as this histocompatibility antigen is not only an important antigen in alloreactivity but also in xenogeneic responses [33–35]. The reduction in lymphokines and chemokines was variable which might be related to different cell populations producing these molecules; i.e., lymphokines like IL6 and IL8 were strongly reduced, and chemokines produced by macrophages to a less extent (IL1b, MCP-1, Mip2). Also for apoptotic and stress markers a reduction was noted, which appeared to be less than that in the other panels, particularly the series of lymphocyte markers. However, in this interpretation it has to be noted that in general the expression of apoptotic and stress markers was highest and that of lymphocyte markers lowest, so that the actual reduction is quite high for apoptosis and stress markers when compared to lymphocyte markers. Quite remarkable in this respect is the substantial reduction in expression of Hsp70, which exceeds the reduction in Hsp27: the biological relevance of this observation with respect to the necessity of cells to protect from oxidative injury needs to be established.

A most remarkable observation in the present study is the higher reduction in gene expression for cultures at 37°C than for cultures at 22°C. This apparently contradicts literature data presented in the introduction, which could be related to differences in species, and also differences in culture conditions like the use of flasks with gas-permeable silicone rubber membranes. The higher effect of 37°C culture than culture at 22°C is nicely illustrated by the results of culture condition C: a one-day culture at 37°C at the end of the culture period resulted for almost all genes in an expression level in between that of 37°C and 22°C. Thus, under the assumption of a beneficial effect of reduced gene expression in the three series for islet quality in transplantation, the present data do not support a beneficial effect of culture at low temperature.

In conclusion, culturing adult porcine islets for seven days after isolation has a beneficial effect on OCR/DNA as a marker for fractional viability, and results in a substantial reduction in expression of genes encoding lymphocyte markers, lymphokines and chemokines, and markers for apoptosis and stress. This effect is most pronounced for culture at 37°C during the full culture time, and less in case of culture at 22°C. The reduced effect for culture at 22°C can be partially compensated if the last day of culture is again at 37°C.

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Abbreviations

Ang-1	angiopoietin 1
Bax	BCL2-associated X protein
c-fos	V-fos FBJ murine osteocarcinoma viral oncogene homolog

c-jun	Jun oncogene cellular homolog
FasL	Fas ligand
GB	granzyme B
Hif1a	hypoxia inducible factor 1, α subunit
Hsp	heat shock protein
IF16	interferon α -inducible protein 6
IL	interleukin
IE	islet equivalent
iNOS	inducible nitric oxide synthase 2
MCP	monocyte chemotactic protein
Mig	monokine induced by interferon γ
Mip2	macrophage inflammatory protein 2
OCR	oxygen consumption rate
Perf	perforin
PKC	protein kinase C
SLA	swine leukocyte antigen
SOCS3	suppressor of cytokine signalling 3
SOD2	superoxide dismutase 2
TF	tissue factor
TGM2	transglutaminase 2
TNF	tumor necrosis factor
TRL4	toll-like receptor 4
TXNIP	thioredoxin interacting protein
Vegf	vascular endothelial growth factor

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Fig. 1.

Study design of islet preparations from three different donors, cultured for 7 days at 37°C (condition A), cultured for one-day at 37°C and 6 days at 22°C (condition B), and cultured for one day at 37°C followed by 5 days at 22°C and one day at 37°C (condition C). Day 0 is the day of isolation.

Fig. 2. DNA recovery of islet preparations cultured under various temperature conditions, expressed in percentage relative to day 0. For the description of the conditions A–C, see Figure 1. Data shown are arithmetic mean values from three different donors $\pm$ SD.

Fig. 3. OCR/DNA values of islet preparations before and after culture under various temperature conditions. For the description of the conditions A–C, see Figure 1. Data shown are arithmetic mean values from three donors $\pm$ SD. The value of 175 nmol/min•mgDNA (dashed line) is a generally accepted threshold in release of islet preparations for transplantation.

Fig. 4.

Marker gene expression of islets cultured under various temperature conditions. For the description of the conditions A–C, see Figure 1. Data shown are relative fold – change ratios with respect to day 0 values. Results are presented as mean values (horizontal line) with minimum to maximum (box) from three islet preparations. No change in gene expression ($\pm$ 2-fold) is represented by a horizontal grey bar. For abbreviations, see Table 1.

Fig. 5. Marker gene expression of islets cultured under various temperature conditions. For the description of the conditions A–C, see Figure 1. Data shown are relative fold – change ratios are presented with respect to values for condition A. Results are presented as mean values

(horizontal line) with minimum to maximum (box) from three islet preparations. No change in gene expression (± 2 -fold) is represented by a horizontal grey bar. For abbreviations, see Table 1.

Table 1

Markers used in gene expression analysis

Abbreviation	Name	Forward sequence	Reverse sequence
CyC	Cyclophilin	CCGTCGATGGCGAGCCC	CCCGTATGCTTCAGGATAAAA
<i>Lymphocyte markers</i>			
CD4	CD4 molecule	GAAGGTGGTGCTGAGCTTGA	CCTATCACAGAGGCCAGGAG
CD3e	CD3e molecule (CD3-TCR complex)	TACCTGCACAGTCGGAGAGA	TAGTCTGGATTGGGGACAGG
Perf	Perforin (pore forming protein)	GCTCCACCCTGAGTTCAAGA	AGTCCTCCACCTCCTTGGAT
GB	Granzyme B (cytotoxic T lymphocyte associated serine esterase 1)	GGAGACCCAAAGAAAAAGCA	TATCCAGGGCAGGAAAACTTG
TLR4	Toll-like receptor 4	AGCTGTATCGCCTTCTCAGC	AGCAAAGGCTTCTTGTAGTCG
SLA-DQB	Swine Leucocyte Antigen β 1 domain (MHC class II)	GACTATCTCCCCATCCAAAGG	GGAAACCACTGGACTTTCACC
SLA-DRB	Swine Leukocyte Antigen DRB (MHC class II)	GAAACGGTTCCTCAGAGTGG	CTCATCATCTTGCCCTGAGC
CD40	CD40 molecule (TNF receptor superfamily member 5)	AACAGAAATGCCTTCCTTGC	TCGCAGTATTGTGCTGATG
CD80	CD80 molecule	TGACTTCTGTTCAGGCA TCG	CTCATACTTGGGCCACACCT
<i>Lymphokines and chemokines</i>			
TNF α	Tumor necrosis factor α (TNF superfamily member 2)	TTCCAGCTGGCCCCCTTGAGC	GAGGGCATTTGGCATACCCAC
IL15	Interleukin-15	GCGATGAAGTGCTTTCTCCT	TTCTCCAGCTCCTCACATT
IL1b	Interleukin-1 β	AAGGCTCTCCACCTCCTCA	CGCTTCTCCATGTCCCTCT
IL8	Interleukin-8	GAACGTGAGAGTGATTGAGAGTGA	GCTGTTGTGTGCTTCTCAGTT
IL6	Interleukin-6	ATTAGTACCAAAAGCACTGATCC	TGAGAAATGATCTTTGTGTTCTTC
MCP-1	Monocyte chemotactic protein-1	CGAAGCTTGAATCCTCATCC	TGTATAGCAGCAGGTGACTGG
Mig	Monokine induced by interferon γ	CAAAACATGTCTAAACCCAGATTCA	GCTGACCTGTTTTCTCCCACT
Mip2	Macrophage inflammatory protein 2	GGAATTCACTCAAGAACATCC	TGGCTATGACTTCCGTTTGG
SOC33	Suppressor of cytokine signaling 3	GAGAGCGGCTTCTACTGGAG	GACTGGGTCTTGACGCTGAG
iNOS	Inducible Nitric Oxide Synthase 2	CGTTATGCCACCAACAATGG	GAGCTGAGCGTTCCAGACC
TF	Tissue factor	TCCGGAAGAGTTAACCGAAG	ACCACCGCTCCAACGATG
<i>Apoptotic and stress markers</i>			
IF16	Interferon α -inducible protein 6	TCAAGGTCCGGTGATAAAGC	CACCGCAGGTGTAGAGTAGTAGG
Hif1a	Hypoxia inducible factor 1, α subunit	TTACAGCAGCCAGATGATCG	ATTGTCTTTTGTCTCCGTTCC
Vegf	Vascular endothelial growth factor A	CCTTGCCCTTGCTGCTCTACC	CGTCCATGAACATCACCACTTC

Abbreviation	Name	Forward sequence	Reverse sequence
SOD2	Superoxide dismutase 2, mitochondrial	GATTGCCGCTTGTTCTAACC	TTTCAGGTAATCAGGTCTGACG
Ang-1	Angiopoietin 1	ATGCAGTTCAGAAACCACACG	GGGTCTCAACATCTGTCAGC
PKC	Protein kinase C	GTTACCCAGACAGCCTGTGG	CCTACACATTAAATGACAAACTCTGG
c-fos	V-fos FBJ murine osteosarcoma viral oncogene homolog	CAACCTGCTGAAAGGAGAAAGG	CAGGCAGGTCGGGTGAGC
c-jun	Jun oncogene cellular homolog	AGGAAGGTATGAGGAACC	CGAGTTCTGGGCTTTTCAAGG
TXNIP	Thioredoxin interacting protein	CCTCTGCTAGATGACACAGATGG	CACGTTGTGTGTGAGGATGC
TGM2	Transglutaminase 2	GTCTTCCCTGCAAGCCTACC	GCGCTTCAAAAGAGACAGTGG
FasL	Fas ligand (TNF superfamily, member 6)	GGGATGTTTTCAGCTCTTCCA	GTGTCTTCCCAATCCAGAGG
Bax	BCL2-associated X protein	ACATGGAGCTGCAGAGGATG	CCAGTTGAAGTTGCCGTCAG
Hsp70	Heat shock 70kDa protein 1B	GGTGCTGACCAAGATGAAGG	CTGCGAGTCGTGTGAAGTAGG
Hsp27	Heat shock 27kDa protein 1	TCAGCAGCGGGCGTCTCG	CTTGGTCTTGACCGTCAGC

Table 2

Marker expression level score

Gene	Pig #2				Pig #2				Pig #3			
	Day 0	A	B	C	Day 0	A	B	C	Day 0	A	B	C
<i>Lymphocyte surface markers</i>												
CD4	+	+	+	+	+	LD	+	+	+	+	+	+
CD3e	+	+	+	+	+	+	+	+	+	+	+	+
Perf	+	+	+	+	+	LD	LD	LD	+	LD	+	+
GB	+	LD	+	LD	+	+	LD	+	+	LD	LD	LD
TLR4	++	++	+	+	++	++	+	++	++	++	+	+
SLA-DQB	++	++	++	++	++	++	++	++	++	++	++	++
SLA-DRB	+	+	+	+	++	+	+	+	++	+	+	+
CD40	++	++	++	++	++	++	++	++	++	++	++	++
CD80	+	+	+	+	+	+	+	+	++	+	LD	++
<i>Lymphokines and chemokines</i>												
TNFa	+	+	+	+	+	+	+	+	+	+	+	+
IL15	+	+	++	+	++	+	+	+	++	+	++	+
IL1b	+	+	+	+	++	++	LD	LD	++	+	+	+
IL8	++	++	++	++	++	LD	++	++	++	LD	++	++
IL6	++	LD	+	+	++	+	+	+	++	+	+	+
MCP-1	+++	++	++	++	+++	++	++	++	+++	++	++	++
Mig	++	+	+	+	+	+	+	LD	++	+	+	+
Mip2	+++	++	+++	++	+++	++	+++	++	+++	++	+++	++
SOC3	++	+	++	++	++	++	++	++	+++	+	+++	++
iNOS	++	+	++	++	++	+	+	++	+	+	+	+
TF	++	++	++	++	+++	++	++	++	+++	++	++	++
<i>Apoptotic and stress markers</i>												
IF16	++	++	++	+	++	++	++	++	++	++	++	++
Hfla	++	++	+++	++	+++	++	+++	++	++	++	++	++
Vegf	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++

	Pig #2				Pig #2				Pig #3			
Gene	Day 0	A	B	C	Day 0	A	B	C	Day 0	A	B	C
SOD2	++	+	++	++	++	++	++	++	++	++	++	++
Ang-1	+	+	+	+	++	+	+	+	++	+	+	+
PKC	++	+	+	+	++	+	+	+	++	+	+	+
c-fos	++	++	++	++	+++	++	++	++	+++	++	++	++
c-jun	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
TXNIP	+++	++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
TGM2	++	++	++	+++	++	+++	++	+++	++	+++	++	+++
FasL	+	+	+	LD	+	LD	+	+	LD	LD	+	+
Bax	++	++	++	++	++	++	++	++	++	++	++	++
Hsp70	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Hsp27	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Column headings A, B, and C represent three culture conditions: for the description of these conditions, see Figure 1.

Δ Ct values (mean Ct value minus the cyclophilin mean) were scored as follows: +, low expression, Δ Ct > 10; ++ moderate expression, Δ Ct between 3 and 10; +++ high expression, Δ Ct < 3; LD, limit of detection (Ct values $\geq$ 38 or undetermined).