




Characterization of MDCK cells and evaluation of their ability to respond to infectious and non-infectious stressors

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Abstract The Madin-Darby Canine Kidney (MDCK) cell line is widely used as epithelial cell model in studies ranging from viral infection to environmental pollutants, and vaccines production. However, little is known about basal expression of genes involved in innate immunity, and the ability to respond to infectious and non-infectious stressors. Therefore, the aims of our study were to evaluate the basal level of expression of pivotal genes in the innate immune response and cell cycle regulation, as well as to evaluate the ability of this cell line to respond to

infectious or non-infectious stressors. As surmised in our working hypothesis, we demonstrated the constitutive expression of genes involved in the innate immune response and cell defense alike, including TLRs, Interleukins, *Myd88*, *p65/NF- κ B* and *p53*. Moreover, we described the ability of this cell line to respond to LPS and cadmium (Cd²⁺) in terms of gene expression and cytokine release. These data confirm the possibility of using this cell line as a model in studies of host/pathogen interaction and response to non-infectious stressors.

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Introduction

The Madin-Darby Canine Kidney (MDCK) cell line is widely used as epithelial cell model. Indeed, its properties of apico-basolateral polarity, cell junctions and in vitro growth rate are well known (Dukes et al. 2012). MDCK cells are also used in studies of viral infections, and more recently in vaccine production (Gregersen et al. 2011). However, little is known about basal expression of genes involved in innate immunity, DNA repair, cell cycle regulation, as well as in the secretion of cytokines, and the response to infectious and non-infectious stressors. Moreover, no

data are available about the effect of cell passages and aging of confluent monolayers. These gaps may hinder a correct use of these cells for vaccine production and studies on host/pathogen interaction. Owing to the above, the aims of our study were: 1-to evaluate the basal level of expression of pivotal genes in the innate immune response and cell cycle regulation. 2-to evaluate the ability of this cell line to respond to infectious or non-infectious stressors.

In our study we decided to consider cell monolayer aging, a concept differing from cellular senescence; indeed, whereas the phenomenon known as replicative senescence is due to the ability of cells obtained from primary cultures to reduce their number of divisions until a complete stop within few weeks of culture (Ogrodnik et al. 2019), an immortalized cell line can indefinitely replicate in the presence of satisfactory environmental conditions (e.g. nutrients, temperature). However, after monolayer confluence the concentration of Damage-associated molecular patterns (DAMPs, such as self DNA) in supernatants can increase and cause changes of gene expression; indeed sensing such DNAs leads to activation of interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF- κ B), respectively (Sok et al. 2018).

In this study, we chose cadmium (Cd²⁺) as a non-infectious stressor. This heavy metal is widely known for its toxic and carcinogenic effects. Cd²⁺ toxicity has now been shown in nearly every organ and tissue of the body and the kidney is a critical organ for Cd²⁺ accumulation and toxicity. Indeed, exposure to Cd²⁺ has been implicated in renal dysfunction (Bernard 2008). Many studies demonstrated the ability of Cd²⁺ to modulate the activity of cellular enzymes, to suppress mitochondrial functions, to initiate oxidative stress, and to disrupt calcium homeostasis (Tchounwou et al. 2012; Rusanov et al. 2015). Moreover, in our previous study we demonstrated the ability of this heavy metal to modulate innate immune responses in IPEC-J2 cells as a function of both time and concentration (Razuoli et al. 2018). Furthermore, Cd²⁺ exposure has been reported to alter a variety of immune cell functions in terms of both protein expression patterns and immune cell functions (Maret and Moulis 2013). The major mechanism behind Cd²⁺ toxicity is oxidative stress that induces damages in organs such as kidney, liver, lung, brain and testis. This is due to the inflammatory response caused by Cd-driven tissue damage, including leukocyte

activation and recruitment. In particular, Cd²⁺ induces expression of IL-1 β , TNF- α , IL-6, IL-8, which underlies the amplification of the inflammatory response (Milnerowicz et al. 2015). Moreover, low levels of Cd²⁺ may be present in environment contaminants like dust (Tan et al. 2016), plastic and glass (Turner 2019).

To mimic infectious stressors, we chose Lipopolysaccharide (LPS), present in the external wall of Gram-negative bacteria and largely responsible for their toxicity. LPS is recognized by TLR4-co-receptors MD2 and CD14, which recognize the lipid A core of LPS. TLR4 transduces the signal via MyD88 and TRIF adapters to activate NF- κ B- and/or IRF3-mediated transcription of genes encoding pivotal molecules of the immune system, including cytokines and chemokines, which mimics major inflammatory response models (Poltorak et al. 1998; Rathinam et al. 2019). Moreover, LPS is of concern since it is a common contaminant of cell cultures. It is an amphipathic molecule which adheres to hydrophobic materials like plastics and glassware. Therefore, cross-contamination between experiments might occur if LPS molecules were not effectively removed. If LPS is introduced into laboratory equipment, its persistence can determine a memory effect provoking inflammatory responses or endotoxin tolerance. The control of LPS contamination is difficult due to its ubiquity in nature, toxicity and stability in solutions (stable depending on pH, ions and surfactants), and even sterilization at temperatures > 180 °C (Gorbet and Sefton 2005). This can cause contamination or cross-contamination between experiments if LPS is not effectively removed from the system (Schwarz et al. 2014).

As for the relevance of our study, MDCK cells are widely used in many laboratories for viral isolation, vaccine development and production; furthermore, as they derive from renal tubule cells, they can be used *in vitro* to evaluate host–pathogen and host-chemical interaction. In this study, for the first time the basal expression of genes involved both in the inflammatory response and in the cell cycle was evaluated; furthermore, we investigated the effects of passage level and aging on the expression of the same genes. These parameters can affect the expression of cytokines, as shown, e.g. in IPEC-J2 cells (Razuoli et al. 2013). Furthermore, we evaluated the ability of MDCK cells to interact with stressors of various nature.

Materials and methods

Cell culture

MDCK cells (Madin-Darby Canine Kidney, IZSLER biobank OIE code BS CL 64) were grown in Minimum Essential Medium (MEM, Carlo Erba Reagents S.r.l., Milano, cat FA30WL0440500) enriched with 10% (v/v) fetal calf serum (FCS, GIBCO™, Thermofisher scientific, Milano, cat 10437-036) and a mixture of antibiotics (penicillin and streptomycin, 1% v/v, Carlo Erba Reagents S.r.l., Milano, cat FA30WL0022100). Cells were seeded into 12-well tissue culture plates (2 ml per well, 2.5×10^5 cells/ml) and incubated at 37 °C in 5% CO₂ until confluence (16–22 h). Cells were tested at the 33rd, 34th and 40th passages; then, cells at confluence (40th passage) were further incubated for 24 h to evaluate the effects of aging. Each experiment was repeated ten times.

Cadmium treatment

Monolayer cells were treated with 20 μM Cadmium (Carlo Erba reagents srl, Milano, cat 505548). The choice of the exposure level was based on a previous study that defined low and moderate cadmium exposure levels (Luevano and Damodaran 2014). In our study, we treated MDCK with 20 μM Cd²⁺, which was equivalent to 1.9% of the 4-h DL100 (105 mg/kg) value after oral administration in dogs (Venugopal and Luckey 1975). Cd²⁺ was dissolved in MEM, and cells were stimulated for 3 or 24 h at 37 °C in 5% CO₂. We set up 3 biological replicates; cells treated with medium only were used as negative control (3 biological replicates, as well). Each experiment was performed trice.

Cd²⁺ uptake

We tested the ability of MDCK to adsorb Cd²⁺ at different times after exposure: 1, 3 and 24 h. Cells were treated with 20 μM Cadmium (4 μg/10⁶ cells); then, intracellular concentration of Cd²⁺ at different time of exposure was checked using a Graphite Furnace Atomic Absorption Spectroscopy (model ZEE nit 650 P, Analytik-Jena, Germany) as previously described (Razzuoli et al. 2018). Each Cd²⁺ concentration was checked in quintuplicate and intracellular Cd²⁺ was expressed as μg Cd²⁺/10⁶ cells.

Lps treatment

Monolayer cells were treated with 1 μg/mL LPS from *E. coli* O111:B4 (Sigma-Aldrich, Inc., Milano, cat L4391). LPS was dissolved in MEM, and cells were stimulated for 3 or 24 h at 37 °C in 5% CO₂. We set up 3 biological replicates; cells treated with medium only were used as negative control (3 repeats). Each experiment was performed thrice.

Gene expression

In this study, we evaluated the expression of the following genes in untreated cells: TNF-α, iNOS, STAT-5a, IFN-γ, IL1-β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17, IL-18, IL-23, IL-27, MYD88, NF-kB/p65, TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR10, MD2, CD14, CD44, CXCR4, RAD51, p53, HPRT1, PTEN, ErbB2, B2M, GAPDH, ACTB. To this purpose, we used primer sets described in previous studies (Table 1), as well as others (Table 1) designed in our laboratory using the BLAST function of PUBMED (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The expression of the above genes was assessed by RT-qPCR. Total RNA was extracted from MDCK cells using RNeasy Mini Kit (Qiagen, Milano, cat No 74106) using DNase digestion protocol, RNA concentration was evaluated by UV absorbance (Biophotometer, Eppendorf, Milan) and 30 ng of RNA (10 ng/μl) were added to the reaction mix for cDNA synthesis as previously described (Razzuoli et al. 2011). EVA Green RT-qPCR amplification were performed a CFX96™ Real-Time System (Bio-Rad, Milano) as previously described (Razzuoli et al. 2014). To evaluate the basal level of expression, PCR-negative samples were given a Ct 39 fictitious value, whereas the positive ones showed Ct values < 38. After LPS or Cd²⁺ treatment we evaluated the expression of the following genes: IL-8, IL-6, IL-1β, TLR1, TLR3, TLR5, TLR9, iNOS, CD14, MyD88, NF-kB/p65, TLR4, MD2, IL-18. To normalize the results, we tested B2M, B-ACT, HPRT1 and GAPDH as possible housekeeping genes by NormFinder algorithm (Peletto et al. 2011). In each sample, the relative expression of the selected genes was calculated using the formula $2^{-\Delta\Delta Ct}$, where Ct is short for cycle of threshold and $\Delta Ct = Ct$ (target gene) – Ct (housekeeping). ΔCt values are the mean of three test

Table 1 Primer sets for Evagreen quantitative, RT Real-Time PCR amplification of canine genes

Gene	Primer		Source
B2M	Forward	TCCTCATCCTCCTCGCT	Selvarajah et al. (2017)
	Reverse	CCTGCTCATTGGGAGTGAA	
ACTB	Forward	ACGGAGCGTGGCTACAGC	Sauter et al. (2005)
	Reverse	TCCTGATGTCACGCACGA	
GADPH	Forward	TTCCACGGCACAGTCAAG	Menezes-Souza et al. (2011)
	Reverse	ACTCAGCACCAGCATCAC	
IL1- β	Forward	TGCAAAACAGATGCGGATAA	Jalilian et al. (2012)
	Reverse	GTAACCTGCAGTCCACCGATT	
IL-2	Forward	CCTCAACTCCTGCCACAATGT	Kim et al. (2013)
	Reverse	TGCGACAAGTACAAGCGTCAGT	
IL-4	Forward	TGCAGAGCTGCTACTGTACTGCGGC	Peeters et al. (2005)
	Reverse	CATGCTGCTGAGTTCTCTGT	
IL-5	Forward	GCCTATGTTTCTGCCTTTGC	Menezes-Souza et al. (2011)
	Reverse	GGTCCCATCGCCTATCA	
IL-6	Forward	TCCAGAACAACTATGAGGGTGA	Cavalcanti et al. (2015)
	Reverse	TCCTGATTCTTTACCTTGCTCTT	
IL-8	Forward	TGATTGACAGTGGCCACATTGTG	This paper
	Reverse	GTCCAGGCACACCTCATTTTC	
IL-10	Forward	CGACCCAGACATCAAGAACC	Peeters et al. (2005)
	Reverse	CACAGGGAAGAAATCGGTGA	
IL-12	Forward	TGGAGGTCAGCTGGGAATACC	Sauter et al. (2005)
	Reverse	TGCAAAATGTCAGGGAGAAGTA	
IL-15	Forward	ACTTGCATCCAGTGTCTACTT	Choi et al. (2006)
	Reverse	CGAGCGAGATAACACCTAAC	
IL-16	Forward	CCAGTCCAAGGGGATTACAG	Vanherberghen et al. (2012)
	Reverse	TGAGAATGAGCGGTTGTG	
IL-17	Forward	ACTCCAGAAGGCCCTCAGATTA	Nascimento et al. (2015)
	Reverse	GATTCCAAGGTGAGGTAGATCG	
IL-18	Forward	CTCTCCTGTAAGAACAAAATTTTCCTT	Kurata et al. (2004)
	Reverse	GAACACTTCTCTGAAAGAATATGATGTCA	
IL-23	Forward	ACAGAACGGACAGCATCAGG	This paper
	Reverse	CGCTGCCTGCTTCTCAAATC	
IL-27	Forward	TTACTGCTCTCCCTGCTCCT	This paper
	Reverse	TTGAACTCCCTCCGCAACTC	
TNF- α	Forward	CGTCCATTCTTGCCCAAAC	Menezes-Souza et al. (2011)
	Reverse	AGCCCTGAGCCCTTAATTC	
IFN- γ	Forward	CCAGATCATTCAAAGGAGCA	Cavalcanti et al. (2015)
	Reverse	CGTTCACAGGAATTTGAATCAG	
TLR1	Forward	GGTCCATAAGCGCAGGGAAT	This paper
	Reverse	TGCAGGATAACGGAGACACG	
TLR2	Forward	AACGCTTTCTCGAAGGAGCA	This paper
	Reverse	AGTGGCACAGGTAGTCTCTT	
TLR3	Forward	TGTCACTTGCTCAGTCTCCTTT	Hosein et al. (2015)
	Reverse	GTCCAATTTCAATTAAGGCCAAG	

Table 1 continued

Gene	Primer		Source
TLR4	Forward	GCTGGATGGTAAACCGTGGA	This paper
	Reverse	AGCACAGTGGCAGGTACATC	
TLR5	Forward	CCAGGACCAGACGTTTCAGAT	Turchetti et al. (2015)
	Reverse	GCCCAGGAAGATGGTGTCTA	
TLR7	Forward	GGAAGACCCAAGGGAGAAAC	Turchetti et al. (2015)
	Reverse	GCTGTATGCTCTGGGAAAGG	
TLR8	Forward	TCAGTACAATGCACACTACTTCC	Grano et al. (2018)
	Reverse	ACGCTTCTCAGGTCTTGCTC	
TLR9	Forward	CTAGTGAAGTTCAGCCGGAG	This paper
	Reverse	CACATCAGGCTCGGGGTA	
TLR10	Forward	GGGGACTCTGCTAAAGGACG	This paper
	Reverse	GCATCCTGAGATACCAGGGC	
p53	Forward	CGTTTGGGGTTCCTGCATTC	This paper
	Reverse	CACTACTGTCAGAGCAGCGT	
CD14	Forward	GCCGGGCCTCAAGTACT	Silva et al. (2010)
	Reverse	TCGTGCGCAGGAAAAAGC	
MD2	Forward	GGGAATACGATTTTCTAAGGGACAA	Silva et al. (2010)
	Reverse	CGGTAAAATTCAAACAAAAGAGCTT	
MYD88	Forward	GAGGAGATGGGCTTCGAGTA	This paper
	Reverse	GTCCACCAACACGTCGTC	
NF- κ B/p65	Forward	TGTAAAGAAGCGGGACCTGG	Ishikawa et al. (2015)
	Reverse	AGAGTTTCGGTTCCTACTCGGC	
STAT5	Forward	TTGACTCTCCTGACCGCAAC	This paper
	Reverse	TCCGTCTACTGCTTTAGCGA	
iNOS	Forward	AGACACACTTCACCACAAGG	Kaim et al. (2006)
	Reverse	TGCTTGGTGGCGAAGATGAGC	
CD44	Forward	CAAGGCTTTCAACAGCACCC	This paper
	Reverse	TACGTGTCGTAAGGGAGGT	
CXCR4	Forward	GCGTCTGGATACTGCTCTC	This paper
	Reverse	GATACCCGGCAGGATAAGGC	
RAD51	Forward	GGAGAAGGAAAGGCCATGTA	Klopfleisch and Gruber (2009)
	Reverse	GGGTCTGGTGGTCTGTGTT	
HPRT1	Forward	TGCTCGAGATGTGATGAAGG	Klopfleisch and Gruber (2009)
	Reverse	TCCCCTGTTGACTGGTCATT	
PTEN	Forward	GTGAAGCTGTAATTCACAA	Kanae et al. (2006)
	Reverse	CTGGGTCAGAGTCAGTGGTG	
ErbB2	Forward	CTGAGGGCCGATATACCTTC	Da Costa et al. (2011)
	Reverse	TCACCTCTTGGTTGTTTCAGG	

replicates \pm 1 standard deviation and $\Delta\Delta Ct = \Delta Ct$ (different passages, 34th and 40th) – ΔCt (control, 33rd passage).

IL-6 elisa

Canine IL-6 was measured in MDCK supernatants by an ELISA kit (R&D system, cat No CA6000)

Table 2 Gene expression in MDCK cells

Gene	Expression	$\Delta\text{Ct} \pm \text{SD}$
GAPDH	+	HK
HPRT1	+	3.6 \pm 0.2
ACTB	+	- 1.81 \pm 0.6
B2M	+	0.74 \pm 0.4
IL-1 β	\pm	19.4 \pm 1.5
IL-2	\pm	20.0 \pm 1.2
IL-4	-	NA
IL-5	+	14.6 \pm 0.5
IL-6	+	15.8 \pm 0.6
IL-8	+	8.5 \pm 0.7
IL-10	-	NA
IL-12	\pm	21.2 \pm 0.7
IL-15	-	NA
IL-16	+	14.2 \pm 0.5
IL-17	-	NA
IL-18	+	16.4 \pm 0.7
IL-23	+	10.8 \pm 0.7
IL-27	-	NA
TNF- α	\pm	20.6 \pm 0.7
IFN- γ	-	NA
TLR1	+	14.2 \pm 0.5
TLR2	\pm	19.4 \pm 1.5
TLR3	+	9.0 \pm 0.6
TLR4	\pm	18.4 \pm 0.6
TLR5	+	15.8 \pm 0.7
TLR7	\pm	20.8 \pm 2.1
TLR8	\pm	21.0 \pm 2.3
TLR9	\pm	20.8 \pm 1.3
TLR10	\pm	20.6 \pm 1.8
p53	+	12.6 \pm 1.1
CD14	+	10.5 \pm 1.1
MD2	+	7.0 \pm 0.6
Myd88	+	6.6 \pm 1.2
NF-kB/p65	+	6.3 \pm 0.7
STAT-5a	+	14.2 \pm 0.5
iNOS	+	9.8 \pm 0.8
CD44	+	4.3 \pm 0.7
CXCR4	+	13.2 \pm 1.3
RAD51	+	6.1 \pm 0.8
PTEN	+	18.4 \pm 1.3
ErbB2	+	10.0 \pm 1.4

Data are expressed as: + all samples were positive; - no positive samples were detected; \pm only some samples tested positive. GAPDH was shown to be most stable, and hence chosen to calculate the ΔCt values

NA not available, HK housekeeping gene

according to the manufacturer's directions. IL-6 was revealed by adding 50 μL /well of ortho-phenylenediamine (OPD) and 0.02% H_2O_2 as substrate. The reaction was blocked after 15 min of incubation by adding 50 μL /well of 2 N H_2SO_4 ; then, plates were read spectrophotometrically at 492 nm. Cytokine concentration was calculated from a standard curve, created using eight, twofold dilutions of canine recombinant IL-6. Data were analyzed by software Prism 5, (Graph Pad Software); the LOQ (limits of quantification) corresponded to 10 $\mu\text{g}/\text{mL}$. Data were expressed as average \pm 1 standard deviation.

Statistical analyses

Data sets were submitted to a Kolmogorov–Smirnov test to check normality of distributions; significant differences within normal distributions were checked by one-way ANOVA followed by a Dunnett's test for gene expression, cytokine release and $\text{Cd}2+$ uptake. A *Student's t* test was applied to investigate the effect of aging on gene expression. The significance threshold was set at $P < 0.05$ (Prism 5, GraphPad Software).

Results

Housekeeping genes

First, GAPDH, ACTB, HPRT1 and B2M were evaluated as possible housekeeping genes (Brinkhof et al. 2006). Results were evaluated by NormFinder, this test evaluate the stability of genes under study according to their expression (ρ). Lower values are assigned to the most stable genes. Our results showed a good stability value for all the housekeeping genes under study; in particular, B2M showed $P = 0.00175$, GAPDH $P = 0.00174$, B-ACT $P = 0.00476$ and HPRT1 $P = 0.00233$. Therefore, GAPDH was chosen to calculate the ΔCt values.

Basal gene expression

We demonstrated the expression of all the genes under study except for IL-4, IL-10, IL-15, IL-17, IL-27 and IFN- γ (Table 2). Concerning TLRs, MDCK expressed TLR1, TLR3 and TLR5 in all samples, while for the other TLRs we obtained the following results: TLR2 was expressed in 96.6% of samples; TLR4 in 93.3%;

TLR7 in 80%; TLR8 in 66.6%; TLR9 in 96.6%; finally, TLR10 was expressed in 86.6% of analysed samples. NF- κ B/p65, MyD88, CD14 and MD2 were expressed in all samples (Table 2). As for cytokines, we showed expression of IL5, IL6, IL8, IL16, IL18 and IL23 in all samples (Table 2). IL1- β and IL-2 were expressed in 93.3% of samples; IL-12 tests indicated expression in 86.6% of samples; TNF- α was unexpressed in 23.3%. Concerning the other genes under study, we observed the expression of INOS, STAT-5a, p53, RAD51, CXCR4, ErbB2, PTEN and CD44 in all the test samples (Table 2).

Modulation of gene expression at different cell passages and effect of cell aging

Our results showed modulation of gene expression at the 34th and 40th passages (Fig. 1). In particular, at passage 34 we observed an increase of TLR7 and a decrease of B2M gene expression. Regarding the 40th passage, IL-2, IL-5, IL-12, IL-18, TLR2, TLR7, TLR9, TLR10, p53, CXCR4, MD2 and HPRT1 gene expression was up-regulated. On the contrary, IL-6, TLR3 and TLR5 were down-regulated. After 24 h aged cells, at passage 40, showed decrease of NF- κ B/p65 and B-ACT gene expression. MyD88, IL-18 and CXCR4 were not modulated (data not shown) while

other genes under study were up-regulated (Fig. 2; $P < 0.05$).

Cadmium uptake and gene expression modulation

Our data showed a significant increase of intracellular Cd²⁺ after 24 h of exposure with respect to the levels at 1 h. In particular, we observed 0.08%, 0.11% and 1.1% Cd²⁺ absorption after 1, 3 and 24 h of exposure, respectively, using 20 μ M Cd²⁺ (4 μ g of Cd²⁺/10⁶ cells; Fig. 3). 20 μ M Cd²⁺ after 3 h of exposure caused up-regulation of IL-1B, IL-6, IL-8, INOS, TLR1, TLR3, TLR5 and TLR9 gene expression. On the contrary, CD14 was down-regulated (Fig. 3). After 24 h of exposure we observed up-regulation of IL-18, NF- κ B/p65 and MYD88; at the same time, INOS, IL-1B, MD2, TLR5 and TLR9 were down-regulated. Other genes under study were not significantly modulated. Regarding IL-6 release, we observed a significant increase of this cytokine in cell supernatants after 3 h (54 \pm 3.3 pg/ml) and 24 h (51 \pm 6.8 pg/ml) of Cd²⁺ exposure with respect to untreated cells (< 10 pg/ml).

Effects of LPS treatment

Treatment with 1 μ g/mL LPS for 3 h caused up-regulation of MyD88 gene expression; at the same

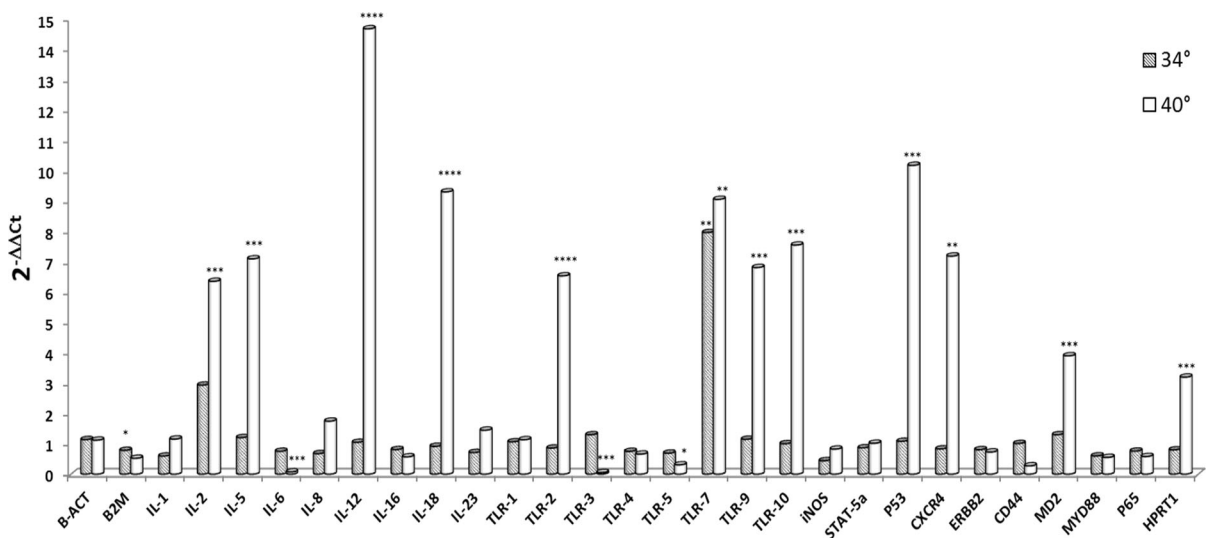


Fig. 1 Gene expression at different passage levels. Data are expressed as $2^{-\Delta\Delta C_t}$ where $\Delta C_t = C_t$ (target gene) – C_t (housekeeping); values are the mean of three test replicates \pm 1 standard deviation and $\Delta\Delta C_t = \Delta C_t$ (34th or 40th

passage) – ΔC_t (control, 33rd passage). Negative samples were given a C_t 38 fictitious value. Asterisks indicate significant differences between controls and different passages cells: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

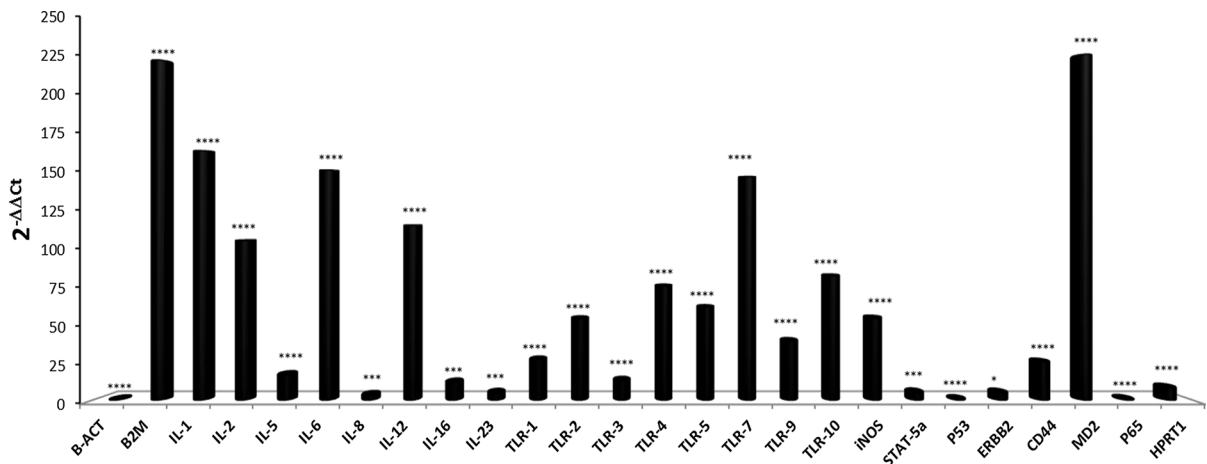


Fig. 2 Modulation of gene expression due to cell aging. Data are expressed as $2^{-\Delta\Delta Ct}$ where $\Delta Ct = Ct$ (target gene) – Ct (housekeeping); values are the mean of three test replicates ± 1 standard deviation and $\Delta\Delta Ct = \Delta Ct$ (40th passage at confluence plus 24 h of incubation at 37 °C) – ΔCt (control 40th passage

at confluence). Negative samples were given a Ct 38 fictitious value. Asterisks indicate significant differences between control and aging cells evaluated by Student's t-test. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$

time, INOS, IL-1B, CD14, TLR5 and TLR9 were down-regulated (Fig. 4). After 24 of treatment no significant modulation of gene expression was observed (Fig. 4). Concerning IL-6 release, all samples of the LPS study tested negative (IL-6 < 10 pg/ml; Data not shown).

Discussion

Madin-Darby Canine Kidney (MDCK) is a continuous cell line of distal tubules of canine kidney widely used for different purposes, such as isolation of influenza viruses, production of flu vaccines, pathogenicity studies of bacterial strains, cytotoxicity tests. Although several studies showed satisfactory results in MDCK cells for these purposes, none of them described their characteristics in terms of both gene expression and involvement in the innate immune response. Therefore, our study focused on the basal levels of protein release and gene expression in this cell line under physiological conditions and after exposure to diverse stressors. Accordingly, we developed a RT-qPCR to evaluate the expression of a selected group of 41 genes coding for molecules of different typology and function. Our results demonstrated the expression of all the genes under study with the exception of IL-4, IL-10, IL-15, IL-17, IL-27 and

IFNG; this is in agreement with other studies that reported no expression of these genes in epithelial cells (Bianchi 2007; Groeger and Meyle 2019). We also investigated the basal expression level of TLRs, a family of Pattern Recognition Receptors (PRRs), expressed by many cell types, in order to recognize microorganisms (e.g. bacteria, viruses and fungi) and to induce the innate immune response (Velloso et al. 2015). The expression in MDCK of these molecules is in line with the routine use of this cell line: evaluation of the virulence of some bacterial strains, including bacterial penetration, adhesion and cytotoxicity (Chou et al. 2016; Lin et al. 2017). The expression in all samples of p65, one of the major members of the NF- κ B protein family, along with MyD88, IL-1 β , IL-6, IL-8 and IL-16, implies the ability of this cell line to mount an inflammatory response. This assumption is confirmed by our results. In particular, Cd2+ exposure caused an inflammatory response characterized by up-regulation of gene expression and IL-6 release; this was associated to Cd2+ uptake, in agreement with previous studies conducted on Enterocytes (Razzuoli et al. 2018). MDCK cells showed the ability to absorb low levels of Cd2+, causing an innate immune response, although this cell line is more resistant to Cd2+ exposure, compared with other cell lines like LLC-PK1 (Bonham et al. 2003). Also, a previous study showed that Cd2+ treatments at concentrations

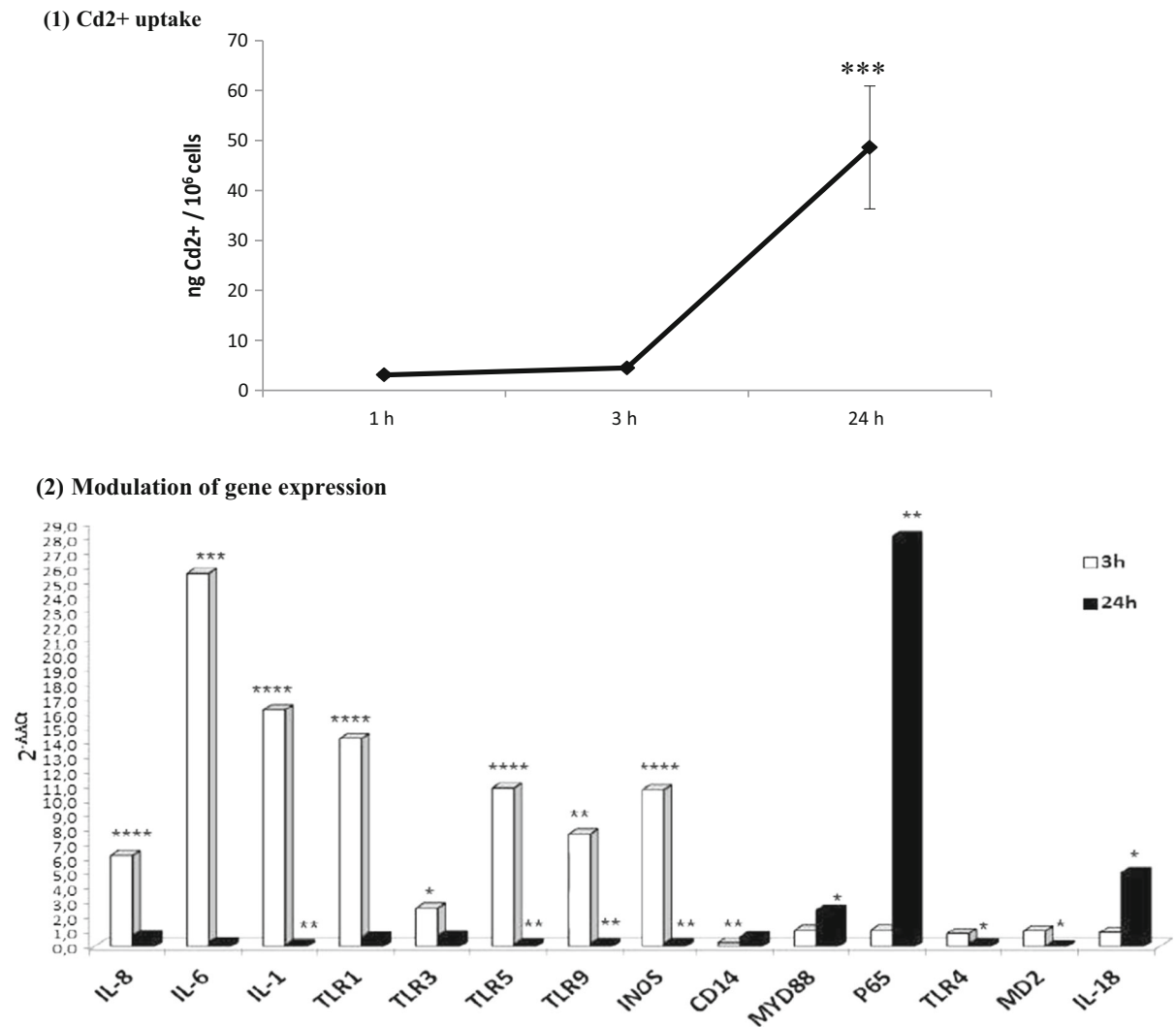


Fig. 3 Cd²⁺ treatment. Cd²⁺ uptake into MDCK cells was measured after 1, 3 and 24 h of exposure. Cells were treated with 20 μ M Cadmium (4 μ g/10⁶ cells); then, intracellular concentration of Cd²⁺ was measured at different times of exposure. Data are shown as mean \pm 1 SD. Asterisk indicate significant differences ($P < 0.0001$) with respect to 1 h of exposure, determined by one-way ANOVA. Gene expression was measured after treatment of MDCK cells with 20 μ M cadmium

for 3 and 24 h at 37 $^{\circ}$ C in 5% CO₂. Data are expressed as 2^{- $\Delta\Delta$ Ct} where Δ Ct = Ct (target gene) – Ct (housekeeping); values are the mean of three test replicates \pm 1 standard deviation and $\Delta\Delta$ Ct = Δ Ct (Cd treatment) – Δ Ct (untreated control). Negative samples were given a Ct 38 fictitious value. Asterisks indicate significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

equal to or greater than 10 μ M (LLC-PK1) or 25 μ M (MDCK) reduce cell vitality and induce cell death (Bonham et al. 2003).

The basal levels of IL-2 and IL-5, involved in the immune response, suggest the possibility to use this cell line in interaction studies between microbial agents and host (Ma et al. 2015; Krishnamoorthy et al. 2016). In our study, the exposure of MDCK to Cd²⁺ caused up-regulation of iNOS, TLR1, TLR3, TLR5

and TLR9 gene expression after 3 h of exposure. The genes that are expressed in response to the activation of TLRs encode important proteins at various levels of the innate immune response, including not only cytokines, but also proteins involved in the mechanisms of bacterial killing, such as the inducible nitric oxide synthetase iNOS (Tsutsuki et al. 2012). This response could ease the replication of viral agents in MDCK cells; in this respect, Checconi et al. (2013)

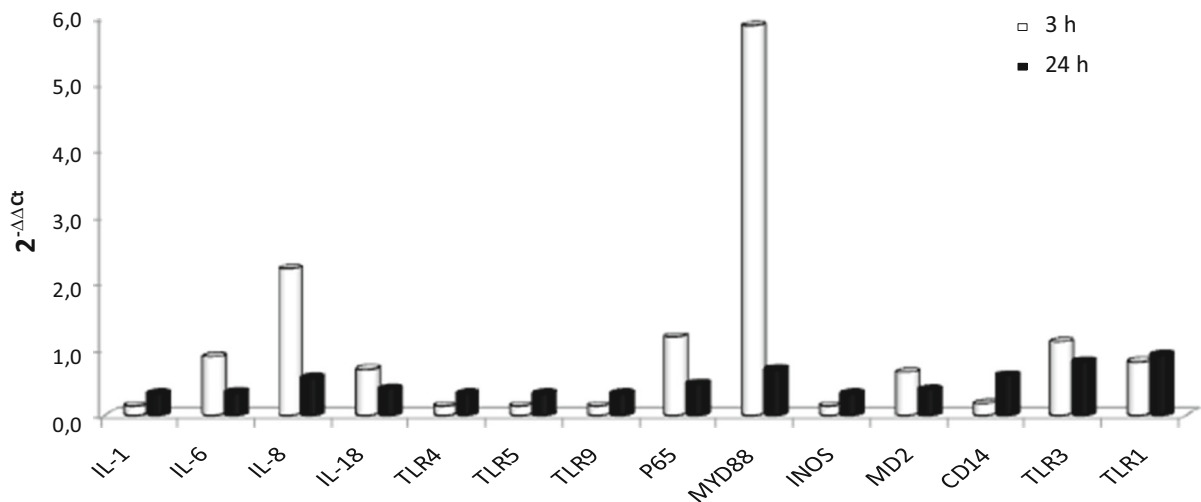


Fig. 4 Effect of LPS treatment. Gene expression was measured after treatment of MDCK cells with 1 $\mu\text{g}/\text{mL}$ LPS for 3 and 24 h at 37 $^{\circ}\text{C}$ in 5% CO_2 . Data are expressed as $2^{-\Delta\Delta\text{Ct}}$ where $\Delta\text{Ct} = \text{Ct}(\text{target gene}) - \text{Ct}(\text{housekeeping})$, values are the mean of three test replicates ± 1 standard deviation and

$\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{Cd treatment}) - \Delta\text{Ct}(\text{untreated control})$. Negative samples were given a Ct 38 fictitious value. Asterisks indicate significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

demonstrated that the exposure to low levels of cadmium (1–50 μM) increased influenza virus replication in a dose-dependent manner. Moreover, the exposure to LPS caused in this study a down-regulation of iNOS, CD14, TLR5 and TLR9, which confirms the ability of cells to react to both infectious and non-infectious stressors.

Treatment with LPS caused no significant effects in terms of TNF- α , IL-1 β , and IL-6 gene expression and protein release in agreement with our previous study (Razzuoli et al. 2013). However, these results differ from data obtained on myeloid cells like human macrophages and monocytes, where LPS causes early pro-inflammatory responses through TLR4/NF- κB signaling (Petes et al. 2018). The absence of IL-1 β and IL-6 responses to LPS in MDCK cells could be accounted for by a form of constitutive endotoxin tolerance (Lotz et al. 2006) in these epithelial cells.

Concerning the Cd $^{2+}$ treatment, we found modulation of gene expression and IL-6 protein release in MDCK cells as a function of time. In particular, we observed at different times of incubation with 20 μM Cd $^{2+}$ the modulation of some important pro-inflammatory cytokines and chemokines (IL-8, IL-6, IL-1 β , IL-18), and transcription factor genes (NF- κB /p65). Similar results were obtained in IPEC-J2 (Razzuoli et al. 2018) and Caco-2 cells, in which Hyun et al.

(2007) demonstrated the activation of an inflammatory response through the NF- κB pathway. These data outline the negative repercussions of lab contamination by LPS or Cd $^{2+}$ on experiments carried out on these cells.

In this study, we also demonstrated the effect of cell passages on MDCK gene expression; this is a very important issue that must be considered not only during gene expression studies but above all in host/pathogen interaction studies because both passage level and aging can alter TLRs and cytokine gene expression.

Conclusion

In conclusion, our study showed the constitutive expression of genes involved in the innate immune response and cell defense in MDCK cells. Albeit our findings refer to two major stressors only, we confirmed the capacity of this cell line to respond to infectious and non-infectious stressors in terms of gene expression and cytokine release. Finally, this data suggests the possibility of using this cell line as a model in studies of host/pathogen interaction and response to environmental pollutants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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