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Discrimination and evaluation of lactoferrin and delta-lactoferrin gene expression levels in cancer cells and under inflammatory stimuli using *TaqMan* real-time PCR

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Abstract The lactoferrin gene is known to be expressed either constitutively or under inducible conditions such as hormonal stimuli or inflammation. Its transcription from alternative promoters leads to two products, lactoferrin (Lf) and delta-lactoferrin (ΔLf) mRNAs the expressions of which are altered during oncogenesis. The comparison of the two enhancer/promoter regions revealed that the two isoforms might be differentially trans-activated. Nevertheless, concomitant expression of both transcripts has been found in some normal tissues and in a subset of breast cancer cell lines and biopsies. Moreover, we found putative inflammatory response elements in both P1 and P2 promoter regions suggesting that both Lf and ΔLf might be upregulated under inflammatory stimuli. Therefore, a duplex Taqman gene expression assay has been developed and used to profile mRNA expression of the Lf gene in the case of cancer and under inflammatory conditions. Discrimination between the two transcripts is achieved by using a primer pairs/probe set within exon 1β for ΔLf and a primer pairs/probe set within exon 1 and exon 2 for Lf. In this study, we confirmed that Lf/ΔLf Taqman gene expression assay is a powerful tool to investigate the expression of both Lf and ΔLf transcripts. We also showed that lymphocytes and leukocytes isolated from fresh human blood expressed an extremely high level of ΔLf messengers. An extensive series of cancer cell lines has been studied confirming that both P1 and P2 promoter regions of the Lf gene are downregulated or silenced in the case of cancer. Furthermore, using stimulation by bacterial lipopolysaccharides (LPS), we showed that in MDA-MB-231 and HT-29 epithelial cells, Lf expression is strongly increased with a higher expression level in MDA-MB-231 whereas ΔLf expression is not. These results suggest that the NF-κB/cRel response elements present in the P1 promoter region are functional whereas those present in the P2 promoter region are not and show that ΔLf is not regulated in inflammatory conditions.

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Abbreviations

Lf	Lactoferrin
ΔLf	Delta-lactoferrin

LPS	Lipopolysaccharides
FCS	Fetal calf serum
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
NF-κB	Nuclear factor κB
STAT3	Signal transducer and activator of transcription 3

Introduction

The Lf gene appeared in mammals and is highly conserved among species with an identical organization (Teng 2002). It is mapped to human chromosome 3 at 3p21.3 (McCombs et al. 1988). Its transcription leads mainly to two products, lactoferrin (Lf) and delta-lactoferrin (ΔLf) mRNAs, which result from the use of alternative promoters, the P1 promoter for Lf and P2, located in the first intron of the Lf gene, for ΔLf (Siebert and Huang 1997; Liu et al. 2003). Therefore, alternative splicing concerns the first exon; exon 1 which characterizes the Lf mRNA is replaced by exon 1β in the ΔLf messenger (Siebert and Huang 1997). Selection of the P2 promoter and the use of the first available AUG codon in frame produce an alternative N-terminus. Thus, compared to Lf, its secretory counterpart, ΔLf is a cytoplasmic protein able to enter the nucleus (Mariller et al. 2007). The secretory Lf isoform is a powerful antimicrobial agent with immunodulatory activity which is also able to protect organisms against cancer and metastasis (Ward et al. 2005; Legrand et al. 2008; Pierce et al. 2009) whereas ΔLf, its intracellular counterpart, is a transcription factor that controls cell cycle progression and survival (Mariller et al. 2007; Pierce et al. 2009).

The two promoter regions of the Lf gene contain numerous constitutive and inducible regulatory elements that modulate both Lf and ΔLf transcriptions (Siebert and Huang 1997; Teng 2002, 2006; Liu et al. 2003). The P1 promoter is highly sensitive to nuclear receptors such as the estrogen receptor in the reproductive organs and to retinoic acid receptors. Concerning the P2 promoter, potential upstream regulatory elements different from those of P1, elevated expression in lymphoid cells and upregulation by Ets have been described (Siebert and Huang 1997; Liu et al. 2003). However, in many normal tissues Lf and ΔLf mRNAs coexist but are expressed at differential levels (Siebert and Huang 1997). In breast cancer cell lines and biopsies, both transcripts were always observed (Benaïssa et al. 2005). Taken together these data suggest that some regulatory elements might direct both transcriptions.

Lf expression is also increased during early steps of embryogenesis, following oxidative damage and in response to infection (Ward et al. 2005) and we previously demonstrated that Lf expression by human microglia was up-regulated in inflammatory conditions and under oxidative stress (Fillebeen et al. 2001). Moreover, the Lf gene is regulated by innate immune stimuli and cRel/NF-κB binding sites have been identified in the promoter of the bovine Lf gene and proved to be highly responsive to LPS induction (Zheng et al. 2005). Recently, it has been shown that Lf expression is induced in the bovine mammary gland in response to *Staphylococcus aureus* and *Escherichia coli* infection (Griesbeck-Zilch et al. 2008) and in normal murine mammary gland epithelial cells by LPS, a major component of the outer membrane of gram negative bacteria, or dsRNA which mimics viral infection (Li et al. 2009). Until now, no data are available concerning the putative presence of inflammatory response elements and their eventual functionality in the human Lf and ΔLf promoter regions.

Downregulation of the expression of Lf and ΔLf has been found in numerous tumors (Siebert and Huang 1997; Liu et al. 2003; Benaïssa et al. 2005; Ward et al. 2005). This is mainly due to structural alterations such as mutations, allelic loss of part of chromosome 3 and modification of the degree, as well as the pattern of methylation (Teng et al. 2004; Iijima et al. 2006). This genetic and epigenetic inactivation of the Lf gene in cancer may therefore provide the tumor cells with a selective growth advantage. Interestingly, we previously showed, using RT–PCR, that the expression level of either Lf or ΔLf mRNAs was of good prognosis value in human breast cancer with high concentrations being associated with longer relapse-free and overall survival, suggesting the usefulness of the detection of either Lf or ΔLf transcripts as markers for the follow-up of breast cancer patients (Benaïssa et al. 2005).

Since Lf isoforms may coexist in the same cells, be expressed under the same regulatory elements, downregulated in case of cancer and used as

prognosis tools, we have developed a very specific and highly sensitive method to discriminate and evaluate both Lf isoform transcripts in the same sample using duplex TaqMan real-time PCR.

Materials and methods

Cell culture and reagents

Human cervical carcinoma HeLa cells (ATCC CCL-2), human prostate cancer cell line DU 145 (ATCC HTB-81) and human hepatocarcinoma cell line Hep G2 (ATCC HB-8065), were a kind gift from Pr. T. Lefebvre (UGSF, UMR 8576 CNRS, Villeneuve d'Ascq, France). Human breast cancer MDA-MB-231 (ATCC HTB-26) and MCF7 (ATCC HTB-22) cell lines were kindly provided by Pr X. Lebourhis (INSERM U908, Villeneuve d'Ascq, France). Human melanoma cells SK-MEL-28 (ATCC HTB-72), human breast cancer BT-20 (ATCC HTB-19) and T-47D (ATCC HTB-133) cell lines were a generous gift of Pr. P. Delannoy (UGSF, UMR 8576 CNRS, Villeneuve d'Ascq, France). Human gastric cancer cells KATO III (ATCC HTB-103) were provided by Dr. A. Hardouin (UGSF, UMR 8576 CNRS, Villeneuve d'Ascq, France). Human colon carcinoma HT-29 cells (ATCC HTB-38), human promonocytic leukemia THP-1 cells (88081201, ECACC), normal human lung epithelial cells NL20 (ATCC CRL-2503) and lung adenocarcinoma cells Calu-3 (ATCC HTB-55) were a kind gift from Drs. C. Masselot and S. Degroux (UGSF, UMR 8576 CNRS, Villeneuve d'Ascq, France). Human leukemia acute T Jurkat cell line (ATCC TIB-152) and MCF 10A normal-like human breast cells (ATCC CRL-10318) were purchased from the American type culture collection (ATCC).

All these cell lines were routinely grown in Dulbecco's modified Eagle's medium or RPMI, containing 10% (v/v) FCS, 2 mM L-glutamine and 1% (w/v) penicillin/streptomycin. Cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. The DAMI megakaryocytic cells were kindly provided by Dr S. Greenberg (Greenberg et al. 1990) and cultured as described (Nillesse et al. 1994).

Cell culture materials were obtained from Dutscher, and culture media and additives from Cambrex Corporation and Invitrogen. Mouse monoclonal FITC-conjugated anti-human CD14 antibody and mouse IgG isotype were purchased from Miltenyi Biotec and BSA from Sigma.

LPS induction

Cells were plated the day before induction in 6-well plates at a density of 10^6 cells/well. Prior to induction, cells were gently washed by fresh medium supplemented or not with FCS. Cells cultured without FCS were grown 24 h with FCS-free medium prior LPS treatment in FCS-free medium. Cells were then exposed or not to LPS (*E. coli* 055B5, Sigma) at a concentration of 100 ng/well during 24 h (Li et al. 2009). All experiments were conducted in triplicate. After washing with PBS, cells were harvested, centrifuged and lysed in an appropriate buffer for RNA assays.

RNA extraction and cDNA preparation

Total RNA extract from human normal mammary gland (HNMG) was purchased from Clontech. Total RNA from all cancer cell lines was isolated from cells using a NucleoSpin RNA II kit, according to the instructions of the manufacturer (Macherey–Nagel). The purity and integrity of each extract were checked using the nanodrop ND-1000 spectrophotometer (Labtech International) and the Bioanalyzer 2100 (Agilent Technologies). Reverse transcription was performed from 2 µg of total RNA with an oligo-dT primer and M-MLV reverse transcriptase (Promega). First strand cDNA from isolated human blood cells (leukocytes, lymphocytes, macrophages) were a kind gift of Dr. A. Denys (UGSF, UMR 8576 CNRS, USTL, Villeneuve d'Ascq, France).

RT–PCR conditions

RT–PCR assays were performed in triplicate as already described (Mariller et al. 2007). Negative control reactions were performed using sterile water instead of cDNA template. Contaminations of genomic DNA were excluded by performing 35 cycles of amplification without retrotranscription. GAPDH was used as internal control. Primer pairs were synthesized by Eurogentec. RT–PCR conditions specific to each primer pair are reported in Table 1. PCR products were separated onto a 1.5% agarose gel stained with

Table 1 Oligonucleotides used for TaqMan Q-PCR and RT–PCR

Method	Target DNA	Oligonucleotides (5′ → 3′)	Tm (°C)	Cycle number	Amplicon size (pb)
Q-PCR	Lf primers	Forward: cagaccgcagacatgaaacttg	60	40	117
		Reverse: gatacggcgcaccactgaa			
	Lf probe	ccctcggactgtgtct			
Q-PCR	ΔLf primers	Forward: ctgaagtttgaatcctgcagtcaat	60	40	66
		Reverse: aagggttgcaatggcacttt			
	ΔLf probe	tggtggcttgtaccc			
Q-PCR	HPRT primers	Forward: gccctggcgtcgtgatt	60	40	101
		Reverse: ctcagcataatgattaggtatgcaaaa			
	HPRT probe	tgatgatgaaccaggttat			
RT–PCR	GAPDH	Mariller et al. (2007)	69	16	240
RT–PCR	TLR4	Erridge et al. (2007)	57	25	336
RT–PCR	TNF-α	Satoh et al. (2000)	60	33	266

ethidium bromide and image acquisition was performed by UV transillumination using a Gel Doc 1000 system (Bio-Rad).

TaqMan Real-time PCR

Duplex Taqman Q-PCR and subsequent analyses were performed using the MX4000 Multiplex Quantitative System equipped with v3.0 software (Stratagene). Using Primer Express software v3.0 (Applied Biosystems) primers and probes were designed and their specificity checked using the BLAST algorithm on major nucleotide databases. Lf isoform primer pairs/probe sets are shown in Fig. 1. The transcript of HPRT (hypoxanthine guanine phosphoribosyltransferase) was used as a control to normalize the expression of genes of interest. The HPRT primer pair was designed to hybridize within exon 1 and exon 2. The nucleotide sequence of primer pairs and probes is presented in Table 1. Primer pairs were synthesized by Eurogentec and the TaqMan probes were from Applied Biosystems. The ΔLf and Lf probes were 5′-FAM-labeled and the normalizing HPRT gene probe was 5′-VIC-labeled. The 3′ non-fluorescent quencher (NFQ) (Applied Biosystems) was used for each probe. PCR reactions were performed with 12.5 µl of the QuantiTect Multiplex PCR Kit Mix (Qiagen),

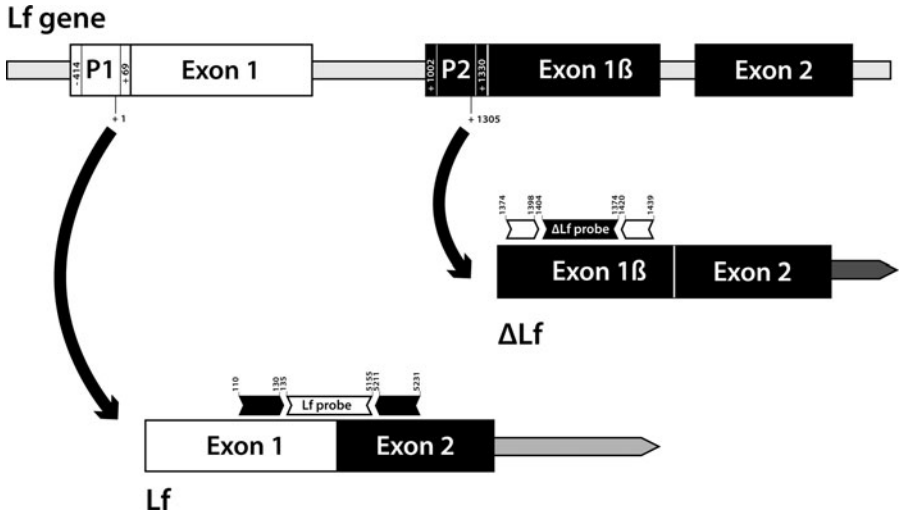

Fig. 1 Diagramatic representation of Lf and ΔLf primer pairs and TaqMan probe localization on the Lf gene. ΔLf primer pairs/probe set was designed to hybridize within exon 1β whereas for Lf, the primer pairs/probe set was designed to amplify a short region around the exon 1/exon 2 junction. The sense primer hybridizes within exon 1, the forward primer within exon 2 and the probe within the exon 1/exon 2 junction

0.6 μM of each primer, 0.2 μM of Taqman probe and 2 μl of cDNA in a final volume of 25 μl. After 95°C for 15 min, 40 PCR cycles were performed as follows: 94°C for 1 min, 60°C for 1 min (hybridization and elongation). Each TaqMan Q-PCR assay was performed in triplicate in 96-well plates. Two to three separate RNA extractions were carried out for each sample and each cDNA was analysed at least three times by Q-PCR. The amplification efficiency was generated for each primer pairs/probe set separately and in duplex with the primer pairs/probe set of the normalizing gene HPRT using 2-fold serial dilutions of cDNA. The fluorescence data were measured at the end of each cycle. The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample, and the average Ct of triplicate samples was used for further calculations. In order to check amplification efficiency (E) of all three primer pairs/probe (Lf, ΔLf and HPRT), a validation experiment was performed using serial dilutions of each sample. Standard curves are obtained and the slopes of curves (*slope*) calculated. Efficiency is then established using the formula $E = 10^{[-1/slope]}$. Relative quantities of Lf and ΔLf mRNA were calculated as described (Pfaffl 2001) and expressed normalized to HPRT.

Flow cytometry analysis

THP-1, MDA-MB-231 and HT-29 cells were washed twice by centrifugation for 5 min at 1,100 rpm in cold Dulbecco's phosphate buffer saline (DPBS) (Sigma) and resuspended in DPBS supplemented with 0.5% bovine serum albumin (BSA) to obtain a final concentration of 10^6 cells/ml. Each pellet was resuspended in DPBS-BSA added with mouse monoclonal FITC-labelled anti-human CD14 (1/1,000) or mouse IgG isotype control (1/1,000) for 30 min at 4°C in the dark. Data were monitored on a Becton–Dickinson FACScan flow cytometer. The light-scatter channels were set on a linear gain, and the fluorescence channels were set on a logarithmic scale. Cells were gated for forward- and site-angle light scatters, and 10,000 fluorescent particles of the gated population were analyzed. The data collected with logarithmic amplification were analyzed by the CellQuest Pro v6.0 software.

Bioinformatics tools and promoter modelling

The P1 promoter of the Lf gene (Genbank NT_022517.18, *Homo sapiens* chromosome 3 genomic contig, 46450953-46446654) and the P2 promoter (Genbank NT_022517.18, *Homo sapiens* chromosome 3 genomic contig, 46446314-46445095) were analysed by checking matrix family assignment. All software employed were part of the Genomatix Suite/GEMS Launcher software package (http://www.genomatix.gsf.de). Potential control elements and transcription factor binding sites were analysed using MatInspector Professional software (Quandt et al. 1995). In order to ensure selection of high-quality binding sites throughout the library of binding-site matrices, MatInspector applies individually optimized matrix thresholds. The inflammatory response modules were defined as follows: NF-κB/cRel element (family V$NF-κB) with a core similarity of 1 and minimum matrix similarity of 0.87; STAT3 elements (family V$STAT) with a core similarity of 1 and minimal matrix similarity of 0.9.

Results and discussion

Design of Taqman probe and primer pairs and validation experiments

Since both human Lf and ΔLf transcripts have been often found in the same cell type at the same time, and at a low level in cancer cells, it was crucial to use a sensitive and highly specific method in order to discriminate them and assay their expression. We chose HPRT as an internal control gene since we previously checked that its expression was not altered in the cancer cell lines we used or during cell exposure to LPS. Therefore, we developed a duplex TaqMan real-time PCR assay which measures PCR-product accumulation during the exponential phase of the PCR reaction using labeled fluorogenic probes (Gibson et al. 1996). Minor groove binder (MGB) probes that incorporate a 5′ reporter dye (FAM or VIC) and a 3′ non-fluorescent quencher (NFQ) were used. The NFQ offers the advantage of a lower background signal which results in better precision in quantification (Kutyavin et al. 2000). FAM and VIC fluorescent reporter dyes were used to get a narrow spectrum and maximum of emission. Therefore, Lf

and ΔLf probes were FAM-labeled whereas HPRT was VIC-labelled at the 5′ end. Discrimination between the two transcripts was achieved as shown in Fig. 1.

Optimization of the assay was performed in order to obtain maximal efficiency of the primer/probe set. The maximum of efficiency was obtained using 600 nM primer pairs and 200 nM probe for each transcript. Efficiencies, determined for all the three primer pairs/probe sets, are 97.8% for ΔLf, 98% for Lf and 96.7% for HPRT. We confirmed that the efficiency was the same when duplex TaqMan Q-PCR assays (Lf/HPRT and ΔLf/HPRT) were carried out.

We next investigated whether the duplex TaqMan Q-PCR assay was sensitive enough to evaluate the respective expression of Lf and ΔLf transcripts in the case of downregulation as in cancerous cells and in the case of overexpression such as in inflammatory conditions.

Evaluation of Lf and ΔLf mRNA contents in cancerous and normal cells

Since both Lf and ΔLf are expressed in a wide variety of tissues, have antitumoral properties, are good prognosis markers and might act as tumor suppressors, detection of their expression levels should be extremely useful to follow tumor development. Therefore, we studied Lf and ΔLf expression levels in breast, lung, stomach, prostate, colon, skin, cervix, hepatocyte, megakaryocyte, monocyte and lymphocyte cancer cell lines, and seven normal human cells or tissues (Fig. 2) using the duplex TaqMan real-time reverse transcription PCR assay we developed.

We demonstrated that Lf mRNA was downregulated in three of the four breast cancer cell lines tested when compared with normal breast tissue (HNMG) or MCF 10A normal-like cell line. We found that only MDA-MB-231 cells exhibit a higher level of Lf mRNA compared to normal counterparts. Lf was at a very low level expressed by T-47D and BT-20 cells. Of the other cancer cell lines tested, only HT-29 and HeLa cells produced detectable Lf mRNA expression levels. Lf was expressed neither in normal (NL20) nor cancerous lung cells (Calu-3). For each sample the Lf relative expression normalized to HPRT is $\geq$0.5 confirming strong gene silencing. Lf mRNA was found in the lymphocyte population and not in the leukocyte population. The absence of Lf expression in leukocytes, which produce and store it in their secondary granules (Masson et al. 1969), is in accordance with its synthesis occurring only during granulocytic differentiation (Rado et al. 1987).

Concerning ΔLf, its mRNA was only absent from T-47D, THP-1 and Jurkat cells among the fourteen cancer cell lines tested. Its expression levels were very low with the highest expression found in DU 145 prostate cancer cell line. For each sample the ΔLf relative expression normalized to HPRT is $\geq$1.5 also confirming strong silencing of the P2 promoter. The sensitivity of our technique shows that ΔLf mRNA is detectable at very low levels in all tumour cell lines analyzed whereas it was found to be absent in all tumour cell lines examined using classical RT–PCR (Siebert and Huang 1997). Among the seven normal human tissues or isolated blood cells tested, both the leukocyte and lymphocyte populations expressed an extremely high level of ΔLf mRNA, with the hippocampus expressing a slightly lower level. The ΔLf relative expression normalized to HPRT is $\geq$60 for leukocytes and 25 for lymphocytes. The strong activity of the P2 promoter in these two cell types might be due to the presence of numerous functional Ets binding sites (Liu et al. 2003). Indeed, the proto-oncogene c-Ets, which is preferentially expressed in lymphoid cells and thymus (Chen 1985) was shown to strongly activate the P2 promoter (Liu et al. 2003). Therefore, the presence of Ets family member, which are involved in various cellular events such as cell growth, transformation, T-cell activation, hematopoietic cell differentiation, and development (Wasylyk et al. 1993), might be critical for ΔLf expression.

Presence of cRel/NF-κB/STAT3 promoter modules in the regulatory regions of the P1 and P2 promoters of the Lf gene

Bovine and murine Lf expressions are significantly up-regulated by LPS in a dose-dependent manner (Griesbeck-Zilch et al. 2008; Li et al. 2009). Recently, LPS-responsive modules were localized in the promoter region of the bovine Lf gene such as cRel/NF-κB, STAT3 and AP1 (Zheng et al. 2005). LPS through the Toll-like receptor (TLR) mediated-signaling pathway (Muzio and Mantovani 2000) triggers pro-inflammatory stimuli such as TNF-α and IL-1β (Karin 1999; Mercurio and Manning 1999) and activation of cRel/NF-κB transcription factors.

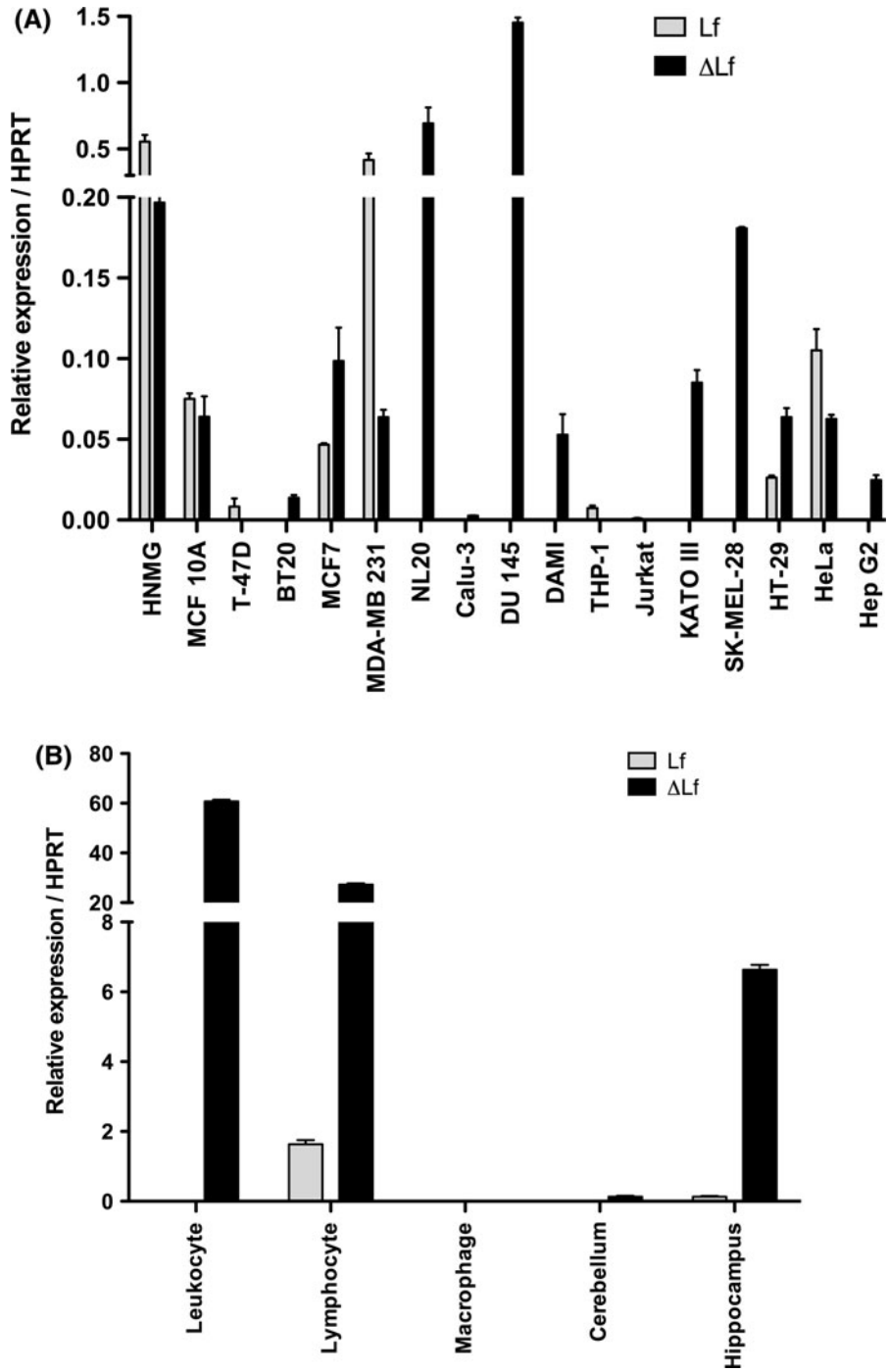

Fig. 2 Lf and ΔLf mRNA expression levels of various human cancer cell lines (**a**) and normal cells and tissues (**b**) using duplex Taqman Q-PCR. *Black* bars, ΔLf transcript; *grey* bars, Lf transcript. Values are normalized to HPRT gene expression. Data are means ± SD of n ≥ 6

The cRel/NF-κB transcription factor family regulate several physiological processes such as cellular homeostasis (Barkett and Gilmore 1999; Baldwin 2001) and the host immune response (Hayden and Ghosh 2008) by controlling the expression of an extremely high number of target genes (Pahl 1999). STAT3 is known as an acute phase response factor (Wegenka et al. 1994) and is also implicated in a variety of cellular functions, including inflammatory processes (Leonard and O'Shea 1998). AP1

regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections and in turn controls a number of cellular processes including differentiation, proliferation, and apoptosis.

Therefore, we investigated the Lf gene for the presence of such inflammatory response elements and subsequently checked whether Lf and/or ΔLf were overexpressed following LPS stimulation. The data presented in Fig. 3 show that potential NF-κB/cRel/STAT3 promoter modules are present in the regulatory regions of the P1 and P2 promoter regions of the Lf gene. Three NF-κB sites, five cRel sites and four STAT3 sites were found in the P1 promoter (Fig. 3a). The P2 promoter region also contains potential LPS-responsive elements, including two NF-κB sites, two cRel sites and two STAT3 sites (Fig. 3b). Our findings suggest that the human Lf promoters may respond to infection *via* the NF-κB pathway but further investigations will be required to ensure the biological significance of these putative responsive elements.

No AP1 site was found in either the P1 and P2 promoter regions although it was present in the bovine Lf promoter region (Zheng et al. 2005). BLAST alignment of the human and the bovine Lf promoter regions showed that neither the presence nor the localization of these elements is conserved (data not shown).

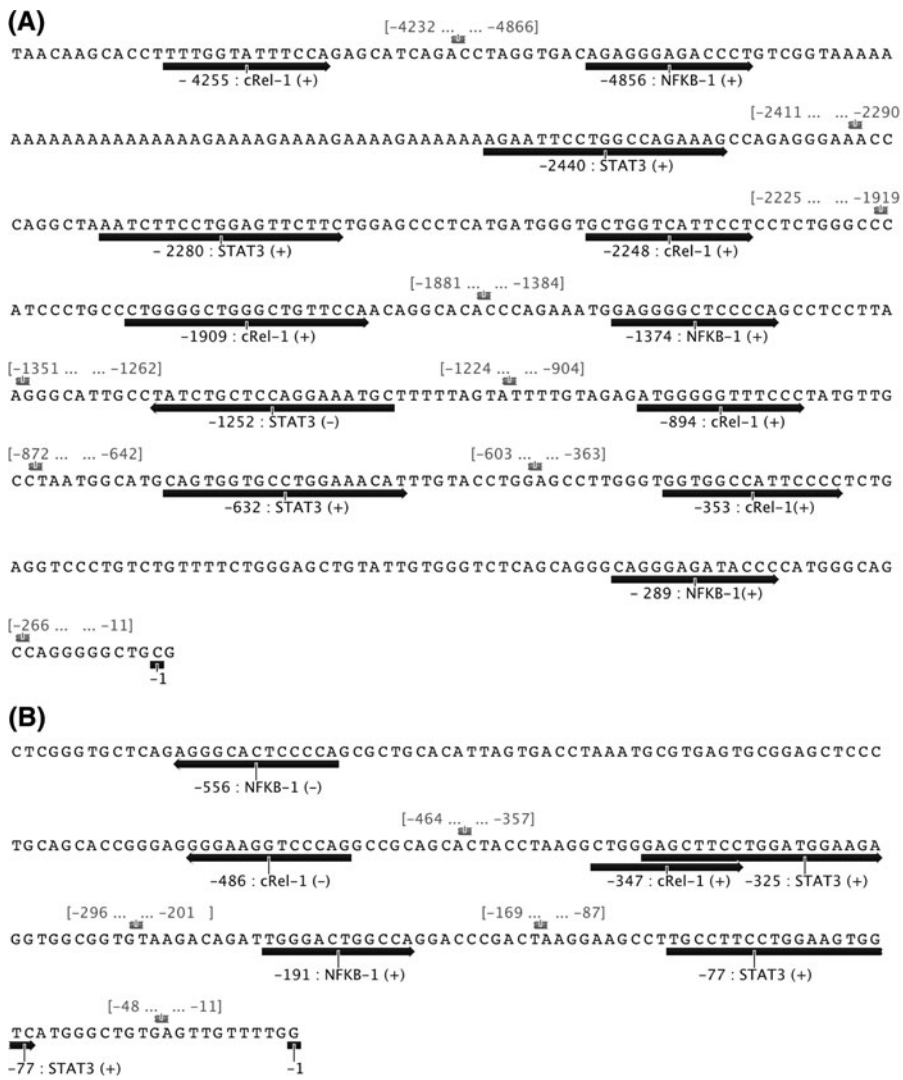

Fig. 3 Putative inflammatory response elements in the human Lf gene. The human P1 (**a**) and P2 (**b**) promoter regions were analyzed using MatInspector (Genomatix). Binding sites of infection responsive transcription factors (NF-κB, cRel1 and STAT3) are *underlined*. (+) plus strand, (−) minus strand. For easier reading, omitted sequences are presented in *grey* between brackets above sequence

LPS induces Lf but not ΔLf expression in human mammary gland epithelial MDA-MB-231 cells and in human colonic carcinoma HT-29 cells

Since inflammatory response modules are present in both the P1 and the P2 promoter regions we next investigated whether or not both human Lf isoforms are upregulated in inflammatory conditions. LPS is recognized by Toll-like receptor 4 (TLR4) (Akira and Hoshino 2003; Beutler 2005) that interacts with different proteins such as LPS binding protein (LBP), soluble CD14 (sCD14) or membrane CD14 (mCD14) to induce a signaling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines such as TNF-α and IL-8 leading to a strong inflammatory response (Arditi et al. 1993; Hailman et al. 1994; Underhill and Ozinsky 2002; Pålsson-McDermott and O'Neill 2004). Therefore, human mammary gland epithelial MDA-MB-231 cells (Zaks-Zilberman et al. 2001) and human colonic epithelial HT-29 cells (Cario et al. 2000; Böcker et al. 2003; Lee et al. 2005) which are known to respond to LPS stimuli, were studied. The optimal LPS concentration (100 ng/10^6 cells) was chosen from previous experiments (data not shown) and in accordance to the literature (Li et al. 2009).

We first determined whether both cell lines expressed the CD14 epitope using flow cytometry. THP-1 were used as a known source of mCD14 (Fig. 4a, black shaded region). Unshaded and grey shaded regions overlapped the isotype control showing that MDA-MB-231 and HT-29 cell surfaces lack mCD14. Therefore, the next experiments were conducted with and without fetal calf serum (FCS) since normal plasma is a source of sCD14.

We next confirmed LPS-cell responsiveness by following TNF-α induction using RT–PCR. As shown in Fig. 4b, TNF-α is overexpressed following LPS induction compared to GAPDH. In both cell types the overexpression of TNF-α is higher in the presence of FCS confirming the importance of providing plasma sCD14 which acts as an opsonin that captures pathogenic microbes, facilitating recognition and binding of LPS to mCD14-negative intestinal and mammary gland epithelia (Pugin et al. 1993).

We then investigated whether TLR4 was overexpressed in the presence of LPS on these two cell lines.

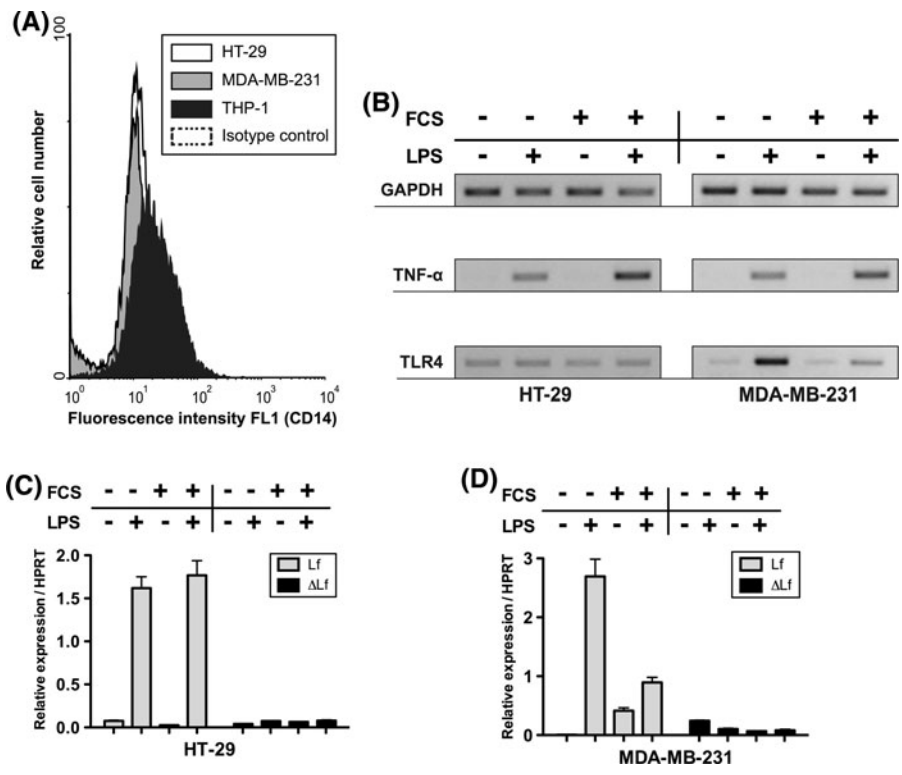

Fig. 4 Lf and not ΔLf is overexpressed under LPS stimulation. **a** Absence of mCD14 on HT-29 and MDA-MB-231 cells compared to differentiated THP-1 by flow cytometry. **b** Expression levels of TNF-α and TLR4 mRNAs compared to the expression level of the housekeeping gene GAPDH using RT–PCR. **c–d** Lf and ΔLf mRNA expression levels expressed as relative intensity compared to HPRT gene expression using duplex TaqMan Q-PCR in HT-29 (**c**) and in MDA-MB-231 (**d**) cells

Responsiveness of HT-29 cells to LPS is known to be correlated with the presence of TLR4 (Böcker et al. 2003). Here, we showed that TLR4 expression is not modulated in the presence of LPS and/or FCS suggesting that it might be constitutively expressed (Fig. 4b). Breast cancer cell responsiveness to LPS has been described previously. TLR4 has been described as being present using a DNA array assay (Merrell et al. 2006) or absent using RT–PCR (Xie et al. 2009) in MDA-MD-231 cells. Here, we showed that TLR4 is effectively expressed but at a very low level and overexpressed following LPS exposure in MDA-MB-231 cells. Expression was higher in the absence of FCS while the inflammatory response was not increased in the same conditions (Fig. 4b).

We further determined whether the human Lf gene was upregulated in inflammatory conditions using our duplex TaqMan Q-PCR assay. Figure 4c shows that Lf expression is poorly expressed without LPS stimulation but is increased 20-fold when HT-29 cells are exposed to LPS in the absence of FCS and 70-fold in the presence of FCS and LPS. Our results confirm that the human Lf gene, like its mouse and bovine counterparts, is upregulated following LPS induction and that the presence of FCS containing sCD14 favors this upregulation. ΔLf, which is also feebly expressed in HT-29 cells, was not upregulated in inflammatory conditions.

Figures 2a and 4d showed that Lf expression is poorly expressed in the absence of stimulation in MDA-MB-231 cells. Overexpression is very marked when cells are exposed to LPS in the absence of FCS (450-fold) whereas this overexpression is dramatically reduced (2-fold) in the presence of FCS. This result is mainly due to the 70-fold higher expression of the Lf gene in the MDA-MB-231 cells cultured in FCS. Lf upregulation may be correlated to TLR4 inducible expression higher in serum free media and LPS exposure. In all cases, Lf is upregulated in presence of LPS whereas under the same experimental conditions, no upregulation of ΔLf expression is detectable.

Our data show that the human MDA-MB-231 mammary gland epithelial cell line and the human colonic HT-29 cancer cell line might be used to study responses to innate immune stimuli. We also confirmed that the inflammation-responsive elements present in the P1 promoter of the Lf gene are functional. Moreover we showed for the first time that even if cRel/NF-κB/STAT3 response modules are present in the P2 promoter, they are not functional and that ΔLf expression is not regulated in response to bacterial infection. We also demonstrated that our duplex TaqMan Q-PCR assay is able to evaluate extremely variable quantities of Lf isoform transcripts.

Our results are in accordance with the function of both Lf isoforms. Lf, which is a multitasking protein, is mainly a powerful antimicrobial agent with immunodulatory activities involved in host defense (Legrand et al. 2008; Pierce et al. 2009). Its production under LPS stimulation will reduce LPS and its proinflammatory effects (Li et al. 2009). In contrast ΔLf is a transcription factor involved in the control of cell cycle progression, mRNA decay and apoptosis (Mariller et al. 2007, 2009) the expression of which is not influenced in response to inflammatory conditions.

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