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LACTOFERRIN DECREASES INFLAMMATORY RESPONSE BY CYSTIC FIBROSIS BRONCHIAL CELLS INVADED WITH *BURKHOLDERIA CENOCEPACIA* IRON-MODULATED BIOFILM

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In cystic fibrosis (CF) high iron concentration in airway secretion plays a pivotal role in bacterial multiplication and biofilm formation as well as in inflammatory response. *Burkholderia cenocepacia*, an opportunistic facultative pathogen responsible for chronic lung infections and cepacia syndrome, recurrently infects CF patients. Lactoferrin (Lf), an iron binding multifunctional glycoprotein synthesized by exocrine glands and neutrophils, has been found at higher concentration in the airway secretions of infected CF patients than in healthy subjects. Here the influence of milk derivative bovine lactoferrin (bLf), an emerging important regulator of iron and inflammatory homeostasis, on invasiveness of *B. cenocepacia* iron-modulated biofilm, as well as on inflammatory response by infected CF bronchial (IB3-1) cells, is reported. bLf did not significantly affect invasion efficacy by biofilm-forming *B. cenocepacia* clinical strains. Conversely, the addition of bLf to cell monolayers during infection significantly decreased the pro-inflammatory Interleukin (IL)-1β and increased the anti-inflammatory IL-11 expression compared to that observed in cells infected in the absence of bLf. The bLf ability to modulate genes expressed following *B. cenocepacia* infection seems related to its localization to the nucleus of infected IB3-1 cells. These results provide evidence for a role of bLf in the protection of infected CF cells from inflammation-related damage, thus extending the therapeutic potential of this multifunctional natural protein.

Cystic fibrosis (CF) is an autosomal recessive disease characterized by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) channel (1) leading to abnormalities in ion transport across the epithelial cells. Particularly relevant is the malfunctioning of respiratory tract, and high iron concentrations are found in airway secretions (2-3).

A hallmark of CF is a chronic lung infection due to opportunistic bacteria inducing sustained neutrophil-mediated inflammation (4-5). Aggressive antibiotic and anti-inflammatory treatments may ameliorate CF patient symptoms in the short-term (6), but they do not consistently reduce the bacterial load, which may be related to persistently increased airway iron concentrations (3). As a matter of fact, high iron

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availability induces bacterial multiplication and biofilm formation as well as inflammatory processes (7-9).

Burkholderia cenocepacia is an opportunistic, facultative intracellular pathogen causing a chronic lung infection in CF patients. The cepacia syndrome, a rapid necrotizing pneumonia often resulting in death, is associated to ET12 lineage of *B. cenocepacia* characterized by high transmissibility and patient-to-patient diffusion (10-11). Noteworthy, infection with *Burkholderia* species is typically considered a contraindication to lung transplantation in CF (12).

Lactoferrin (Lf), a cationic glycoprotein able to chelate two Fe^{3+} ions per molecule with high affinity, is synthesized by exocrine glands and neutrophils in infection and inflammation sites (9). In the CF airway secretions, the high concentrations of pro-inflammatory cytokines, including Interleukin (IL)-8, recruit neutrophils which synthesise and secrete Lf (13). As a consequence, Lf is found at higher concentrations in airway secretion of CF patients than in healthy humans (up to 0.1 and 0.01 mg/ml, respectively) (13). Lf exerts multiple antimicrobial functions both dependent on and independent of its iron-withholding ability. Among the functions independent of iron-binding property, Lf inhibits the host cell invasion by some facultative intracellular bacteria through its binding to bacterial and/or cell surfaces (9, 14). Finally, although the mechanism of action is not fully elucidated, Lf demonstrates anti-inflammatory activity, which contributes to protection of the mucosa from inflammation-related damage (15-17).

As a forerunner of future clinical studies, we analyzed the *in vitro* effect of bovine milk derivative Lf (bLf) on the inflammatory response by CF bronchial cells experimentally infected with different biofilm-forming clinical strains of *B. cenocepacia*. The results suggest bLf may have a pivotal role in decreasing inflammatory response by infected CF cells.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions

B. cenocepacia LMG 16656 (ET12 lineage type strain; pattern of pathogenicity markers: cable pili, *cblA*+; *B. cepacia* Complex Epidemic Strain Marker, BCESM+) was from Belgium Coordinate Collection for Microbe Strain (BCCM/LMG). *B. cenocepacia* 6L (*cblA*; BCESM+) and PV1 (*cblA*+; BCESM-) were clinical isolates from lung sputum of CF patients (7, 18). Strains were streaked on Burkholderia cepacia medium (BCM) plates (Oxoid LTD, England) before the experiments. Chemically defined medium containing 1 and 100 μM ferric ions as $(Fe)_2(SO_4)_3$ (IL1-CDM and IL100-CDM, respectively) were prepared as previously described (7). To prepare inoculum, the strains were grown overnight at 37°C in IL1- and IL100-CDM. Broth cultures were diluted 1:50 in IL1- and IL100-CDM and incubated for an additional 2.5 h to reach an OD_{600} of 0.025 corresponding to about $1.0\pm0.5x10^7$ colony forming units (CFUs)/ml.

Bacterial lifestyle detection

Samples of overnight bacterial cultures in IL1- and IL100-CDM were stained with BacLight®LIVE/DEAD viability probe (Molecular Probes). Bacterial lifestyle was evaluated by epifluorescence microscopy (Leitz, Dialux 20 EB) (7).

Production of N-acylhomoserine lactones

The production of N-acylhomoserine lactones (AHLs) with *N*-acyl side chains of 4-8 and 6-12 carbons was evaluated by using *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 (pZLR4) bacterial reporters, respectively (19-20). *B. cenocepacia* were grown as described above. After 24 h of incubation, the spent supernatants containing AHLs were harvested by centrifugation (16.000 g, 20 min) and sterilized by filtration using 0.2 μ filters (Millipore Corp., Bedford, MA). The AHL plate assays were performed by using 20 μl of filtered spent supernatants. After 24 h of incubation, violacein production by *C. violaceum* CV026 and β-galactosidase activity by *A. tumefaciens* NTL4 (pZLR4) were detected to demonstrate AHL production.

Host cells

CFTR mutated IB3−1 (ATCC CRL−2777) and isogenic CFTR wild−type C38 (ATCC CRL−2779) cell lines were grown as semi-confluent monolayer in LHC-8 medium (Gibco, Invitrogen, NY, USA) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 0.1 mg/ml of streptomycin, and 10% heat-inactivated fetal calf serum (FCS) in a 5% CO_2 incubator at 37°C. Two hours before infection, cells were washed with LHC-8, and then cultured in fresh media with 2% FCS without antibiotics.

Lactoferrin

Highly purified bovine milk derivative Lf (bLf) was kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan). The purity of bLf was checked according

to Siciliano et al. (21). Lipopolysaccharide (LPS) contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, PBI International, Italy), was equal to 0.6±0.08 ng/mg of bLf. The bLf iron saturation was about 20% (7). Before biological assays, bLf was sterilized by filtration (Millipore). BLf concentrations ranging from 0.1 to 1 mg/ml were not toxic for IB3-1 and C38 cells and did not show antibacterial activity (14).

Infection of host cells

IB3-1 and C-38 semi-confluent monolayers were infected at multiplicity of infection (MOI) of 10 bacteria per cell with *B. cenocepacia* strains grown in IL1- and IL100-CDM in the absence and presence of 0.1 mg/ml of bLf. After 3 hours at 37°C in CO_2 atmosphere, cells were washed and fresh medium containing amikacine (1 mg/ml) and ceftazydime (1 mg/ml) was added to kill extracellular bacteria (7). Invasion efficiency values were calculated as the percentage of the ratio between the numbers of intracellular versus infecting bacteria. The mean values ± standard deviations were obtained from five independent experiments. Values of $P \leq 0.05$ were considered significant.

Gene expression of IB3-1 cells

DNA microarray mRNA profiling was performed for gene expression studies of IB3-1 cells infected with *B. cenocepacia* strains grown in IL100-CDM in the absence and presence of 0.1 mg/ml of bLf. Parallel experiments were performed treating uninfected IB3-1 cells with 0.1 mg/ml of bLf or 0.1 ng/ml of *Escherichia coli* 055:B5 LPS (Sigma-Aldrich Co.).

Total RNA was extracted after three hours of infection at 37°C, purified by using the 5PRIME PerfectPure RNA Purification Kit (5PRIME, Italy), and checked for purity and integrity by agarose gel electrophoresis and measuring the absorbance at $A_{260/280}$ nm, respectively. A total of 5 µg of total RNA was used to synthesize biotin-labelled double stranded cDNA (Eppendorf DualChip Microarrays Silverquant Detection Method, Eppendorf, Italy). Biotin-labelled cDNAs were used for hybridization onto DualChip® Human Inflammation Microarray slide (Eppendorf, Italy) containing 310 spotted genes of inflammatory pattern. After 16 h of incubation at 60°C at 1400 rpm in thermoblock for slides DC (Eppendorf), glass slides were processed by using Silverquant Detection Kit (Eppendorf). Data were acquired by Silverquant Scanner and analysed by Silverquant software (Eppendorf). Gene expression in infected IB3-1 cells in the absence or presence of bLf was considered up- or down-regulated if there was at least a 1.5-fold difference compared to that of uninfected cells.

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Quantitative reverse transcription-RealTime PCR (qRT-PCR) was performed by using as template 2 µg of total RNA and the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was quantified by relative quantitative qRT-PCR performed with 10 µl reactions by using the 1X Fast SYBR®Green Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primers (3'→5') selected by Primer Express Software and purchased from IDT-Tema Ricerca (Bologna, Italy) were: IL-8 (AF385628): Fwd GACCACACTGCGCCAACA and Rev GCTCTCTTCCATCAGAAAGTTACATAATT; IL-6 (NM_000600): Fwd CGGTACATCCTCGACGGC and Rev CTTGTTACATGTTCTCCTTTCTCAGG; IL-1 (BT007213): Fwd CTCCACCTCCAGGGACAGGA and Rev GGACATGGAGAACACCACTTGTT; IL-11 (NM_000641): Fwd CTGCACAGCTGAGGGACAAA and Rev AATCCAGGTTGTGGTCCCC. Calibrator genes were GAPDH (NM_002275; Fwd GTGGAGTCCACTGGCGTCTT and Rev GCAAATGAGCCCAGCCTTC) for IL-8 and IL-6; EEF1γ (NM_001404; Fwd AAACTGTGTGAGAAGATGGCCC and Rev GGGTCTCTGCAAACTTTTTAGCA) for IL-1β; and CK-15 (NM_002275; Fwd GGCTGGCTGCGGACG and Rev GCAGGGCCAGCTCATTCTC) for IL-11 quantifications. Amplification conditions were: initial denaturation/activation step of 95°C for 2 min, followed by 40 repeats of 95°C for 15 s and 60°C for 30 s. Results were collected with Sequence Detection software (version 1.3; Applied Biosystems) and quantified by using the comparative threshold (C_T) method (Applied Biosystems *User Bulletin 2*). Changes in mRNA expression level were calculated following normalization to the calibrator gene. The ratios obtained following normalization are expressed as the fold change over non-treated samples.

The qRT-PCRs were performed in triplicate for both target and normaliser genes.

Cytokine production

ELISA were performed to determine IL-8, IL-6, IL1-β and IL-11 levels on supernatants of uninfected and infected IB3-1 cells with *B. cenocepacia* strains grown in IL100-CDM in the absence or presence of 0.1 mg/ml of bLf by using Human IL-8, IL-6, and IL-1β Elisa Max Deluxe Sets (BioLegend, San Diego, USA) and Human IL-11 Immunoassay (R&D Systems, Minneapolis, USA).

Immunocytochemistry confocal laser scanner microscopy

IB3-1 cells cultured in CultureSlide wells (BD Falcon, BD Biosciences, Erembodegem, Belgium), were uninfected or infected with *B. cenocepacia* LMG 16656 grown in IL100-CDM (MOI 10) in the absence or presence of bLf (0.1 mg/ml). After 3 h of incubation at 37°C, the cells were fixed in PFA% for 30 min at room temperature, washed in saline solution and treated with 0.1% BSA- 0.2% Triton X100 (Sigma-Aldrich) for 60 min at room temperature to minimize the non-specific binding. The cells then were then exposed to a 1:50 dilution of fluorescein isothiocyanate-conjugated IgG Polyclonal Rabbit Anti-Human lactoferrin antibody (DakoCytomation, Denmark A/S) for 1 h at room temperature. The cells were rinsed with PBS, treated with the nuclear fluorescent probe TO-PRO-3 (1 mM) (Molecular Probes), and mounted in buffered glycerol (pH 9.0). Control experiments were performed omitting anti-lactoferrin antibody. Samples were analyzed by using a Leica confocal laser scanner microscopy (CLSM) (Laser Scanning TCS SP2) equipped with Ar/ArKr and He/Ne lasers. Laser line was at 488 and 633 nm for FITC and TO-PRO-3 excitation, respectively. The images were scanned under a 40X oil immersion objective.

Statistical analysis

Data derived from at least three independent experiments were expressed as mean values ± standard deviation. Statistical analysis was performed by using the Student's *t* test for unpaired data. *P* values of ≤ 0.05 were considered significant.

RESULTS

Influence of iron availability on Burkholderia cenocepacia biofilm development

Three *B. cenocepacia* strains with different patterns of pathogenicity markers (see Materials and Methods section) were cultured under different iron availability to evaluate bacterial growth and lifestyle (i.e. free, aggregated and biofilm). Results showed that media containing 1 and 100 μM Fe^{3+} (IL1-CDM and IL100-CDM, respectively) did not affect significantly bacterial multiplication (Fig. 1, Panel A), but significantly modulated the bacterial aggregation and biofilm matrix formation. In particular, bacterial aggregates surrounded by extracellular matrix were more numerous in the presence of high than of low iron concentrations (100 and 1μM Fe^{3+}, respectively) (Fig. 1, Panel B).

The production of AHLs in spent supernatants of *B. cenocepacia* cultures was monitored to confirm biofilm mode of growth (22). AHLs production was assessed by using *C. violaceum* CV026 and *A. tumefaciens* NTL4 (pZLR4) reporter strains (Fig. 1, Panels C and D) (19, 20, 22). The spent supernatants deriving from IL100-CDM stimulated AHL-mediated responses at higher extent than those from IL1-CDM cultures. Spent supernatants of *B. cenocepacia* PV1 and 6L stimulated both *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) responses while *B. cenocepacia* LMG 16656 induced only *A. tumefaciens* NTL4 (pZLR4) response.

Taken together, the data demonstrated that iron availability influenced the lifestyle of *B. cenocepacia* strains. In particular, the low and high iron concentration significantly induced planktonic and biofilm lifestyle, respectively.

Influence of bovine lactoferrin on the invasion ability of Burkholderia cenocepacia strains

Iron-modulated planktonic and biofilm *B. cenocepacia* invaded at higher efficiencies IB3-1 than C38 cells ($P \leq 0.05$). The invasion efficiency of *B. cenocepacia* LMG 16656 and 6L strains in planktonic and biofilm lifestyle did not significantly differ, while that of *B. cenocepacia* PV1 biofilm was ten-fold lower in respect to its planktonic counterpart. In particular, iron-modulated biofilm of *B. cenocepacia* LMG 16656 showed the highest (12.1±7.1 and 7.6±0.4 of IB3-1 and C38 cells, respectively), PV1 the lowest (0.8±0.5 and 0.7±0.1 of IB3-1 and C38 cells, respectively), and 6L intermediate invasion efficiencies (5.7±2.8 and 2.4±0.9, of IB3-1 and C38 cells, respectively). The addition of 0.1 mg/ml of bLf to the cells at the moment of the infection did not affect the invasion efficiency of either iron-modulated planktonic or

biofilm *B. cenocepacia* strains.

Influence of bovine lactoferrin on inflammatory response of IB3-1 cells infected with iron-modulated biofilm of Burkholderia cenocepacia strains

To mimic the *in vivo* situation, IB3-1 cells were infected with *B. cenocepacia* iron-modulated biofilm and the putative anti-inflammatory influence of bLf on inflammatory response of IB3-1 infected cells was evaluated by using microarray, qRT-PCR and ELISA assays.

Firstly, the influence of bLf on gene expression of uninfected IB3-1 cells was evaluated by using microarray assay. The results showed that bLf did not change the gene expression of IB3-1 in respect to untreated cells (data not shown). Secondly, LPS contamination of bLf was found equal to 0.06±0.008 ng/0.1 mg of bLf. IB3-1 cells exposed to a similar LPS concentration did not change the gene expression in respect to untreated IB3-1 cells (data not shown).

The gene expression of IB3-1 cells infected with *B. cenocepacia* iron-modulated biofilm in the absence and presence of bLf in respect to uninfected cells was analyzed by microarray.

IB3-1 cells infected with iron-modulated biofilm of *B. cenocepacia* clinical strains regulated several different patterns of genes in the absence than in the presence of bLf (Table I). The highest number of genes was modulated by infection with *B. cenocepacia* LMG 16656 and PV1 strains and the lowest with *B. cenocepacia* 6L.

The expression of IL-6, IL-8, IL-1, and IL-11 was further analyzed, based on the importance of these cytokines in CF lung disease (23). In cells infected with *B. cenocepacia* strains, the IL-6 expression was not changed either in the absence or presence of bLf while the addition of bLf partially influenced IL-8, significantly decreased IL-1β, and significantly increased IL-11 as compared to that detected in the absence of bLf ($P \leq 0.05$) (Figs. 2, 3). The expression of the cytokines by uninfected cells did not change following bLf addition (Figs. 2, 3).

Localization of bovine lactoferrin in IB3-1 cells

BLf localization in IB3-1 cells uninfected and infected with iron-modulated biofilm of *B. cenocepacia* LMG 16656 was detected by LSCM by using FITC-conjugated anti-lactoferrin antibody (Fig. 4). In control experiments (i.e. in the absence of anti-bLf antibody), IB3-1 cells did not demonstrate green fluorescence (data not shown). Following administration of bLf, LSCM images of the optical sections recovered from the nucleus level of uninfected and infected IB3-1 cells revealed green fluorescent bLf (Fig. 4, Panels B and F) and blue fluorescent nucleus (Fig. 4, Panels C and G). The merged images (Fig. 4, Panels D and H obtained overlaying panels A-C and E-G, respectively) revealed that green and blue fluorescence was at the same optical section and that bLf was localized to the cytoplasm and co-localized to the nucleus.

DISCUSSION

To evaluate the potential therapeutic effects of bLf in CF, we assessed the effects of bLf on both the ability of biofilm-forming *B. cenocepacia* clinical strains to invade CF bronchial epithelial cells and the inflammatory response by infected cells.

B. cenocepacia strains were grown in the presence of high iron concentrations like that observed in infected CF airway secretions (3). The iron availability strongly influenced *B. cenocepacia* strain lifestyle inducing biofilm development (Fig. 1) (7-8). Therefore, CF bronchial cells were infected with iron-modulated biofilm of *B. cenocepacia* clinical strains. While the invasion ability of *B. cenocepacia* had already been described (7, 11), here we report for the first time the invasiveness of *B. cenocepacia* iron-modulated biofilm. Interestingly, higher invasion efficiencies were recorded in CF (IB3-1) than in non-CF (C38) cells ($P \leq 0.05$) and the highest invasion efficiencies were shown by *B. cenocepacia* LGM 16656, belonging to the high infective ET12 lineage (10). To assess the effect of bLf on invasion ability, bLf at a concentration comparable to that recovered in airway secretions of infected CF patients (0.1 mg/ml) (13) was added to the cells at the moment of the infection. In these experimental conditions, bLf did not exert a significant anti-invasive effect as comparable values of invasion efficiencies were recorded both in the absence and in the presence of bLf. These data not agree with previously reported findings showing the anti-invasive activity of bLf (7, 9).

Table I. *Differentially expressed inflammatory genes of IB3-1 cells infected with Burkholderia cenocepacia iron-modulated biofilm in the absence or presence of bovine lactoferrin.*

Gene	GenBank accession number	*B. cenocepacia* LMG 16656[a]		*B. cenocepacia* PV1		*B. cenocepacia* 6L	
		None	bLf	None	bLf	None	bLf
ALOX5	NM_000698	5.8[b]	4.6	-	-	-	-
ALOX15	NM_001140	-	-	-	-	-	-1.9
ATF2	NM_001880	2.0	-	-	-	-2.2	-
BAD	NM_004322	-	-	-	-	-3.2	-
BCL2	NM_000633	4.5	2.4	-	-	-	-
BCL3	NM_005178	-	-	-	-	-2.5	-
CFB	NM_001710.3	2.5	1.6	-	-	-	-
BMP7	NM_001719	11.7	7.6	-	-	-	-
BMPR2	D50516	1.7	1.8	-	-	-1.5	-
BSG	NM_001728	-	-	-1.5	1.8	-	-
CASP1	M87507	2.2	1.7	-	-	-	-
CAV1	NM_001753	-	-	-	-	-5.2	-
CD3E	NM_000733	-	-	1.6	-1.6	-5.4	-
CDKN2B	U17075	-	-	-	-	-1.8	-
CEBPB	NM_005194	-	-	-	-	-	1.8
COL1A1	NM_000088	-	-	-	-	-	-2.8
CRHR1	NM_004382	3.2	3.6	-	-	-	-
CSF2	M11220	1.8	2.5	-	-	1.8	4.3
CSF3	X03438	-	-	1.7	-	-	-
CSF3R	M59818	-	-	1.7	-	-	-
CXCL2	NM_002089	18.4	18.8	2.8	1.9	7.9	7.0
EGR1	NM_001964	2.6	2.2	-	-	-	-
EPHB1	NM_004441	6.8	5.8	-	-	-	-
EPHB2	AF025304	2.5	3.0	-	-	-	-
ERBB3	M29366	1.6	2.5	-	-	-	-
FASLG	NM_000639	1.5	-	-	-	-	-
FCGR3A	NM_000569	3.2	3.5	1.7	-	-1.7	-1.6
FGF2	NM_002006	2.1	3.9	-	-	-	-
FGF3	NM_005247	4.1	4.1	-	-	-	-
FGF5	AF535149	2.6	2.5	-	-	-	-
FGF8	U36223	9.4	9.1	1.9	2.4	-	-
FGF9	NM_020996	3.6	2.7	-	-	-	-
FST	NM_006350	-1.8	-2.3	-	-	-	-
GATA3	NM_002051	4.4	3.5	-	-	-	-
HRAS	NM_005343	-2.2	-1.8	-	-	-	-
ICAM2	NM_000873	1.9	1.8	1.6	-	-	-
IGFBP2	M35410	-2.2	-3.8	1.6	-	-	-
IGFBP3	X64875	-1.7	-	-	-	-	-
IL10	NM_000572	-	-	-	-	-	1.6
IL10RA	NM_001558	4.0	3.0	-	-	-	-
IL11	NM_000641	-	-	-	1.5	-1.6	-
IL13	NM_002188	2.0	-	3.5	2.1	-	-
IL1β	M15330	2.3	-	2.1	-	-	-
IL2RB	NM_000878	2.2	1.7	-	-	-	-
IL2RG	NM_000206	1.7	-	-	-	-	-
IL3	M20137	-	-	5.6	3.7	-	-
IL6	NM_000600	8.3	7.0	1.8	2.3	6.3	6.6
IL8	NM_000584	11.9	12.7	3.8	2.8	12.7	9.6
IRF1	NM_002198	-	-	-	-	-1.6	-
ISGF3G	NM_006084	-	-	1.7	1.6	-	-
ITGB2	NM_000211	1.7	-	1.5	-	-	-

JUN	NM_002228	-	-	-2.0	-	-	-
KIT	NM_000222	1.9	-	-	-	-	-
KLK3	NM_001648	-	-	4.2	2.3	-	-
LIF	NM_002309	-	1.9	-	-	2.0	3.7
LILRB4	NM_006847	-	-	1.8	-	-	-
MAP3K1	XM_042066	4.5	4.7	-	-	-	-
MAP3K14	NM_003954	2.2	2.3	3.3	5.2	-	-
MAP3K8	NM_005204	-	-	2.1	-	-	-
MAPK14	AF100544	-	-	-	-	-1.9	-1.8
MIF	NM_002415	-	-	-	-	-1.7	-1.9
MMP7	NM_002423	-	-	4.9	4.3	-	-
MYC	NM_002467	-	-	-	-	-	-2.7
NCAM1	NM_181351	4.1	2.8	-	-	-	-
NEG1	M94165	3.3	2.5	-	-	-	-
NTF3	NM_002527	-	-	2.3	-1.8	-	-
ORM1	NM_000607	-	-	-1.5	-1.6	-	-
OSM	NM_020530	-	-	-2.2	-	-	-
PDGFA	X06374	-	-	-1.5	-	-	-
PDGFB	NM_002608	-	1.6	-2.1	-	-	1.9
PF4	NM_002619	-	-	1.7	-1.7	-	-
PGF	NM_002632	-	-	-	-	-3.4	-
PPBP	NM_002704	2.3	1.5	-	-	-	-
PTN	NM_002825	-	-	2.2	-	-	-
RENBP	NM_002910	1.5	-	-	-	-	-
RUNX1	D43968	-	-	-	-	-	2.5
TGFBI	NM_000358	-	2.3	-	-	-	-
TNFAIP3	NM_006290	4.3	5.2	2.1	1.8	7.6	8.9
TNFRSF8	NM_001243	1.6	-	-	-	-	-
TNFRSF9	NM_001561	1.6	1.7	-	-	-	-
TNFSF8	NM_001244	2.3	1.5	-	-	-	-
VEGF	AF022375	-	1.7	-1.8	-	-	3.3
VEGFB	U43368	-	-	2.0	-	-	-

[a]: IB3-1 cells were infected with iron-modulated biofilm of B. cenocepacia LMG 16656, PV1, and 6L clinical strains in the absence (None) or in the presence of 0.1 mg/ml of bovine lactoferrin (bLf); [b]: gene expression was expressed as fold-change of gene transcription of infected respect to uninfected IB3-1 cells; numerical values ≤-1.5 and ≥ 1.5 were considered to indicate significant decreased and increased transcription, respectively; [c] -: normo or not expressed genes. See Materials and Methods Section for details.

These discrepancies may be related to the different experimental protocols and, especially, to the higher concentration of bLf employed (≥0.5 versus 0.1 mg/ml) (7, 9).

To evaluate the effect of bLf on the inflammatory response of infected IB3-1 cells with iron-modulated biofilm of *B. cenocepacia*, bLf was employed at the concentration of 0.1 mg/ml not affecting the invasion efficiency.

BLf added to uninfected cells did not induce any significant change of inflammatory gene expression in respect to cells in the absence of bLf in accordance with previously reported data (15, 17). Also LPS at the concentrations found to contaminate bLf was unable to change the gene expression of uninfected IB3-1 in respect to control cells (15, 17).

Similarly to what was observed with planktonic *B. cenocepacia* (24), the infection with iron-modulated biofilm induced a strong inflammatory response of IB3-1. Different patterns of genes were regulated depending on the infecting strain and bLf presence (Table I).

We also examined the effect of bLf on the regulation of IL-6, IL-8, IL-1β, and IL-11, based on the importance of these cytokines in CF lung disease (23). IL-6, IL-8 and IL-1β cytokines were expressed

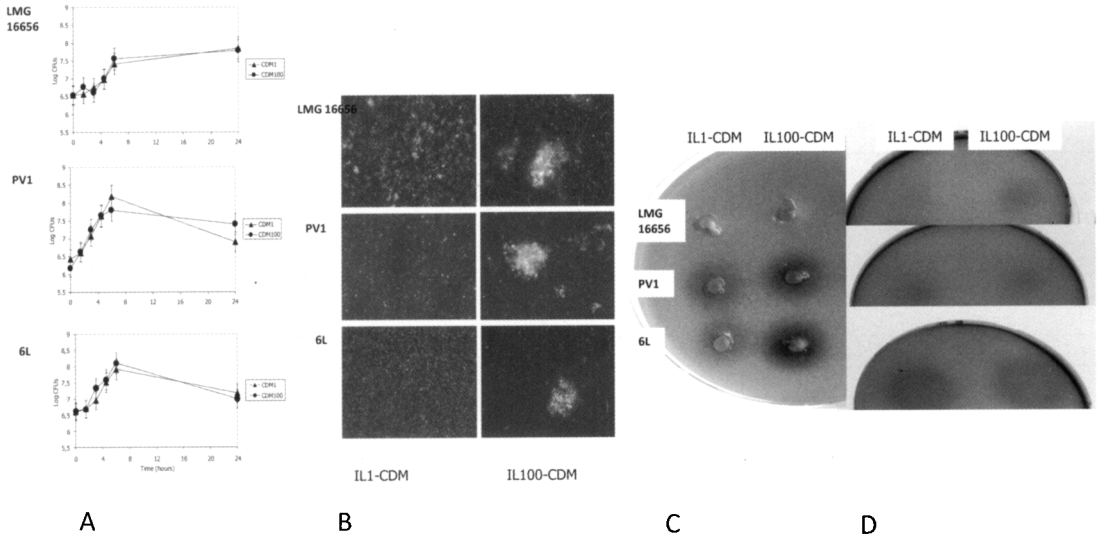

Fig. 1. *Effect of iron availability on growth (**A**), lifestyle (**B**) and production of N-acylhomoserine lactones (**C**) by Burkholderia cenocepacia clinical strains. Panel A: growth curves of B. cenocepacia LGM 16656, PV1, and 6L strains in IL1-CDM (▲) and IL100-CDM (●) containing 1 μM and 100 μM iron as ferric sulphate, respectively; Panel B: epifluorescence micrographs of B. cenocepacia strains after 24 hours of incubation in IL1- and IL100-CDM; Panels C and D: detection of N-acylhomoserine lactones (AHLs) in spent supernatants of 24-hour cultures of B. cenocepacia strains grown in IL1- and IL100-CDM through violacein production by Chromobacterium violaceum CV026 (Panel C) and β–galactosidase activity by Agrobacterium tumefaciens NTL4 (pZLR4) (Panel D). See Materials & Methods section for details.*

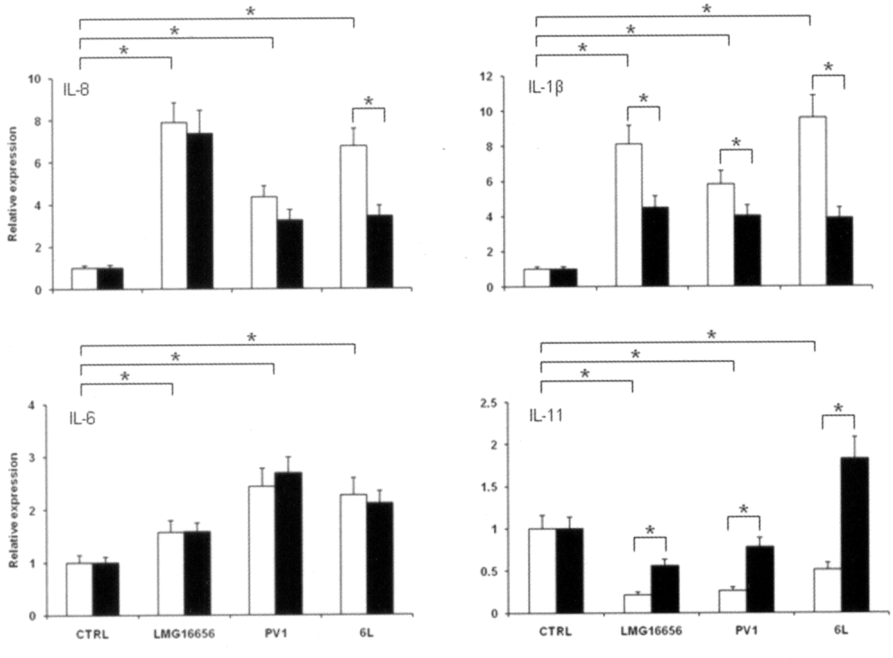

Fig. 2. *Relative gene expression of IB3-1 cells infected with iron-modulated biofilm of Burkholderia cenocepacia clinical strains in the absence and presence of bovine lactoferrin. Relative gene expression was determined by qRT-PCR experiments. Legend: infected IB3-1 cells in the absence (white columns) and presence of bovine lactoferrin (0.1 mg/ml) (black columns); *: significant values ($P \leq 0.05$). See Materials and Methods section for details.*

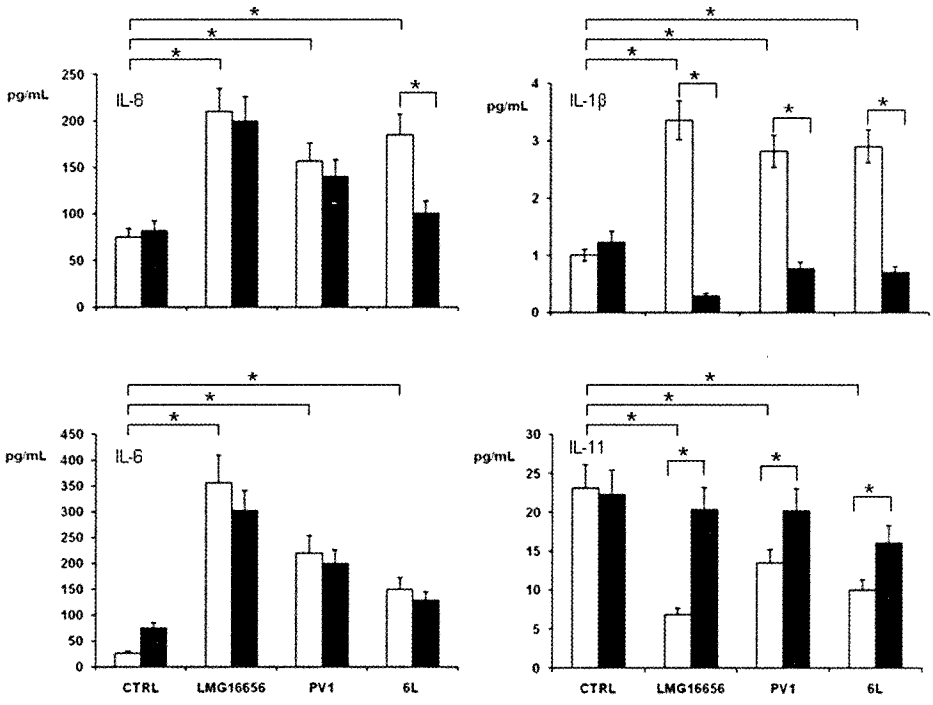

Fig. 3. *Cytokine levels in supernatants of IB3-1 cells infected with iron-modulated biofilm of Burkholderia cenocepacia clinical strains in the absence and presence of bovine lactoferrin. Cytokine levels were determined by ELISA. Legend: infected IB3-1 cells in the absence (white columns) and presence of bovine lactoferrin (0.1 mg/ml) (black columns); *: significant values ($P \leq 0.05$). See Materials and Methods section for details.*

at significantly higher and IL-11 at significantly lower levels by infected IB3-1 cells in respect to uninfected IB3-1 cells (Figs. 2, 3).

The addition of bLf to infected cells did not affect the expression of IL-6 and induced significant or partial reduction of IL-8 (Figs. 2, 3). Conversely, the addition of bLf significantly decreased the expression of IL-1β to levels similar to those recovered in the supernatants of uninfected cells (Figs. 2, 3). IL-1β is an important inflammatory cytokine associated with cell death and tissue destruction (25). The malfunction of the CFTR channel produces exacerbated levels of IL-1β by murine macrophages in response to *B. cenocepacia* infection (26). The bLf-induced decrease of IL-1β expression may be considered crucial in CF lung infections as the reduction of IL-1β decreases the related cell damages (27).

Recently, human cells infected with virulent bacteria showed a significant down-regulation of IL-11 (28). In our *in vitro* model the highest decrease of IL-11 expression was recorded in IB3-1 cells infected with *B. cenocepacia* LMG 16656, the most virulent among the tested strains (Figs. 2, 3). It is interesting to underline that bLf added to infected cells increased IL-11 to levels similar to those of uninfected cells, independently of the infecting strain. This result showing the *in vitro* bLf's ability to increase IL-11 expression is consistent with bLf-mediated increase of IL-11 in an inflamed mouse model (29).

It has been hypothesized that bLf localization to the nucleus influenced gene expression regulation in uninfected cells (30-31). On the contrary, here we show that even if bLf reaches the nucleus of uninfected CF cells, the expression of inflammatory genes is not changed, confirming previously reported observations (15, 17).

To our knowledge this is the first demonstration

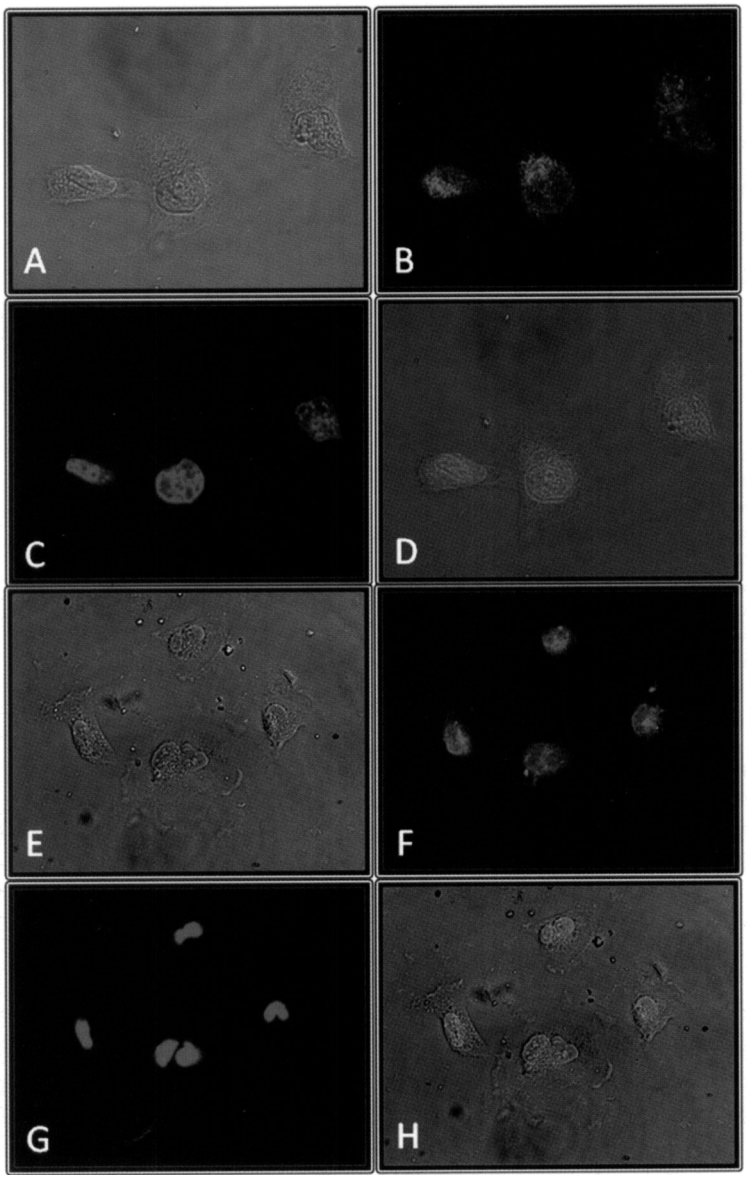

Fig. 4. *Localization of bovine lactoferrin in IB3-1 cells. Laser scanner confocal microscopy images of the optical sections recovered to the nucleus level of IB3-1 cells uninfected or infected with iron-modulated biofilm of Burkholderia cenocepacia LMG 16656. Legend: bright field microscopy images of uninfected (Panel **A**) or infected (Panel **E**) IB3-1 cells; green fluorescent bLf recovered in uninfected (Panel **B**) or infected (Panel **F**) IB3-1 cells; blue fluorescent nucleus recovered in uninfected (Panel **C**) or infected (Panel **G**) IB3-1 cells; merged images showing bLf localization in uninfected (Panel **D**, merged image of Panels A, B, and C) or infected (Panel **H**, merged image of Panels E, F, and G) IB3-1 cells.*

of bLf localization to the nucleus of infected cells. As bLf changed the expression of inflammatory genes of infected cells, it should be argued that the bLf modulates only genes regulated in response to bacterial invasion.

From clinical trials, bLf is emerging as a safe and potent anti-inflammatory natural molecule (32). Our data confirm the relevant influence of bLf in decreasing inflammatory response thus encouraging further studies on bLf protection from inflammation-

related damages in CF.

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