CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Catalogo dei prodotti della ricerca

HLA-G expression and regulation during *Pseudomonas aeruginosa* infection in cystic fibrosis patients

Roberta Rizzo[1]*, Gabriella Bergamini[1-2]*, Daria Bortolotti[1], Teresinha Leal[3], Ciro D'Orazio[4], Emily Pintani[4], Loredana Melchiorri[1], Eleonora Zavatti[5], Baroukh Maurice Assael[4], Claudio Sorio[2°], Paola Melotti[4].

[1]Medical Sciences Department, Section of Microbiology and Medical Genetics, Ferrara University, Italy

[2]Cystic Fibrosis Translational Research Laboratory "Daniele Lissandrini", Department of Pathology and Diagnostics, University of Verona, Italy

[3]Louvain Centre for Toxicology and Applied Pharmacology, Université Catholique de Louvain, Belgium

[4]Cystic Fibrosis Center, Azienda Ospedaliera Universitaria Integrata di Verona, Italy

[5]Azienda Ospedaliero-Universitaria S. Anna, Ferrara, Italy

* Equal contribution to this study, °Corresponding author

Corresponding author

Claudio Sorio MD, PhD

Phone number: +39 0458027668

Fax number: +39 0458027127

e-mail: claudio.sorio@univr.it

Abstract

Background Deregulated immune response fails to control biofilm-forming bacteria, as *Pseudomonas aeruginosa*, in the lung of Cystic Fibrosis (CF) patients. Human Leukocyte Antigen (HLA)-G is an immune-modulatory molecule with a possible role in respiratory diseases and infections. **Methods** We performed HLA-G mRNA and protein analysis in plasma and exhaled breath condensate (EBC) from CF patients undergoing intravenous antibiotic treatment, CF cell line and murine model. **Results** Antibiotic therapy normalizes plasmatic levels of HLA-G in CF patients hospitalized for bacterial respiratory infection suggesting and anti-inflammatory role at the systemic level while in CF lung microenvironment, higher expression of HLA-G is associated with *P. aeruginosa* infection. CF cell line and CF murine model expressed higher HLA-G molecules in the presence of *P. aeruginosa*. **Conclusions** HLA-G expression and regulation in plasma and lung microenvironment suggest a role in reducing systemic inflammation and supporting the establishment and persistence of *P. aeruginosa* infection in the respiratory system.

Keywords: *Pseudomonas aeruginosa,* HLA-G, cystic fibrosis

Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are the most common pathogens colonizing lungs of patients with cystic fibrosis (CF) [1, 2]. Central for *P. aeruginosa* and *S. aureus* survival in the lung environment is their ability to adapt and switch between free-living (planktonic) and surface-attached (biofilm) life-styles [3, 4].

There are several lines of evidence that support the critical importance of immune response and, specifically, professional phagocytic cells as key determinants in the ability of the host to control biofilm forming bacteria [5]. As a proof of concept, patients with CF are well characterized to generate a deregulated innate phagocytic and/or signaling pathway insufficient for effective host control of bacterial infection and for sterilizing immunity [6]. However, the underlying mechanisms are largely unclear.

Recent studies have shown that Human Leukocyte Antigen (HLA)-G molecules play a role in airway immune responses [7-9], in particular in asthmatic patients there is an increase in local and circulating HLA-G expression that could be an attempt to restore a proper balance in inflammatory cells and cascades that have been activated in chronic asthma, or be a part of the on-going pathogenesis of chronic asthma by the repression of selected classes of immunologically active cells [10].

Compared to Class Ia HLA, the non-classical Class I HLA-G antigen has low allelic polymorphism, highly restricted distribution in tissue and alternative mRNA splicing. The latter creates distinct membrane-bound (HLA-G1 to G4) and soluble (HLA-G5 to G7) variant isoforms [11]. In addition, a soluble HLA-G1 isoform (sHLA-G1) can be generated by membrane HLA-G1 proteolytic cleavage [12]. In healthy tissues, HLA-G1, HLA-G5, and sHLA-G1 are the most frequently reported isoforms. Their structure are similar to those of classical HLA Class I molecules. A tolerogenic function for HLA-G has been suggested, based on its ability to inhibit activated CD8+ T, natural killer and dendritic cells, to stimulate

T regulatory cells and to block T lymphocyte allo-response [11]. These functions are mediated by interactions with specific, inhibitory immune cell receptors ILT-2, ILT-4, CD8 and KIR2DL4. HLA-G synthesis is controlled by several polymorphisms that modify the affinity of gene-targeted sequences of transcriptional or post-transcriptional factors [13]. A 14 base pair (14 bp) insertion/deletion (ins/del) polymorphism (rs66554220) in exon 8 affects mRNA stability and protein expression while the ins allele is characterized by mRNA destabilization and lower protein production [14] and associates with pathological events such as pregnancy failure [15], autoimmune diseases [12], organ transplant failure [16], increased susceptibility to viral infection [17] and tumor progression [18]. *HLA-G* is regarded as a potential asthma and bronchial hyper-responsiveness susceptibility gene [8, 9, 11]; it is expressed by airway epithelium [10], is detectable in bronchial-alveolar lavage (BAL) samples from asthmatic patients [19] and is involved in lung development [20].

On the basis of these observations, we hypothesized an implication of HLA-G molecules in CF disease and bacterial airway infections and designed a study to investigate its expression and modulation in this context.

Materials and Methods

Human subjects

Plasma (n=49) and Exhaled Breath Condensate (EBC) samples (n=28) were collected from CF patients with bacterial respiratory infection and treated with intravenous (IV) antibiotics (beta-lactamic, aminoglycosides) for 14 + 2 days at recommended doses [21] (**Table I**) for acute exacerbations. We selected to evaluate *P. aeruginosa* and *S. aureus* infection status, as the two main representative bacteria in CF respiratory infections. Patients with CF were considered infected by *P. aeruginosa* and *S. aureus* when it was isolated in at least three sputum cultures at intervals longer than one month in a period of six months or when mucoid colonies were present in the sample [21]. A cohort of 195 patients with CF and of 230 sex and age-matched non-CF individuals was tested for HLA-G ins/del 14bp polymorphism. Healthy control individuals (CTRLs, n=76) were tested for HLA-G protein levels in plasma and for Exhaled Breath Condensate (EBC, n=7). Written informed consent was obtained from all subjects enrolled in the study approved by the Institutional Review Board of AOUI Verona as project 1849.

To assess pulmonary function the forced expiratory volume in one second (FEV1) was assessed and expressed as percentage of the predicted value for age, sex and height [22]. The inflammatory biomarker C-reactive protein (CRP) was measured in patients with CF by enzyme immunosorbent assay (Cell Biolabs, San Diego, CA, USA). The kit has detection sensitivity limit of 1 ng/mL human CRP.

Cell lines

HLA-G expression was studied in CF IB3-1 cells and in the corresponding isogenic controls (C38 cells), a kind gift from Pamela Zeitlin, Johns Hopkins University, Baltimore, USA,

cultured as previously described [23]. Replicates of $5x10^5$ cells were exposed for 6, 12, and 24 hours to 5 or 10 ng/ml lipopolysaccharide from *P. aeruginosa* (LPS; Sigma-Aldrich, St. Louis, MO, USA) or to 10% conditioned medium (CM)[23]. JEG-3 cells (ATCC, HTB-36) cultured in RPMI medium (Sigma-Aldrich) containing 10% fetal calf serum were used as a positive control since they constitutively express HLA-G.

CF mouse model

Sex- and weight-matched 129/FVB mice, homozygous for the F508del-CFTR mutation, and wild-type littermates were housed at the animal facility of the Université Catholique de Louvain (Brussels) and anesthetized with an intra-peritoneal mixture of 100 mg/kg ketamine (Pfizer, NY, USA) and 15 mg/kg xylazine (Bayer, Leverkusen, Germany). Bronchoalveolar lavage (BAL) was collected using a laryngoscope and a fine pipette tip, in the presence or in the absence of induction of inflammatory reaction by LPS (50 µl volume, 100 µg/25g body weight) instilled in the trachea. The local Animal Care and Use Committee approved the experiments (2013/UCL/MD/012).

HLA-G assay

HLA-G levels were measured in 100 µl of cell CM, and in plasma and EBC samples. EBCs were collected using a condenser (TURBO-DECCS, Medivac, Parma; Italy) and concentrated by evaporation with a SpeedVac Concentrator SVC100H (Savant™ Universal SpeedVac™ Vacuum System; Thermo Scientific, Waltham, MA, USA). A bead array Bio-Plex system (BioRad, Hercules, CA, USA) was used to assay sHLA-G with anti-HLA-G MoAbs conjugated beads: MEM-G9 for sHLA-G1 and HLA-G5 isoform, and 5A6G7 for HLA-G5 isoform (Exbio, Vestec, Czech Republic), respectively. sHLA-G1 levels were calculated as the difference between total sHLA-G and HLA-G5 [24]. The sensitivity of the method is 1

pg/mL. Plasma samples were analyzed for sHLA-G levels by enzyme immunosorbent assay [24]. The limit of sensitivity was 1.0 ng/ml.

Immunoblotting. JEG-3, IB3-1 and C38 cell samples of conditioned medium, murine splenocytes (C57B1/6 mice) and BALs were biotinylated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) and immunoprecipitated for 2 hours with anti-HLA-G MoAb (MEMG9) or anti-Qa2 MoAb (e-Bioscience, San Diego, CA, USA). Immunoprecipitates, previously normalized for the total protein content to 1 mg/mL were loaded and separated in 10% TGX-Pre-cast gel (BioRad), transferred onto a PVDF membrane (Merck Millipore), incubated with horseradish peroxidase (HRP)-conjugated anti-mouse MoAb (GE Healthcare Europe GmbH, Milan, Italy) and developed by enhanced chemi-luminescence (ECL kit, GE Healthcare). Densitometric analysis was then performed with a Geliance Imaging System (Perkin Elmer, Waltham, MA, USA).

Flow cytometry

The expression of HLA-G was analyzed in cells by direct immunofluorescence with anti-HLA-G AlexaFluor-conjugated MoAb (87G) (Exbio) and flow cytometry performed with FacsVantage (Becton Dickinson).

Quantification of HLA-G transcripts and HLA-G 14bp ins/del polymorphism typing

Total RNA (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) was reverse transcribed (SuperScript™ System; Life Technologies) and quantitative PCR was performed using the endogenous control RNaseP eukaryotic gene (MGB, Applied Biosystems®, Milan, Italy).

Genomic DNA, extracted from EDTA blood (Nucleon; GE Healthcare) was genotyped for HLA-G 14bp ins/del polymorphism by Real Time-PCR [25].

Statistical analysis

According to data distribution (Kolmogorov-Smirnov test), we applied parametric statistics (StatView software, SAS Institute Inc, USA). A significance level (P-value) ≤ 0.05 was considered significant.

Results

Intravenous antibiotic therapy influences HLA-G expression in CF patients.

In this study we evaluated the possible role of HLA-G molecules in CF and airway bacterial infections. In Table 1 the main characteristics of CF and control (CTRL) subjects are summarized. A less negative Height Z score and the absence of diabetic subjects were found in the CTRL group ($p=0.042$ and $p<0.0001$, respectively). We followed *P. aeruginosa* and *S. aureus* infection status as the two main representative bacteria implicated in CF respiratory infections. At the time of admission, 49% of sputum cultures from patients with CF resulted positive for *P. aeruginosa* and 45% were positive for *S. aureus.* Nine patients (18%) scored positive for both strains (Table II). We then examined sHLA-G expression in the plasma sample from CTRLs and patients with CF during hospitalized for respiratory exacerbation. sHLA-G levels in the plasma of patients with CF (median 2.5 ng/mL) resulted significantly lower than in CTRLs (median 17.05 ng/mL, $p=0.0018$, Student *t* test). All patients with CF underwent intravenous antibiotic (i.v.) therapy for 14+2 days at recommended doses [21]. Plasma samples of patients with CF obtained after i.v. treatment showed an increase in sHLA-G levels (median 13.77 ng/mL, $P < 0.0001$) which reach those levels observed in the plasma of CTRLs (**Figure 1A**).

Clinical and functional laboratory data (FEV1 and CRP variations) at discharge indicated that all CF patients had benefited from antibiotic therapy. Although not statistically significant (p=?? please complete) a trend to a reduced bacterial load appeared. Indeed, an 8% reduction of sputum cultures resulted positive for *P. aeruginosa* and of 19% for *S. aureus* (**Table 2**). The i.v. treatment improved FEV1 values (≥ 5% positive increase of FEV1, **Table** 2) while the acute phase reaction pentraxin C-reactive protein (CRP), an inflammation-related biomarker [26], was drastically reduced (**Table 2**). Interesting, variations of CRP plasma levels were inversely correlated to sHLA-G in 30% of CF patients (Spearman Correlation r=-

0.307; P=0.032), while no significant correlations were observed with FEV1 values and the outcome of microbial infections (data not shown).

HLA-G gene polymorphisms and microbial infection

A 14bp ins/del polymorphism (rs66554220) in the *HLA-G* gene is known to affect its expression, in particular the 14bp del allele is known to stabilize its mRNA and to increase HLA-G protein levels [14]. We analyzed the *HLA-G* ins/del 14bp polymorphism in CF individuals that were categorized in three genotypes (ins/del, ins/ins, del/del). The genotype distribution did not differ between the two cohorts of 195 patients with CF and 230 sex- and age-matched CTRLs (p=0.062) (**Table 3**). Moreover, there was no significant influence of the genotype on plasma levels of sHLA-G in patients with CF (**Figure 1B**) and in non-CF individuals (data non-shown). However, the outcome of microbial infections was associated with an increased risk of chronic *P. aeruginosa* infection in patients with CF displaying the del/del genotype (OR: 3.3; 95% CI: 1.8-6.1) (**Table 4**), which has been associated to an increased HLA-G production [25]. On the contrary, no correlation was observed with *S. aureus* infection status (data non shown). Given the absence of any evident correlation between plasma HLA-G levels and the occurrence of lung infection by *P. aeruginosa,* we tested whether a specific accumulation of HLA-G might occur in the lung microenvironment.

sHLA-G expression in Exhaled Breath Condensate

To better characterize the expression of HLA-G in the lung microenvironment, we examined sHLA-G expression in the Exhaled Breath Condensate (EBC) from CTRLs and patients with CF during hospitalization for respiratory exacerbation. EBC was selected as a non-invasive matrix to monitor sHLA-G molecules in the lung microenvironment [27], where bacteria accumulate. In EBC of patients with CF, sHLA-G levels were significantly higher (median

8.68 pg/mL) than in CTRLs (median 1.7 pg/mL, p=0.016, Student *t* test). After antibiotic i.v. therapy, a decrease in sHLA-G (median 2.3 pg/mL, p=0.0235) was observed with intermediate values when compared with CTRLs (p=0.016) (**Figure 2A**). No significant relationship was observed between the levels of sHLA-G in EBC samples, sHLA-G amount in plasma samples (suggesting a microenvironment-specific regulation of its levels), clinical data and *S. aureus* infection status (data not shown).

HLA-G levels in EBC and *P. aeruginosa* infection

We next examined the association of HLA-G levels in EBC and the infection status of patients with CF. sHLA-G levels in EBC were manifold higher (median 17.3 pg/mL) in patients found positive for the presence of *P. aeruginosa* than in those patients whose sputum culture were negative for the bacterium (median 2.1 pg/mL, p=0.005) or healthy CTRLs (median 1.7 pg/mL, p=0.025) (**Figure 2B**). After antibiotic i.v. therapy, patients with CF who cultured positive for *P. aeruginosa* after antibiotic therapy showed higher HLA-G levels in EBC (median 11.2 pg/mL) than patients whose sputum culture turned negative (median 1.5 pg/mL, $p < 0.001$) or CTRLs (median 1.7 pg/mL, $p < 0.001$) (**Figure 2B**). Since soluble HLA-G could derive from both shedded membrane HLA-G1 isoform and soluble HLA-G5 isoform, we evaluated the amount of the two different isoforms in EBC. Both sHLA-G1 and HLA-G5 isoforms were detected with HLA-G5 molecule representing the main isoform monitored after i.v. antibiotic therapy in patients with CF (**Figure 3**).

***P. aeruginosa* infection induces HLA-G expression in CF bronchial epithelial cells**.

As a bimodal distribution of sHLA-G values in EBC samples was noticed within individuals with CF on the basis of the presence/absence of *P. aeruginosa* infection and an association between *HLA-G* del/del 14bp genotype and the occurrence of infection, we tested the

hypotheses that the bacterium might affect HLA-G expression in CF lung environment. In particular, we focused on bronco-epithelial cells, the airway interface that plays a key role in CF [28]. We measured HLA-G levels in CF (IB3-1) and isogenic, corrected (C38) bronchial epithelial cells in the presence/absence of *P. aeruginosa* culture medium (CM). *P. aeruginosa* CM was able to induce the expression of larger (10-fold) amounts of HLA-G in IB3-1 CF cells compared to the corresponding non-CF C38 cell line, where a lower (4-fold) HLA-G increase was recorded (**Figure 4A**) 24hrs after incubation. These findings were confirmed using *P. aeruginosa* LPS, as a key component of the bacterial wall. In fact, it is known that LPS acts as a powerful inducer for HLA-G expression [14]. The treatment of C38 and IB3-1 cells with *P. aeruginosa* LPS for 12 hours promoted an increase of HLA-G at the mRNA level (**Figure 4B**). No induction of the HLA-G membrane expression was found in any C38 and IB3-1 cells (**Figure 4C**). On the contrary, we observed HLA-G release in both C38 and IB3-1 cells with higher amounts being found in IB3-1 cells in comparison with C38 cell line (**Figure 4D**).

We then evaluated the amounts of HLA-G1 and HLA-G5 isoforms after *P. aeruginosa* LPS treatment. mRNA analysis indicated that only the HLA-G5 isoform is produced by bronchial epithelial cells, thus excluding membrane shedding as the source of the protein found in the CM (**Figure 5A**). ELISA test confirmed the presence of HLA-G5 isoform in the CM (**Figure 5B**). Altogether, these data indicate that bronchial epithelial cells are able to express HLA-G5 and that *P. aeruginosa* soluble molecules, in particular LPS, are able to induce the expression and secretion of the isoform, with the highest levels produced by CF cells.

***P. aeruginosa* infection induces Qa2 expression in CF murine lung**.

To further characterize the differential regulation of HLA-G in CF airway in the presence/absence of *P. aeruginosa* infection, we compared the levels of HLA-G murine

ortholog, Qa2, in the BAL of wild-type (CTRL) and CF mice treated with vehicle (saline) or *P. aeruginosa* LPS [14]. Lower Qa2 levels were found in BAL samples from naïve, non-LPS-stimulated CF mice as compared to wild-type mice ($p=0.0005$). Challenge with LPS was able to increase Qa2 levels only in the BAL of CF mice ($p=0.001$) (**Figure 6**) suggesting that CF is associated to an increased capability to release this important modulator of the immune response.

Discussion

The expression of HLA-G molecules during pathogen infection is an important component of microbial immune escape mechanisms and influences disease severity during viral infections. Indeed, alveolar macrophages collected from patients suffering from acute cytomegalovirus pneumonitis usually express high levels of HLA-G molecules [29], a conditions favouring immune evasion. Altered levels of HLA-G were recorded in airway system in asthma and bronchial hyper-responsiveness [11], suggesting a role of HLA-G molecules in chronic lung inflammation and infections in respiratory tract, a condition typical of CF disease.

Our data showed that plasma sHLA-G levels are lower in hospitalized CF patients in comparison to healthy controls but increase following i.v. antibacterial therapy to levels that are indistinguishable from healthy controls. Interesting, the increased HLA-G in a subgroup of patients with CF was inversely related to levels of CRP, a biomarker of systemic inflammation [26]. An impact of the role of HLA-G molecules in immune-regulation, for example in the creation of a tolerogenic environment at the maternal-fetal interface [15] and in transplanted patients [16, 30], has been already suggested. Our data on plasma samples support the idea of a possible implication of HLA-G molecules in the regulation of systemic inflammation in CF condition, where sHLA-G increase might control an excessive activation of immune cells. On the other hand, we found an increased frequency of the 14bp del/del genotype in CF patients with persistent *P. aeruginosa* infection. Since this genotype is characterized by increased HLA-G expression with respect to the other genotypes [25], this finding was unexpected. However, we need to consider that immune response maybe tightly regulated by local conditions. Indeed, our data showed that there is an association between increased sHLA-G levels in the alveolar microenvironment (assayed in EBCs) and persistence of *P. aeruginosa* infection even after i.v. antibiotic therapy administered to CF patients. In

this context, the immune-regulatory role of HLA-G, by decreasing local immune response, might support the persistence of bacterial infection in the lung microenvironment.

Experiments in mice confirmed that CF condition is associated with a stronger up-regulation of HLA-G expression in the lung microenvironment in response to challenge with LPS. In fact, higher levels of HLA-G murine ortholog Qa2 were found in BALs of CF mice challenged with *P. aeruginosa* LPS in comparison with wild-type mice. Similarly, CF bronchial epithelial cells secreted higher amounts of HLA-G in comparison to isogenic CFTR-corrected cell lines upon challenge with *P. aeruginosa* CM or with LPS. Taken together, these findings indicate that CF disease influences the regulation of Qa2/HLA-G expression, mainly through the secreted isoform HLA-G5, in the presence of bacterial infections.

How the presence of a higher HLA-G concentration in the lung microenvironment might contribute to the development of chronic *P. aeruginosa* infection in CF remains still unknown. We propose that the same mechanism that is beneficial at the systemic level, where it might control the excessive immune response, can support the establishment of *P. aeruginosa* infection reducing the activation of local immune response. Bacteria may inhibit immune cell responses by inducing HLA-G that in turn interacts with immune inhibitory receptors, including ILT2, ILT4 and KIR2DL4, to counteract the host immune system. This can occur at the early stages of bacterial clearance as well as at later time thus supporting long-term bacterial persistence.

Interestingly, we found a correlation between HLA-G expression and *P. aeruginosa* infection status, but not with *S. aureus* presence/absence. This finding is in agreement with the previous results obtained comparing the ability of these two bacteria to induce cytokine secretion [31]. The authors observed that planktonic and biofilm *S. aureus* induced equivalent amounts of

cytokine in human monocytes. In contrast, biofilm-forming *P. aeruginosa* induced a higher production of tumor necrosis factor and interleukin-6 than their planktonic counterpart.

Conclusions

HLA-G differential regulation in the plasma and lung microenvironment in CF suggest, for the first time, a role as an anti-inflammatory molecule at systemic level, whereas in the lung, HLA-G could impair bacterial clearance mechanisms and increase, by a similar mechanism, the probability of developing chronic bacterial airway infection.

Further studies are needed to fully understand the role of HLA-G molecules in this context, but these data suggest that this target deserve additional efforts necessary to define its precise role in CF.

Future perspectives

Additional studies on the role of HLA-G molecules in CF patients and bacterial infections will be relevant to precisely define the mechanisms controlling the deregulated immune response present in patients with CF and responsible for the persistence of bacterial infection in the lung environment. The identification of HLA-G as a key molecule in this process could help in the definition of new therapeutic protocols that could help in the management of CF patients. In spite of aggressive antibiotic treatment, the eradication of bacterial infection is difficult to achieve and often leads to chronic airway infection. The possibility to restore immune response modulating HLA-G expression could be an adjuvant therapy to the standard antibacterial protocols. The current researches on HLA-G expression [32] bode well for the use of HLA-G modulation as a therapeutic strategy in a few years.

Summary points

1. Immune response acts as a key determinant in the control of biofilm-forming bacteria in the lung of patients with Cystic Fibrosis (CF).

2. Human Leukocyte Antigen (HLA)-G is an immune-modulating molecule with a possible role in respiratory diseases and infections.

3. Soluble HLA-G is lower in plasma samples of CF patients in comparison with controls and is normalized after i.v. therapy.

4. Soluble HLA-G is higher in the EBC of CF patients and is normalized after i.v. therapy in CF patients free of *P. aeruginosa* infection.

5. CF cell line and CF murine model expressed higher HLA-G molecules in the presence of *P. aeruginosa*.

6. Higher expression of HLA-G in CF lung microenvironment is associated with *P. aeruginosa* infection.

7. HLA-G might have a role in bacterial immune-escape mechanisms.

References

1. Pritt B, O'brien L, Winn W. Mucoid pseudomonas in cystic fibrosis. *Am J Clin Pathol* 128(1), 32-34 (2007).
2. Rajan S SL. Pulmonary infections in patients with cystic fibrosis. *Semin Respir Infect* 17, 47–56 (2002).
3. Singh Pk SA, Parsek Mr, Moninger to, Welsh Mj, Greenberg Ep. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407, 762–764. (2000).
4. Hoiby N KJH, Moser C, Song Z, Ciofu O, Kharazmi A. Pseudomonas aeruginosa and the in vitro and in vivo biofilm mode of growth. *Microbes Infect* 3, 23–35 (2001).
5. Lovewell RR, Yash R, Berwin B. Mechanisms of phagocytosis and host clearance of pseudomonas aeruginosa. *Am J Physiol Lung Cell Mol Physiol* 306(7), L591–L603 (2014).
6. Sallenave JM. Phagocytic and signaling innate immune receptors: Are they dysregulated in cystic fibrosis in the fight against pseudomonas aeruginosa? *Int J Biochem Cell Biol* 52, 103-107. (2014).
7. Borghi A, Fogli E, Stignani M *et al*. Soluble human leukocyte antigen-g and interleukin-10 levels in plasma of psoriatic patients: Preliminary study on a possible correlation between generalized immune status, treatments and disease. *Arch Dermatol Res* 300(10), 551-559 (2008).
8. Mapp CE, Ferrazzoni S, Rizzo R *et al*. Soluble human leucocyte antigen-g and interleukin-10 levels in isocyanate-induced asthma. *Clin Exp Allergy* 39(6), 812-819 (2009).
9. Rizzo R, Mapp CE, Melchiorri L *et al*. Defective production of soluble hla-g molecules by peripheral blood monocytes in patients with asthma. *J Allergy Clin Immunol* 115(3), 508-513 (2005).
10. White SR, Loisel DA, Stern R, Laxman B, Floreth T, Marroquin BA. Human leukocyte antigen-g expression in differentiated human airway epithelial cells: Lack of modulation by th2-associated cytokines. *Respir Res* 14, 4 (2013).
11. Rizzo R, Bortolotti D, Bolzani S, Fainardi E. Hla-g molecules in autoimmune diseases and infections. *Front Immunol* 5, 592 (2014).
12. Rizzo R, Trentini A, Bortolotti D *et al*. Matrix metalloproteinase-2 (mmp-2) generates soluble hla-g1 by cell surface proteolytic shedding. *Mol Cell Biochem* 381(1-2), 243-255 (2013).
13. Hviid TV, Rizzo R, Christiansen OB, Melchiorri L, Lindhard A, Baricordi OR. Hla-g and il-10 in serum in relation to hla-g genotype and polymorphisms. *Immunogenetics* 56(3), 135-141 (2004).
14. Rizzo R, Hviid TV, Stignani M *et al*. The hla-g genotype is associated with il-10 levels in activated pbmcs. *Immunogenetics* 57(3-4), 172-181 (2005).
15. Rizzo R, Vercammen M, Van De Velde H, Horn PA, Rebmann V. The importance of hla-g expression in embryos, trophoblast cells, and embryonic stem cells. *Cell Mol Life Sci* 68(3), 341-352 (2011).
16. Chiusolo P, Bellesi S, Piccirillo N *et al*. The role of hla--g 14-bp polymorphism in allo-hsct after short-term course mtx for gvhd prophylaxis. *Bone Marrow Transplant* 47(1), 120-124 (2012).

17. Bortolotti D, Gentili V, Rotola A, Di Luca D, Rizzo R. Implication of hla-g 3' untranslated region polymorphisms in human papillomavirus infection. *Tissue Antigens* 83(2), 113-118 (2014).
18. Jiang Y, Chen S, Jia S *et al*. Association of hla-g 3' utr 14-bp insertion/deletion polymorphism with hepatocellular carcinoma susceptibility in a chinese population. *DNA Cell Biol* 30(12), 1027-1032 (2011).
19. White SR, Loisel DA, Mcconville JF *et al*. Levels of soluble human leukocyte antigen-g are increased in asthmatic airways. *Eur Respir J* 35(4), 925-927 (2010).
20. Melen E, Kho AT, Sharma S *et al*. Expression analysis of asthma candidate genes during human and murine lung development. *Respir Res* 12, 86 (2011).
21. Doring G, Conway SP, Heijerman HG *et al*. Antibiotic therapy against pseudomonas aeruginosa in cystic fibrosis: A european consensus. *Eur Respir J* 16(4), 749-767 (2000).
22. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. *Eur Respir J* 6 Suppl 16, 5-40 (1993).
23. Bergamini G, Di Silvestre D, Mauri P *et al*. Mudpit analysis of released proteins in pseudomonas aeruginosa laboratory and clinical strains in relation to pro-inflammatory effects. *Integr Biol (Camb)* 4(3), 270-279 (2012).
24. Fainardi E, Rizzo R, Melchiorri L *et al*. Soluble hla-g molecules are released as hla-g5 and not as soluble hla-g1 isoforms in csf of patients with relapsing-remitting multiple sclerosis. *J Neuroimmunol* 192(1-2), 219-225 (2007).
25. Rizzo R, Bortolotti D, Fredj NB *et al*. Role of hla-g 14bp deletion/insertion and +3142c>g polymorphisms in the production of shla-g molecules in relapsing-remitting multiple sclerosis. *Hum Immunol* 73(11), 1140-1146 (2012).
26. Joshi A, Perin DP, Gehle A, Nsiah-Kumi PA. Feasibility of using c-reactive protein for point-of-care testing. *Technol Health Care* 21(3), 233-240 (2013).
27. Liang Y, Yeligar SM, Brown LA. Exhaled breath condensate: A promising source for biomarkers of lung disease. *ScientificWorldJournal* 2012, 217518 (2012).
28. Pm Q. Physiological basis of cystic fibrosis: A historical perspective. *Physiol Rev* 79(1), S3–S22 (1999).
29. Onno M, Pangault C, Le Friec G, Guilloux V, Andre P, Fauchet R. Modulation of hla-g antigens expression by human cytomegalovirus: Specific induction in activated macrophages harboring human cytomegalovirus infection. *J Immunol* 164(12), 6426-6434 (2000).
30. Lemaoult J, Rouas-Freiss N, Carosella ED. Immuno-tolerogenic functions of hla-g: Relevance in transplantation and oncology. *Autoimmun Rev* 4(8), 503-509 (2005).
31. Ciornei Cd NA, Beloin C, Fitting C, Caroff M, Ghigo Jm, Cavaillon Jm, Adib-Conquy M. Biofilm-forming pseudomonas aeruginosa bacteria undergo lipopolysaccharide structural modifications and induce enhanced inflammatory cytokine response in human monocytes. *Innate Immun* 16(5), 288-301. (2010).
32. Porto I, Mendes-Junior CT, Felício L, et al. Micrornas targeting the immunomodulatory hla-g gene: A new survey searching for micrornas with potential to regulate hla-g. *Mol Immunol.* 65(2), 230-241 (2015).

Acknowledgements: We thank Alice Fox for revision English language revision. We thank the following personnel at the Cystic Fibrosis Centers of Verona: Drs Marianna Passiu and Ilaria Meneghelli for interacting with local Ethics Committee; Elisa Calcaterra for the organization of sample collection; Nicoletta Vardaro for EBC collection; Dr Gloria Tridello for project design assistance; Patrizia Iansa for collaboration on the clinical database; nurses, CF patients, healthy volunteers; staff of the Molecular Pathology Laboratory of the Azienda Ospedaliera Universitaria Integrata di Verona led by Dr. Maria Stella Graziani, for DNA samples storage. We also thank Iva Previati for technical support, Dr. Giuseppe Bellisola for his advice and Prof. O. Baricordi for stimulating and critical discussions and acknowledge the contribution of the personnel of the Clinical Research Center of CFC that managed the relations with the Ethic Committee of AOUI.

Financial Disclosure: This study was in part supported by Italian Cystic Fibrosis Research Foundation (Grants: FFC #06/2010 adopted by Delegazione FFC di V.C.O. Verbania, AssociazioneTrentina FC Onlus FFC Support Group in memory of Silvia Sommavilla, ConsorzioPromotres.c.r.l., Antonio Guadagnin and son, Alessandra Boccanera; FFC #26/2011, project adopted by Donatori SMS Solidale 2011, Delegazione FFC di Varese, Associazione Trentina FC onlus), FFC #6/2013, project adopted by Delegazione FFC di Minerbe Verona, Delegazione FFC di Imola e Romagna and by Lega Italiana Fibrosi Cistica (Italian Cystic Fibrosis League), Lega Italiana Fibrosi Cistica (Italian Cystic Fibrosis League) through Veneto Branch – Associazione Veneta Lotta contro la Fibrosi Cistica Onlus. The support sources were not involved in study design, collection, analysis and interpretation of data, writing or report submission.

Conflict of interest: The authors have no conflict of interest to declare.

Figure legends

Figure 1.

Panel A: Levels of sHLA-G in plasma samples from 49 CF patients before and after i.v.antibiotic therapy and in 76 control subjects (CTRL). Median values are reported. Panel B: sHLA-G plasma levels in 33 CF patients before (pre) and after (post) i.v. antibiotic therapy subdivided accordingly with 14bp ins/del polymorphism (rs66554220) genotypes (13 for genotype E., 8 for genotype I, 12 for genotype D). Mean ± standard deviations are reported. P values were obtained by Student *t* test.

Figure 2.

Panel A: Levels of sHLA-G in EBC samples from CF patients (n=28) before and after I.V. antibiotic therapy and control subjects (CTRL, n=7). Panel B: Levels of sHLA-G in EBC from 26 CF patients before and after i.v. antibiotic therapy and in control subjects (CTRL, n=7). CF patients were categorized according to the presence (Pa+) (n=10) or absence (Pa-) (n=16) of P. aeruginosa infection. Median values are reported. P values were obtained by Student *t* test.

Figure 3. sHLA-G1 and HLA-G5 protein levels in EBC samples before (pre) and after (post) I.V. therapy. Median values are reported. P values were obtained by Student *t* test.

Figure 4. Panel A: Western Blot analysis of IB3-1 and C38 CM untreated or after *P. aeruginosa* CM exposure. Panel B: Q-PCR for total HLA-G mRNA expression in IB3-1 and C38 cell lines after 10ng/ml *P. aeruginosa* LPS treatment for 12 hrs. Results of Q-PCR analysis shown as relative quantities (RQ) of HLA-G transcripts in IB3-1 and C38 cell lines treated or not with LPS compared to those of JEG-3 positive control cell line (assigned an

arbitrary value of 10); *P* values were obtained by Student *t* test. Means ± standard deviations are reported. Panel Panel C: Flow cytometric analysis HLA-G expression on a representative experiment on IB3-1 and C38 cell lines after LPS exposure. Cells were stained with 87G-Alexa Fluor 488 (Exbio, Praha, CZ) for membrane HLA-G expression. Panel D: sHLA-G levels in IB3-1 and C38 left untreated or after treatment for 6, 12 or 24 hrs with 5ng/ml 10ng/ml of LPS; p values were obtained by Student *t* test. Means ± standard deviations are reported.

Figure 5. Panel A: Representative Q-PCR analysis of HLA-G1 and HLA-G5 isoforms mRNA expression in the indicated cell lines treated for 12 hrs with 10ng/ml LPS. Panel B: sHLA-G1 and HLA-G5 protein levels in the indicated cell lines treated with 10ng/ml LPS for the indicated hours (hrs). Means ± standard deviations are reported.

Figure 6.

Western Blot analysis of BALs from CF (CF) and wild type (WT) mice before and after treatment with LPS from *P. aeruginosa.* SP: murine splenocytes, used as positive control for Qa2 expression. One representative western blotting is shown, below are the means ± SD of the densitometric analysis of four independent experiments. Arbitrary OD values were normalized versus the wild-type control samples set to 100%.

Table 1. Demographic and Clinical Characteristics of CF patients enrolled during exacerbation and control subjects (CTRLs).

Patients N	49	CTRL N	76	p value
Male/Female	22/27	Male/Female	35/41	0.888
Age years	18.0±8.9	Age years	19.0±9.1	0.752
Weight Z score	-1.03±1.32	Weight Z score	-0.85±1.30	0.069
Height Z score	-0.65±1.19	Height Z score	-0.23±0.82	0.042*
BMI Z score	-0.61±1.56	BMI Z score	-0.71±1.42	0.532
Diabetes n (%)	17 (35%)	Diabetes n (%)	0 (0%)	<0.0001*

BMI: body mass index

P values were obtained by Fisher exact test and Student *t* test. * significant p values

Table 2. Clinical conditions of CF Subjects before and after I.V. antibiotic therapy.

	Before	After	p value
Lung function			
FEV1 (%)	51.38 ± 19.68	57.16 ± 22.30	<0.001*
Serum values			
CRP (mg/L)	15.56 ± 7.73	2.06 ± 1.15	<0.001*
Sputum Microbiology			
P. aeruginosa	24/49 (49%)	20/49 (41%)	0.5426†
S. aureus	22/49 (45%)	13/49 (26%)	0.091†

*Wilcoxon signed rank test

†Fisher exact test

FEV1: forced expiratory volume in 1 sec; CRP: C-reactive protein

Table 3. CF patients and control subjects subdivided according to HLA-G 14bp ins/del polymorphism.

Genotype 14bp	del/del n (%)	ins/del n (%)	ins/ins n (%)	p value
CF (192)	93 (47)	72 (38)	27 (15)	0.062*
CTRL (213)	80 (38)	90 (42)	43 (20)	

*Chi squared test (3x2 Table)

Table 4. CF patients subdivided according to HLA-G 14bp ins/del polymorphism.

Genotype	14bp del/del	14bp ins/del	14bp ins/ins	p value
Age years	25.5±11.0	25.5±10.1	24.0±10.2	0.34*
Height Z score	-0.5±2.5	-0.5±1.2	-0.4±1.2	0.25*
Weight Z score	-0.7±1.3	-0.7±1.3	-0.5±1.4	0.18*
BMI Z score	0.2±1.0	0.1±0.9	0.2±1.0	0.80*
FEV1 (% predicted)	73.4±22.8	65.5±29.6	70.8±24.0	0.35*
Chronic *P. aeruginosa* infection, n (%)	49 (53)	19 (26)	6 (22)	0.0005†
Diabetes n (%)	14 (15)	11 (15)	3 (11)	0.85†

* Kruskal Wallis test; †Chi squared test

Figure 1.

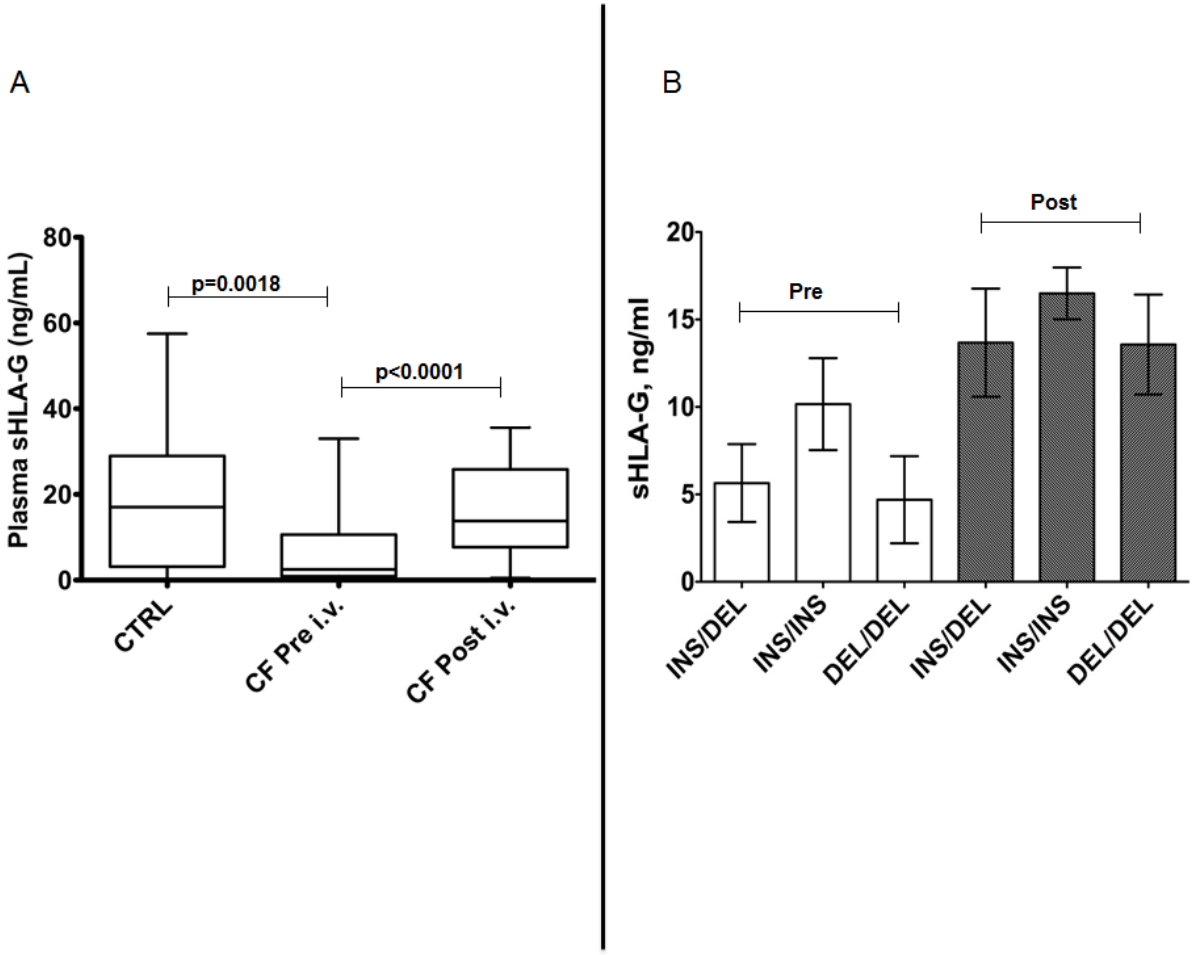

A
Plasma sHLA-G (ng/mL)
80
60
40
20
0
p=0.0018
p<0.0001
CTRL
CF Pre i.v.
CF Post i.v.
B
sHLA-G, ng/ml
20
15
10
5
0
Pre
Post
INS/DEL
INS/INS
DEL/DEL
INS/DEL
INS/INS
DEL/DEL

Figure 2.

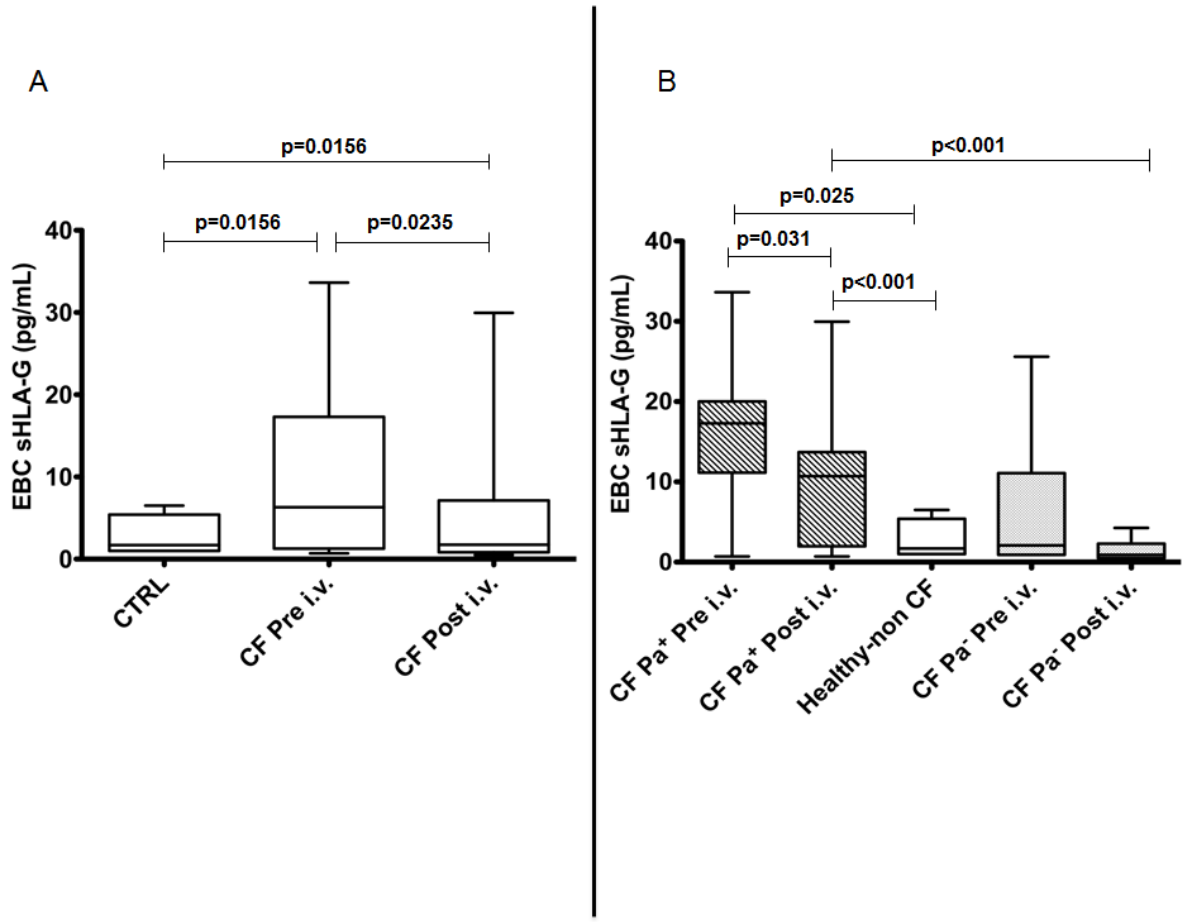

A
p=0.0156
p=0.0156
p=0.0235
EBC sHLA-G (pg/mL)
40
30
20
10
0
CTRL
CF Pre i.v.
CF Post i.v.
B
p<0.001
p=0.025
p=0.031
p<0.001
EBC sHLA-G (pg/mL)
40
30
20
10
0
CF Pa+ Pre i.v.
CF Pa+ Post i.v.
Healthy-non CF
CF Pa- Pre i.v.
CF Pa- Post i.v.

Figure 3.

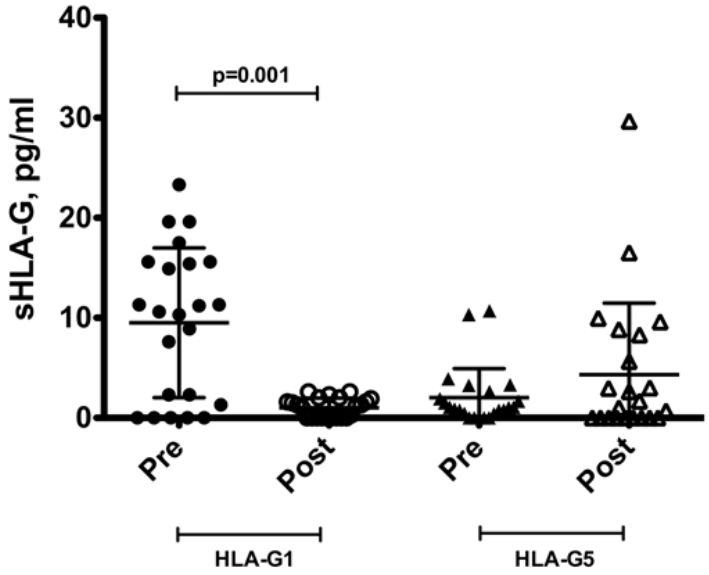


Figure 4.

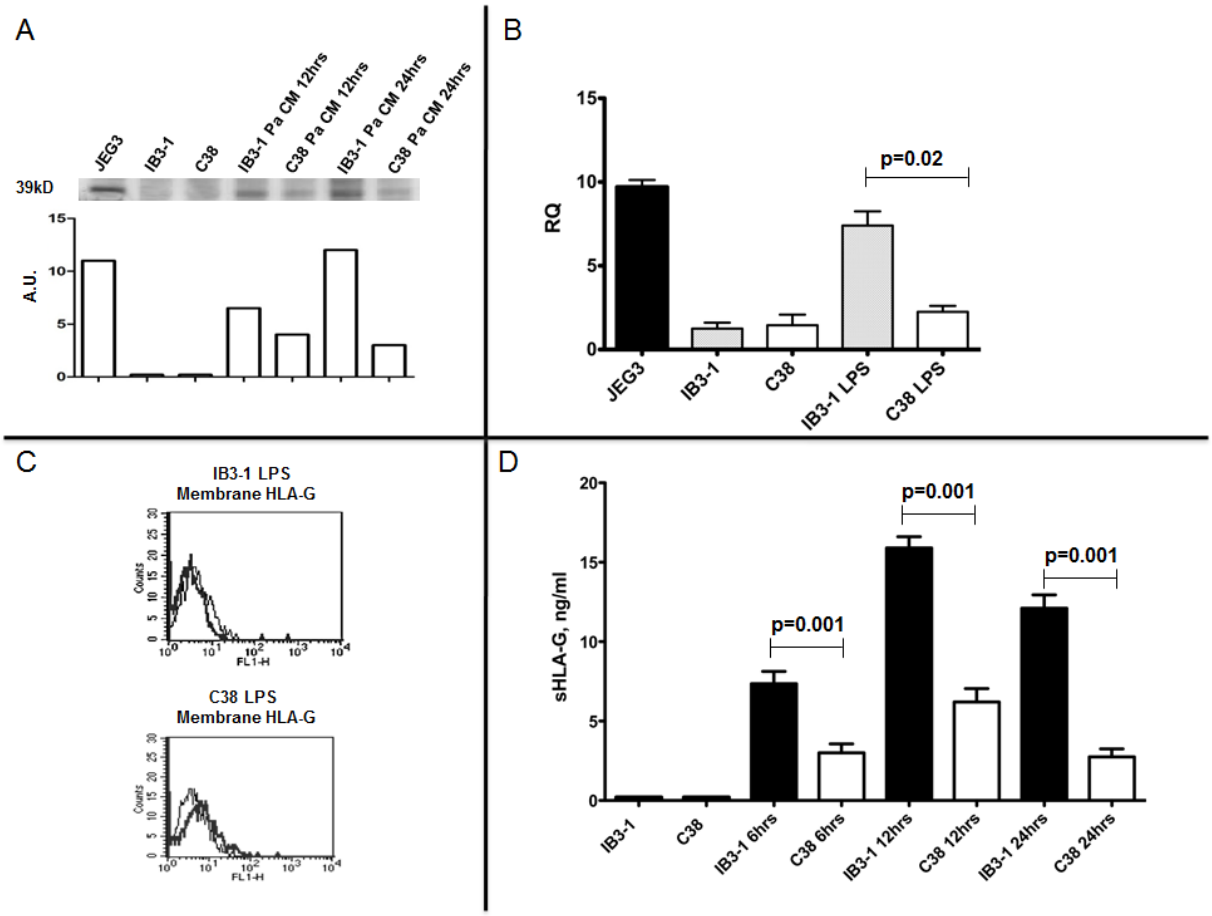

A
JEG3
IB3-1
C38
IB3-1 Pa CM 12hrs
C38 Pa CM 12hrs
IB3-1 Pa CM 24hrs
C38 Pa CM 24hrs
39kD
A.U.
B
RQ
p=0.02
JEG3
IB3-1
C38
IB3-1 LPS
C38 LPS
C
IB3-1 LPS
Membrane HLA-G
C38 LPS
Membrane HLA-G
Counts
FL1-H
D
sHLA-G, ng/ml
p=0.001
p=0.001
p=0.001
IB3-1
C38
IB3-1 6hrs
C38 6hrs
IB3-1 12hrs
C38 12hrs
IB3-1 24hrs
C38 24hrs

Figure 5.

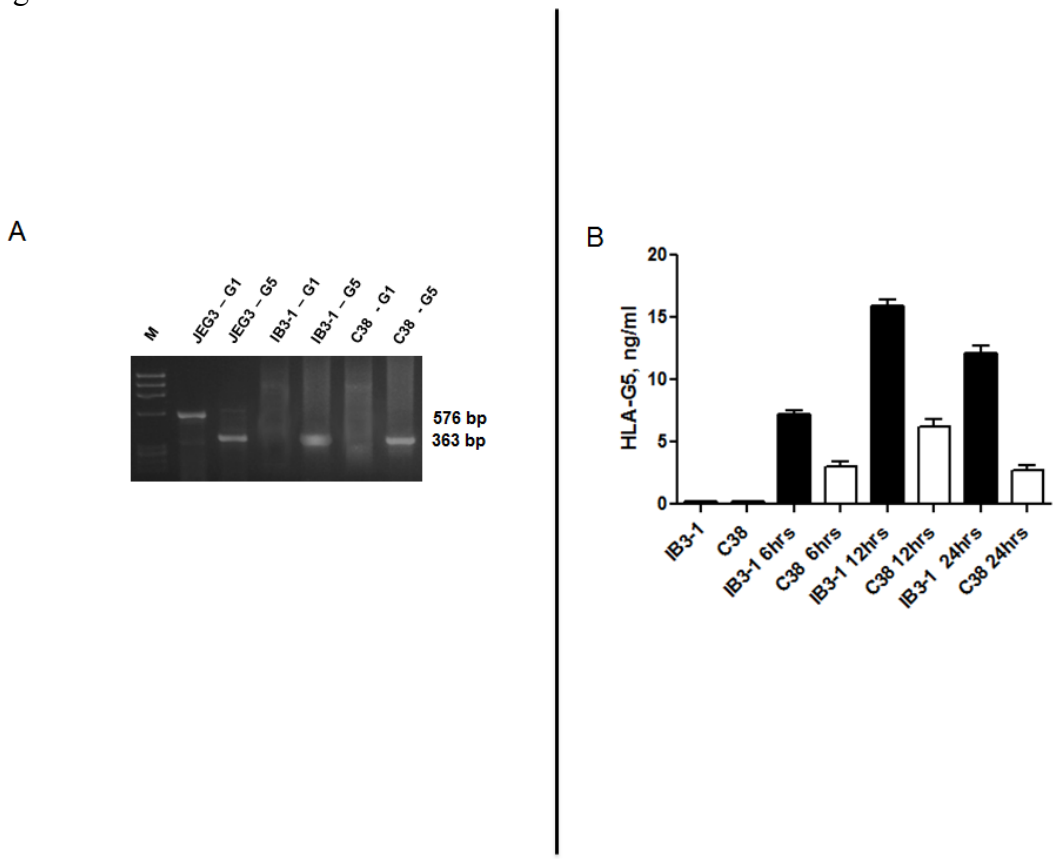
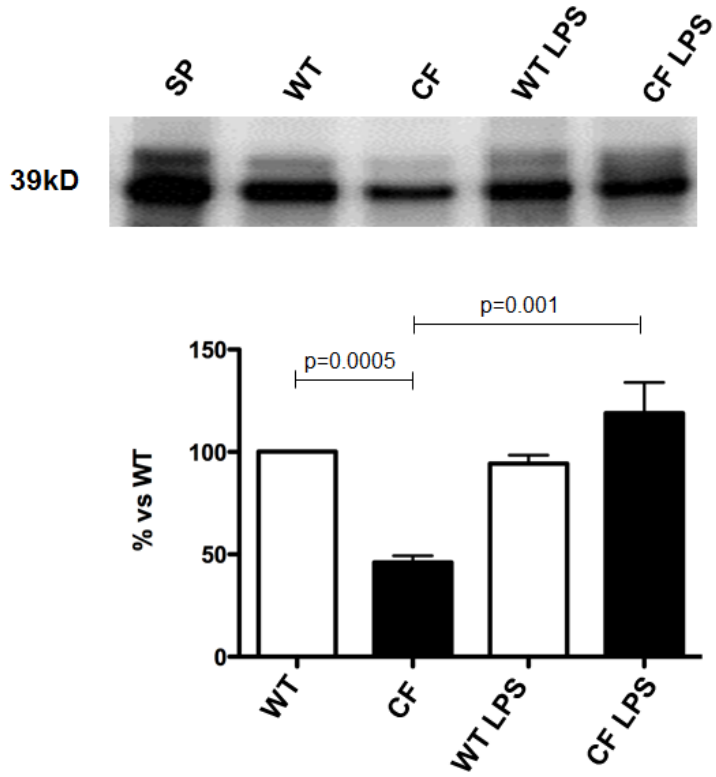

A
M
JEG3 – G1
JEG3 – G5
IB3-1 – G1
IB3-1 – G5
C38 - G1
C38 - G5
576 bp
363 bp
B
HLA-G5, ng/ml
20
15
10
5
0
IB3-1
C38
IB3-1 6hrs
C38 6hrs
IB3-1 12hrs
C38 12hrs
IB3-1 24hrs
C38 24hrs

Figure 6.


SP
WT
CF
WT LPS
CF LPS
39kD
p=0.001
p=0.0005
150
100
50
0
% vs WT
WT
CF
WT LPS
CF LPS