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Holo and apo-transferrins interfere with adherence to abiotic surfaces and with adhesion/invasion to HeLa cells in *Staphylococcus* spp.

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Abstract *Staphylococcus aureus* and *Staphylococcus epidermidis* are the major cause of infections associated with implanted medical devices. Colonization on abiotic and biotic surfaces is often sustained by biofilm forming strains. Human natural defenses can interfere with this virulence factor. We investigated the effect of human apo-transferrin (apo-Tf, the iron-free form of transferrin, Tf) and holo-transferrin (holo-Tf, the iron-saturated form) on biofilm formation by CA-MRSA *S. aureus* USA300 type (ST8-IV) and *S. epidermidis* (a clinical isolate and ATCC 35984 strain). Furthermore *S. aureus* adhesion and invasion assays were performed in a eukaryotic cell line. A strong reduction in biofilm formation with both Tfs was obtained albeit at very different concentrations. In particular, the reduction in biofilm formation was higher with apo-Tf rather than obtained with holo-Tf. Furthermore, while *S. aureus* adhesion to eukaryotic cells was not appreciably affected, their invasion was highly inhibited in the presence of holo-Tf, and partially inhibited by the apo form. Our results suggest that Tfs could be used as antibacterial adjuvant therapy in infection sustained by staphylococci to strongly reduce their virulence related to adhesion and cellular invasion.

Keywords Apotransferrin · Holotransferrin · Biofilm · Cellular invasion · Internalisation

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Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are two of the etiologic agents most commonly isolated from nosocomial and community-associated infections, often persisting through the development of a microbial biofilm (Lindsay and Holden 2004; Benton et al. 2004). Incidences of *S. aureus* and *S. epidermidis* infections are becoming more worrisome with the emergence of multiple-antibiotic-resistant strains such as methicillin-resistant and vancomycin-resistant strains (Conly and Johnston 2002).

The pathogenicity of staphylococci depends on the expression of a variety of virulence factors, either released from the bacteria (e.g., enzymes, exotoxins) or associated with the bacterial cell (e.g. capsular polysaccharides, adhesins). Indwelling medical devices or damaged tissues provide a physiological niche for pathogenic organisms to cause infection. In

fact, implants are rapidly coated from host proteins, such as fibrinogen and fibronectin (Francois et al. 1998), thus becoming prone to bacterial adhesion due to the ability, especially in staphylococci, to bind fibrinogen and fibrin via receptors. Once an implant is colonized and chronic infection develops, implant removal is usually the only intervention capable of resolving the infection.

Both *S. aureus* and *S. epidermidis* produce various microbial surface proteins recognizing adhesive matrix molecules of eukaryotic cells, as well as other adhesive proteins, which enable bacteria to bind different surfaces moieties. Among them fibrinogen, collagen and elastin adhesins (fnbA-B, clfA-B, cna, ebpS), sialoprotein binding proteins (bbp, sdrC, sdrE), extracellular matrix binding proteins (map, eap, sasG), biofilm associated proteins (bap) and adhesins/autolysins (atl) were identified. Although biofilm formation in staphylococci typically relies on the production of extracellular polysaccharide adhesin (PIA/PNAG), strains that are able to produce a protein-dependent biofilm were also identified (Götz, 2002; Rohde et al. 2007; O'Gara 2007; Vergara-Irigaray et al. 2009).

Recently, some host-pathogen interaction studies in various fish species and in swine led to the identification of innate immuno factors important to select for resistance to Gram-negative infections. These studies have pointed the attention on transferrin (Tf) as a candidate gene for disease resistance (Das et al. 2011; Peatman et al. 2007; Raida and Buchmann 2009; Danilowicz et al. 2010). Thus, Tf has come to forefront as an interesting first line of defence against bacterial infections.

Tf is a monomeric glycoprotein structurally divided in two lobes, each capable of binding one iron III ion. The iron depleted form is named apo-Tf while the iron loaded holo-Tf (collectively referred as Tfs). In healthy individuals, blood stream free ionic iron levels are kept very low (10^{-18} M), mainly by the action of two iron-binding proteins: Lactoferrin (Lf) and Tf. The latter (2–4 mg/ml range) is more abundant by about 2,000-fold than the former (0.02–1.5 μg/ml) (Adlerova et al. 2008). In physiological conditions Tf and Lf are 30–40% iron-saturated, with an affinity constant of 10^{36} M^{-1}. Therefore, Tf and Lf are able to capture virtually all the circulating free iron (Bullen et al. 2005).

Tf has been shown to exert both a bacteriostatic and bactericidal effect in vitro on a variety of microbial pathogens (Heine et al. 1996; Oftung et al. 1999; Vardhan et al. 2009; Rooijakkers et al. 2010). The antimicrobial activity has been linked to the iron free or apo-Tf, however, evidence from a number of investigations, using human sera as well as purified proteins, indicated that the antimicrobial mechanism of Tf is more complex than simple nutritional iron deprivation (Modun et al. 1998; Ardehali et al. 2002, 2003; von Bonsdorff et al. 2003). Both Gram-negative and Gram-positive bacteria in fact have developed scavenging mechanisms which specifically target iron withholding host proteins, such as Tf (Griffiths and Williams 1999). In *S. aureus*, some of the host cell actors in the Tf recognition, have been identified: holo-Tf is recognized by the cell wall protein StbA whose expression is regulated by iron level (Taylor and Heinrichs 2002). More recent investigations have shown that staphylococcal surface proteins such as IsdA can also bind Tf, and it is reasonable to think that other NEAr Transporters (NEAT) domains used by bacteria to scavenge iron from host proteins could be implied (Clarke and Foster 2008). Moreover, it is known that in Gram-negative bacteria apo-Tf can damage the outer membrane causing the release of lipopolysaccharide (Ellison et al. 1988, 1990). Interestingly, was recently shown that in *Pseudomonas aeruginosa* respiring cells both Lf and Tf induced bacterial cell death interfering with H^+ translocation (Andrés and Fierro 2010).

Apo-Tf also possesses an inhibitory effect on the adhesion of *S. aureus* and *S. epidermidis* on biomaterials, and that this inhibitory activity has no direct relation with its ability of iron withholding (Ardehali et al. 2002). While the effect of Tf on the adhesion on abiotic biomaterials of Gram-positive bacteria has been previously explored, to the best of our knowledge direct evidence of the Tf effect on the adhesion and internalization mechanism in host cells is still lacking.

In the present study by using both pure holo and apo-Tfs we aim to verify their capacity of inhibition of the adhesion of four staphyloccal strains, namely two each of *S. aureus* and *S. epidermidis*, in planktonic and sessile phase. Then we investigate, for the first time, the effect of Tf, in both apo and holo forms, on the adhesion/invasion ability of *S. aureus* 6538P and USA300 into HeLa cells. Furthermore we present direct evidence of surface proteome expression modulation induced by the action of the apo/holo-Tf binding, and attempt to rationalize our data with alterations induced in the proteome pathway.

Materials and methods

Bacterial strains and culture conditions

Staphylococcus aureus USA300 (clinical strain), *S. aureus* 6538P (DSMZ346, reference strain), *S. epidermidis* O-47 (clinical strain), and *S. epidermidis* RP62A (ATCC35984, reference strain) were used. Tryptic Soy broth (TSB, Oxoid, UK) supplemented with 1% D-glucose was used for biofilm formation in static chamber system at 37°C (TSB-glu). Planktonic cultures were grown in TSB without D-glucose under vigorous agitation (180 rpm).

Cells

HeLa cells were cultured in minimal essential medium with Earle's Salts (MEM/EBSS), supplemented with 10% foetal calf serum (FCS), 1% glutamine and 1% penicillin streptomycin in an atmosphere of 95% air and 5% CO_2. All media were from Euroclone. All incubations were carried out in a 5% CO_2 atmosphere at 37°C. Monolayers were used 48 h after seeding.

Chemicals

Lyophilized human apo and holo-Tfs were obtained both from Kedrion S.p.A., Italy, and from Sigma-Aldrich S.p.A., Italy. Tfs were dissolved in phosphate-buffered saline pH 7.2 (PBS) at a stock concentration of 100 mg/ml and stored at −20°C. All other reagents and solvents were of the highest purity available from Sigma-Aldrich S.p.A., Italy.

MIC assay

MICs of transferrins were determined in snap-cap polystyrene tubes measuring the optical density at the wavelength of 600 nm (OD_{600}). TSB broth was added to each tube and Tfs were added to the first tube and serially diluted (1:2 dilutions). Logarithmic-phase cultures of staphylococci were added to each tube to achieve 10^6 CFU/well. The inoculated tubes were incubated on rotary shaker at 37°C and OD_{600} was recorded after 1 and 24 h. The MICs were defined as the lowest concentrations of transferrins that completely inhibited growth.

Quantification of biofilm formation

Quantification of in vitro biofilm production was based on the method described by Christensen (1985). The wells of a sterile 96-well flat-bottomed polystyrene plate (Falcon) were filled with 90 µl of the appropriate medium containing or not containing Tf concentration ranging 1.0 mg/ml–0.5 µg/ml and 18 mg/ml–8.0 µg/ml for apo and holo-Tf, respectively. 10 µl of overnight bacterial cultures grown in TSB-glu was added into each well. The plates were incubated aerobically with or without the assayed molecules for 24 h at 37°C under static condition. Growth was monitored by measuring the OD_{600}, and after 24 h incubation the ability of the *S. aureus* strain to adhere the polystyrene plates was tested. The content of the plates was then poured off and the wells washed with sterile distilled water. The plates were then stained with crystal violet 0.1% for 5 min. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 20% (v/v) acetone and 80% (v/v) ethanol per well. The OD of each well was measured at 590 nm. Based on the classification reported by Cafiso and co-workers (2007), all strains used in this study were classified as strong biofilm producers ($OD_{590} > 0.4$).

Assay on mature biofilm was also performed. The wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 µl of 1/10 diluted overnight bacterial cultures. The plates were aerobically incubated for 24 h at 37°C. After 24 h the content of the plates was poured off and the wells washed with sterile distilled water to remove the unattached bacteria. The remaining attached bacteria were treated with 100 µl of the appropriate medium containing or not containing 5 mg/ml of Tfs and aerobically incubated for 24 h at 37°C. After 24 h the plates were treated as previously described.

Adhesion-invasion assays (antibiotic protection assays)

Bacteria from 18 h cultures in TSB broth, grown in the absence of Tfs were further subcultured up to $OD_{600} = 0.5$ at 37°C in TSB with or without Tfs at concentrations about threefold higher than the respective biofilm formation Ki value (inhibition constant ~240 and ~3.0 mg/ml for apo and holo-Tf, respectively). HeLa cells were cultured in MEM/EBSS plus

10% FCS in 24-well plates (Falcon) to obtain semi-confluent monolayers (1×10^5 cells/well) and were then inoculated with 0.05 ml of bacterial suspensions in logarithmic-phase growth at a MOI (multiplicity of infection) of about 10 bacteria per cell. The adhesion-invasion assays were carried out in MEM/EBSS plus 10% FCS by infecting cells for 1 h at 37°C at a MOI of about 10 bacteria per cell. Adhesion and invasion were conducted in parallel on twin cellular monolayers. Loosely bound bacteria were removed from the cell monolayers by two washes with PBS. The cells were then lysed with 0.025% Triton X-100 and plated on TSA agar to determine viable bacteria (**A**: cellular adhesion plus invasion). After incubation, to evaluate the infection rates the monolayers were washed with PBS and 0.5 ml of fresh medium containing 200 μg/ml of gentamicin was added to each well and maintained for 1 h at 37°C to kill extra-cellular bacteria. Cells were then lysed by addition of 0.025% Triton X-100 and plated on TSA to count viable intracellular bacteria (**B**: cellular invasion). We further calculated adhesion efficiency by subtraction of **B** to **A**. Adhesion and invasion efficiency were expressed as percentage of the inoculated bacteria that adhered or invaded HeLa cells, respectively.

Surface protein extraction and processing

The surface proteins were extracted as reported. Briefly, after centrifugation of 50 ml of each bacterial culture ($OD_{600} = 0.6$), pellets were washed twice in PBS and then suspended in 500 μl in PBS containing 1% SDS. Samples were incubated at 37°C for 15 min and after centrifugation the supernatants were collected and used for SDS-PAGE and zymogram analyses. The protein content in the samples was determined by the Bradford procedure (Sambrook et al. 1989).

SDS-PAGE and zymogram

SDS-PAGE was carried out by standard methods (Sambrook et al. 1989) with an SDS-polyacrylamide separating gel (10% acrylamide, pH 8.8) and constant voltage (180 V) at room temperature. Following electrophoresis proteins were stained with Coomassie Brilliant Blue (Bio-Rad). Renaturing SDS-PAGE was performed according to the methods of Valence and Lortal (1995), with some modifications. SDS-polyacrylamide separating gel (10% acrylamide, pH 8.8) containing 0.2% (wt/v) lyophilized *Micrococcus luteus* cells provided by Sigma, was used to detect the lytic activities. After electrophoresis, the gels were soaked (2 times, 15 min) in distilled water at room temperature. The gels were then transferred into the renaturing buffer (50 mM Tris–HCl pH 8.0 containing 1% Triton X-100) and shaken at 60 rpm for 2 h at 37°C to allow renaturation. The renatured autolysins appeared as clear translucent bands on opaque background. For each experiment, two gels were prepared from the same stock solution and electrophoresed in the same apparatus at the same time. The presence of *M. luteus* cells in the gel did not evidence differences in the migration of the standards.

Results

Staphylococcal biofilm inhibition by Tfs

We investigated the effect of human apo and holo Tf on biofilm formation by *S. aureus* and *S. epidermidis* by means of a static biofilm assay. First we determined the minimum inhibitory concentrations of both apo-Tf (0.48–0.96 mg/ml) and holo-Tf (>9 mg/ml) to the staphylococcal isolates. Then we tested the ability of the concentrations below the MIC to prevent microbial adhesion on abiotic surfaces. In a preliminary assay biofilm formation was performed in various growth media i.e. TSB supplemented with 1% glucose, MEM/EBSS plus 10% FCS and Dulbecco's Modified Eagle's Medium (High glucose). Then we tested the effect of apo and holo-Tf in these media. No differences in biofilm formation were observed nor in *S. aureus* or *S. epidermidis* (data not shown). Further experiments were performed in TSB supplemented with 1% glucose.

In Fig. 1a–d are shown the inhibition of the adhesion results for both apo and holo-Tf on all considered strains. We found that adhesion of all the strains was inhibited by the apo-Tf, although at different concentrations. The *S. aureus* biofilm formation was 50% inhibited at about 50 and 65 μg/ml, respectively, for 6538P and USA300. The *S. epidermidis* biofilm was inhibited at higher concentrations, namely at 95 and 250 μg/ml for RP62A and O-47, respectively.

The holo-Tf also inhibited biofilm formation in all the strains, however, at very high concentrations. The

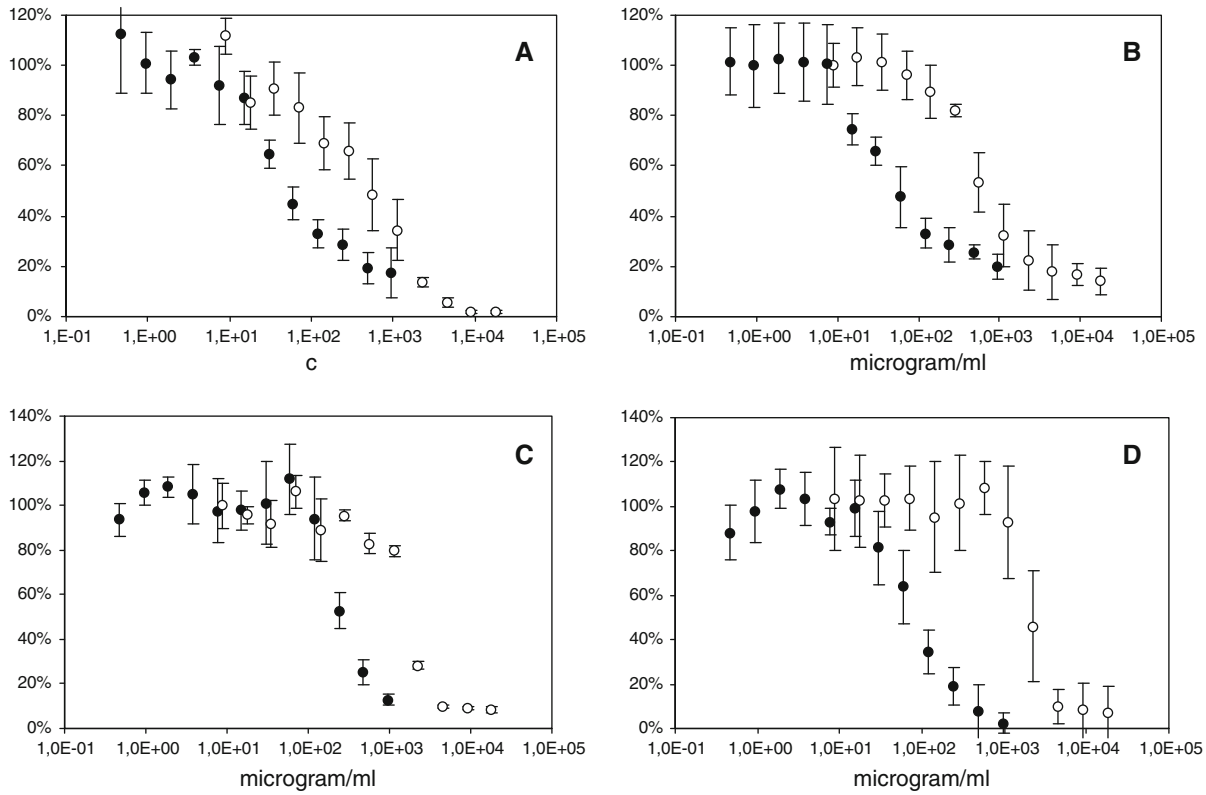

Fig. 1 **a–d** Biofilm formation inhibition by apo and holo-transferrin. Biofilm formation by *S. aureus* **a** 6538P, **b** USA300; by *S. epidermidis*, **c** O-47, and **d** RP62A. In the presence of apo-Tf (*black circles*) and holo-Tf (*empty circles*). *Error bars* are given by standard deviations of four different replicates

S. aureus biofilm formation was 50% inhibited at about 550 and 600 μg/ml for 6538P and USA300, respectively. The *S. epidermidis* biofilm was inhibited at higher concentrations, namely at about 2.0 and 2.5 mg/ml for RP62A and O-47, respectively. Denaturated transferrins were used as control showing any effect on biofilm formation.

Effect of Tfs on *S. aureus* adhesion and invasion of HeLa cells

In Table 1 are shown the results on HeLa cell adhesion and invasion inhibition tests both for apo and holo-Tf. These assays measured adherence by calculating the number of bacteria that, following washing steps, remained adherent to cells incubated with 240 μg/ml and 3.0 mg/ml for apo and holo-Tf, respectively. We did not know if the concentrations, at which an adhesion effect was found on abiotic surfaces, were also effective in inhibiting adhesion to cells. Then we decided to perform the experiments at Tf concentrations which were about threefold above the inhibition threshold previously found for the abiotic surface adhesion experiments, since we wanted to inhibit a conspicuous population of bacteria so that an effect (if any) could be reasonably detected. The invasion assay was performed by infecting HeLa cells with *S. aureus* (either treated with above mentioned concentrations of Tfs or untreated), incubating for 1 h, then half of the samples were treated with gentamicin. The invasion efficiency was calculated by dividing the number of intracellular bacteria in gentamicin treated samples divided by the total number of bacteria in untreated samples.

As one can see in Table 1, apo-Tf effect on both *S. aureus* 6538P and USA 300 is to moderately enhance the adhesion to HeLa cells, by about 30 and 10%, respectively. The effect of holo-Tf is exactly opposite on 6538P and USA 300: while it acts inhibiting the adhesion by about 30% on the former, it increases the adhesion by about the same amount on the latter.

Table 1 Adhesion and invasion assays

	Adhesion			Invasion		
	CTRL (%)	Apo-Tf (%)	Holo-Tf (%)	CTRL (%)	Apo-Tf (%)	Holo-Tf (%)
S. aureus 6538P	100	129 ± 12	73 ± 10	100	42 ± 16	11 ± 8
S. aureus USA300	100	112 ± 10	132 ± 6	100	78 ± 12	31 ± 7

The effect on invasion is more uniform, in fact both apo and holo-Tf inhibit invasion although with different efficacy. Apo-Tf is more effectively attenuating the invasion in HeLa cells by 6538P than by USA300, 42 and 78% invasion is found respectively. The strongest inhibition is found for the holo-Tf which reduces HeLa cell invasion by 6538P to a minimum 11% versus the 31% by USA300. Denatured transferrins did not influenced nor adhesion or invasion of HeLa cells.

Analysis of surface protein pattern following the treatment with Tfs

Lastly, we sought to see if the effect of a reduced invasivity and biofilm adherence inhibition was due to a differential surface protein expression pattern induced by incubation with Tfs. As shown in Fig. 2a, the SDS-PAGE profile shows differences due to the incubation with 240 μg/ml apo and 3.0 mg holo-Tf. Specifically in the region of MW between 50 and 75 kDa. Following, we performed a zymogram analysis to verify if a change in protein profile corresponded to a change in lytic activity. While incubation with apo-Tf did not show any difference (data not shown), the sample incubated with holo-Tf showed marked differences with respect to the control specifically at MW between 75 and 100 kDa and around 37 and 50 kDa (Fig. 2b). These differences evidence a different lytic activity by bacterial autolysins.

Discussion

In the past two decades a number of studies have evidenced that human plasma/sera possessed intrinsic defenses against the growth of microbial pathogens, and Tf was identified among the molecules responsible for this action (Heine et al. 1996; Ardehali et al. 2003; von Bonsdorff et al. 2003; Rooijakkers et al. 2010). The attention then had been shifted towards the understanding of the opportunistic mechanisms developed by microorganisms to take advantage of the Tf as

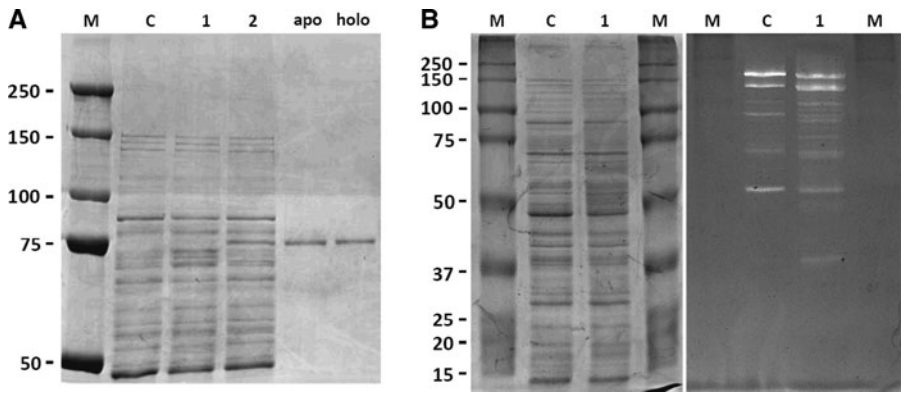

Fig. 2 Electrophoretic analysis of *S. aureus* 6538P surface protein extracts. **a**: SDS-PAGE; cells were grown in the presence of 4 mg/ml of apo-Tf-K or 4 mg/ml of holo-Tf-K. *M* molecular weight marker. *C* Non treated culture; *1* apo-Tf-K treated culture; *2* holo-Tf-K treated culture; **b**: Zymography (*right*) compared to a simultaneous SDS-PAGE (*left*); *M* molecular weight marker; *C* Non treated culture; *1* holo-Tf-K treated culture. Both in SDS-PAGE and zymography, additional bands with apparent mobility different from that of transferrins are visible in treated samples with respect to untreated controls. No differences were noted in the zymographic pattern between untreated control and apo-Tf-K treated culture

an essential iron source for bacterial growth (Alford et al. 1991; Modun et al. 1998, 2000; Taylor and Heinrichs 2002). Recently Tf is experiencing a renaissance of attention by microbiologists, especially in the veterinary field, for its revealed importance in innate immune response to microbial infections (Sahoo et al. 2008; Danilowicz et al. 2010; Das et al. 2011). Apparently Tf may have both a positive and a negative modulating role for microorganisms, depending on the particular specie and situation.

In the present study, our aim was to determine whether Tfs were able to interfere with microbial adherence of *S. aureus* and *S. epidermidis* to abiotic surfaces and to eukaryotic host cell.

While apo-Tf was effective at reducing biofilms of both species at very low concentrations (<200 μg/ml), the reduction seen by *S. aureus* was remarkably more pronounced than in *S. epidermidis* (see Fig. 1a–d). In contrast to other studies that found that only apo-Tf had a role in inhibition of binding (Benson et al. 1996; Ardehali et al. 2002), we find that also holo-Tf exerts an inhibition albeit at much higher concentrations, namely about at 20fold higher concentration than the corresponding action of apo-Tf. In the above mentioned studies the authors did not explore concentrations beyond the 100 μg/ml, and since the effect is apparent only at a much higher dosage, they could not reveal any inhibition. Thus, the surface adhesion inhibiting action that we find is actually at concentrations that are quite comparable with the physiological ones (about 3 mg/ml), well accounting then for the previous observations by several groups of the inhibiting action of plasma on the adhesion of bacteria on different abiotic surfaces (Ardehali et al. 2003; von Bonsdorff et al. 2003).

The presence of apo-Tf contributed to the adhesion of bacteria to HeLa cell, increasing this capacity of about 30 and 10%, respectively, for *S. aureus* 6538P and USA300 (Table 1). On the other hand the holo-Tf was inhibing the adhesion of 6538P by about 30% while was favouring of the same amount the adhesion of USA300.

S. aureus expresses numerous surface proteins that are involved in adherence to host proteins including fibrinogen, fibronectin, collagen and Fc domains of IgG molecules (Foster and Höök 1998). Furthermore, *S. aureus* is able to bind Tf by means of a cell wall-anchored protein, namely StbA, which is also rather abundant (Modun et al. 1994; Taylor and Heinrichs 2002). The direct binding of Tf would thus change the bacterial surface and thus the host cell ability to detect it. On the other side, the host cell possesses a specific Tf receptor. To date it is not clear if the binding of Tf to bacterial StbA would impair its recognition by the host cell Tf receptor. It is possible that bacterial binding of Tfs then produces a synergic action with bacterial adhesins, thus increasing the percentage of adhesion if compared to the control. All our data could be accounted by this effect except for 6538P behaviour, for which a reduction in adhesion is observed with the holo-Tf. We do not have a clear suggestion for this effect, it is however, possible in this case, that the high concentration of holo-Tf used may interfere negatively with the action of the bacterial adhesins resulting in a reduction of the adhesion property. More in general, the results we find somewhat recall the behaviour of the Gram-negative *Neisseria gonorrhoeae* in which the addiction of Tfs significantly increase the adherence of bacteria to human endometrial cells (Heine et al. 1996).

An important feature among host-pathogen interactions is the ability of the pathogen to invade and multiply within the host. The clarification that the availability of iron is a crucial point for this step has been achieved (Bullen and Griffiths 1999). However, iron availability in the host environment is extremely limiting. To overcome this limitation *S. aureus* has developed a very complex mechanism to access iron, kidnapping it directly from Tf (Beasley et al. 2011).

The response to the invasion tests was that Tfs inhibit the invasion process, although with different efficacy. The apo-Tf was actually less effective to inhibit the invasion than the holo-Tf, and the strain 6538P demonstrated more sensitive to inhibition than USA300. The most striking inhibition to invasion (~90% inhibition) was shown by 6538P treated with the holo-Tf. Once the adhesion has occurred, the internalization would proceed through the specific triggering recognition signals (i.e. specific protein–protein interactions). However, can be inferred that a consequence of high concentration of Tf is the saturation of bacterial Tf receptors, this complex then may mask the internalization recognition sites by sterical hindrance. If this is the case, this mechanism would be more effective as the concentration of the bound Tf is raised, and thus, considering the concentrations used in the present study, inhibition should be more evident in the experiment with the holo-Tf than

with the apo-Tf. Moreover, while it has been clearly seen that the bacterial Tf receptors are indeed able to bind efficiently holo-Tf, it is still not clear if the apo form is able to bind and with which efficiency.

Both forms of Tfs alter the membrane/cell wall protein composition and this may be the cause of the altered biofilm formation and reduced invasion. Moreover, holo-Tf exerts an effect also on *S. aureus* autolytic pattern. It has been postulated that the autolysins play an important role in the primary attachment phase of biofilm development (Heilmann 2011). Recently, a novel mechanism involved in staphylococcal internalization by host cells, which is mediated by the major autolysin/adhesins Atl from *S. aureus* was reported (Hirschhausen et al. 2010). However, identification of these proteins, knocking them out via deletion mutation, and measuring an equal reduction in invasion would have to be performed to confirm this link, these preliminary results point toward a direction for further study.

The results of this study suggest that both apo-Tf and holo-Tf could be used as anti-infective molecules in infection sustained by staphylococci to strongly reduce their virulence property related to adhesion and invasion. We found that holo-Tf does play a major role, particularly with the clinically relevant strain of the pathogen *S. aureus.*

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