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# Bi-component Staphylococcal Leukotoxins Induce Chloride Ions Fluxes in Human Neutrophils: Opening of $Ca^{2+}$-activated $Cl^-$ Channels

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**Abstract:** The bi-component leukotoxins; γ-hemolysin and Panton and Valentin Leukocidin (PVL) from *Staphylococcus aureus* induce two independent cellular events *1)* the formation of trans-membrane pores not permeable to chloride ($Cl^-$) ions and *2)* the activation of at least, two modes of chloride fluxes (efflux/influx), including pre-existing $Ca^{2+}$-activated $Cl^-$ channels (CaCC) in human polymorphonuclear neutrophils (PMNs). This was investigated by using spectrofluorometry techniques and the chloride-sensitive quencher fluorescent indicator, MQAE (N-(6-methoxyquinolyl) acetoacethyl ester). The ethidium bromide was used as an indicator for the trans-membrane pores formation by staphylococcal leukotoxins. In the absence of extracellular $Ca^{2+}$, HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV leukotoxins from *S. aureus* induced a massive efflux of chloride ($Cl^-$) ions. Interestingly, in the presence of extracellular $Ca^{2+}$, the HlgA/HlgB γ-hemolysin provoked a biphasic response of $Cl^-$ movements (efflux/influx). Conversely to HlgA/HlgB and LukS-PV/LukF-PV, HlgC/HlgB leukotoxins did not induce any $Cl^-$ movement under this condition (e.g. in the presence of extracellular $Ca^{2+}$). The potent $Cl^-$ channel inhibitor, DIDS, did inhibit significantly the $Cl^-$ fluxes caused by all pairs of staphylococcal leukotoxins tested in both conditions. In the present study, we found that the inhibitory effect of flufenamic acid, known as a $Cl^-$ channel inhibitor, was restricted only to the $Ca^{2+}$-dependent $Cl^-$ influx triggered only by HlgA/HlgB and LukS-PV/LukF-PV leukotoxins. These findings might suggest that, $Cl^-$ fluxes in human neutrophils did involve at least, two different types of $Cl^-$ pathways, depending on the absence or presence of extracellular $Ca^{2+}$. Both $Cl^-$ channels blockers, DIDS and flufenamic acid did not alter the pores formation by staphylococcal leukotoxins. Furthermore, under conditions when the membrane pores formation was blocked by divalent ions ($Ca^{2+}$ and/or $Zn^{2+}$), $Cl^-$ ions movements were still observed. Taken together, our results strongly provide an evidence that: *i)* trans-membrane pores formed by staphylococcal leukotoxins: HlgA/HlgB, HlgC/HlgB (γ-hemolysin) and LukS-PV/LukF-PV (PVL) do not drive $Cl^-$ ions fluxes *ii)* at least, two different types of $Cl^-$ ions pathways are activated, depending on the absence or presence of extracellular $Ca^{2+}$, including $Ca^{2+}$-activated $Cl^-$ channels (CaCC) and, *iii)* $Ca^{2+}$-activated $Cl^-$ channels are mediated only by HlgA/HlgB and LukS-PV/LukF-PV leukotoxins.

**Keywords:** Pore-forming Toxin, *S. aureus*, Leukotoxin, $Cl^-$ channels, γ-hemolysin, Panton-Valentin Leukocidin, Neutrophils, Spectrofluorometry

## 1. Introduction

*S. aureus* secretes a family of bi-component pore-forming leukotoxins whose components belong either to class S or classs F [1]. The Panton-Valentine Leukocidin (class S: LukS-PV; class F: LukF-PV) is secreted by clinical strains associated with furuncles [2-3], and forms one toxic pair LukS-PV/LukF-PV. The γ-hemolysin (class S: HlgA, HgC; class F: HlgB) which forms two toxic pairs HlgA/HlgB and HlgC/HlgB, is secreted by all clinical strains [4]. It has been

shown that, the class S component binding was a pre-requisite to binding of the class F component [5-6]. Both components (S/F) synergistically induce the opening of pre-existing $Ca^{2+}$ channels [7] and then form pores through the membrane of human neutrophils [8]. These membrane pores result from oligomerization of both components (class S/class F) into the membrane and it was proposed from previous studies using synthetic lipidic vesicles that, these two components assembled as heterohexamers [9]. The heterohexamer structure was confirmed by stimulation of the pore construction after solving the crystal structure of LukF-PV [10]. Furthermore, it has been demonstrated that, these membrane pores were not permeable to divalent ions ($Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$) but might be pathways for $Na^+$ and $K^+$ ions [7, 11]. Again, we have previously reported [7, 12] that, bi-component staphylococcal leukotoxins did induce the opening of different types of $Ca^{2+}$ channels, including $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels, as results to a massive internal $Ca^{2+}$ stores depletion. An increase in intracellular $Ca^{2+}$ levels into PMNS might regulate different cellular events and the relevance of such a process, has not been elucidated and remained unclear in the activity of staphylococcal leukotoxins in human neutrophils. A previous study [13], did report that leukotoxins from *S. aureus* were able to control as an early intracellular event in signaling pathways, the activation of the respiratory burst as results to human neutrophils activation, during the immune response against staphylococcal infectious diseases. It is widely known that, chloride ions movements may represent an essential step in the cascade of early intracellular events leading to the human neutrophils pathophysiology. Nevertheless, the impact of staphylococcal leukotoxins on the $Cl^-$ ions permeability in human PMNs has not been explored and a very little is known about the specificity of pores to $Cl^-$ ions.

Chloride movements in human neutrophils may occur through diverse pathways. Thus, several types of $Cl^-$ channels have been reported to be involved in human PMNs activation including, $Ca^{2+}$-activated $Cl^-$ channels [14-15], voltage-dependent $Cl^-$ channels [16], and swelling-activated $Cl^-$ channels [17]. Furthermore, Gallin *et al*. [18] have previously demonstrated that, at least three different types of chloride channels might be activated and which could exhibit differences in their conductance (maxi, medium, small). In this context, attention has been focused in the present research to further investigate whether chloride ions movements through $Cl^-$ channels could be a target during staphylococcal infections, which might probably contribute to cells damage before death.

The objectives of the present study were: *1)* to determine whether the membrane pores formed by leukotoxins were $Cl^-$ anions conductive pathways, *2)* to investigate if pre-existing $Cl^-$ ions pathways were involved in the leukotoxins activity, and *3)* to compare the membrane activity (pores formation and $Cl^-$ ions fluxes) caused by staphylococcal HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV leukotoxins. This was achieved in human neutrophils by using spectrofluorometry techniques and fluorescent probes. The chloride-sensitive fluorescent indicator MQAE was a useful tool to determine the intracellular chloride activity. Interestingly, our data provided evidence that, *i)* membrane pores formed by staphylococcal leukotoxins did not drive $Cl^-$ anions fluxes, *ii)* the time course of leukotoxins-stimulated $Cl^-$ flux differed depending, on the leukotoxins pair tested and *iii)* in the absence of extracellular $Ca^{2+}$, both γ-hemolysin and Panton-Valentin Leukocidin (PVL) leukotoxins induced a massive $Cl^-$ efflux through a specific $Cl^-$ pathway sensitive only to DIDS. Whereas, in the presence of extracellular $Ca^{2+}$, only HlgA/HlgB and LukS-PV/LukF-PV leukotoxins induced the opening of another type of chloride channels, known as *$Ca^{2+}$-activated $Cl^-$ channels* sensitive to DIDS and flufenamic acid, potent blockers of pre-existing $Cl^-$ channels.

# 2. Materials and Methods

### *2.1. Reagents*

N-(6methoxyquinolyl) acetoacethyl ester (MQAE) from Molecular Probes (Eugene, OR) was diluted in $Me_2SO$ to 1 M. J. PREP was from Tech Gen International (Les Ulis, France). Flufenamic acid was from Parke-Davis (Detroit, MI). DIDS, 4, 4'-diisothiocyanato-dihydrostilbene-2, 2,-disulfonic acid and all other reagents were from SIGMA (L'Isle d'Abeau Chesnes, France).

### *2.2. Leukotoxins Purification*

The 5 components of leukotoxins (LukS-PV, LukF-PV, HlgA, HlgC, HlgB) secreted from the *S. aureus* V8 strain (ATCC 49775) kindly provided by S. Thornley (Wellcome Laboratories, London, UK), were purified as described previously [1]. Briefly, they were stored at -80°C before use. In all experiments, class S components (HlgA, HlgC, LukS-PV) were injected at a final concentration 2.2 nM and class F (HlgB, LukF-PV) at 0.85 nM.

### *2.3. Polymorphonuclear Neutrophils Isolation*

Human polymorphonuclear neutrophils (PMNs) were prepared from buffy-coat of healthy donors of either sex, kindly provided by the *Etablissement de Transfusion Sanguine (ETS) - Strasbourg, France*, as previously described [7]. Briefly, human PMNs enriched blood was centrifuged in J. Prep and the pellet was suspended in dextran for sedimentation. Contaminating erythrocytes were removed by an hypotonic lysis. After purification, the PMNs suspension was adjusted to $6.10^6$ cells/ml in the assay medium containing (in mM): 140 NaCl, 5 KCl, 10 glucose, 10 N-2-hydroxyethilpiperazine-N'-2-ethanesulfonic acid, 0.1 ethylene glycol-bis (b-aminoethyl ether)-N, N, N', N'-tetraacetic acid, and 3 Tris-base (pH 7.3). This method led to 98% viable cells as counted after May-Grunwald-Giemsa staining.

### *2.4. MQAE Loading*

MQAE is a chloride ion ($Cl^-$)-quenched fluorescent

indicator considered as a useful tool to measure the dynamic changes in intracellular $Cl^-$ levels [19-20]. It has been found to be a useful fluorescence dye for non-invasive measurements of the intracellular $Cl^-$ concentration. In this study, to investigate the $Cl^-$ movement in human neutrophils, PMNs were incubated with 10 µM MQAE during 60 min in an atmosphere of 95% air/5% $CO_2$ at 37°C in the assay medium. Then, loaded-PMNs were washed twice by centrifugations at 800xg for 10 min to remove the extracellular dye, and were re-suspended in the assay medium at $6\times10^6$ cells/ml.

### *2.5. Fluorescence Determination*

Fluorescence intensity variations of chloride-sensitive indicator MQAE and ethidium bromide were simultaneously recorded with dual excitation and dual emission spectrofluorometer DeltaScan (Bio-Tek Kontron, PTI, St Quentin en Yvelines, France) with slit width set at 4 nm. For fluorescence measurements, one ml of PMNs suspension ($6.10^6$ cells/ml) was added to 1 ml of assay solution continuously stirred in a 4 ml quartz cuvette (1cm light path), thermostated at 37°C.

Relative fluorescence variations of quenched fluorescent indicator MQAE, recorded at $\lambda_{EX}$=360 nm, $\lambda_{EM}$=460 nm, were expressed in all results as arbitrary units (a.u.). An increase in MQAE fluorescence was relative to an efflux of $Cl^-$ ions from human PMNS, whereas a decrease in the MQAE fluorescence intensity during time is proportional to the $Cl^-$ ions influx, indicating an increase in intracellular $Cl^-$ levels into neutrophils.

The fluorescence increase of the ethidium cation (100 µM; $\lambda_{EX}$=340 nm, $\lambda_{EM}$=600 nm) through intercalation in nucleic acids subsequent to the cell penetration, was used as an indicator of the trans-membrane pores formation as described previously [7-8]. The fluorescence intensity obtained at the beginning of the recording was assigned to 0% of pore-formation and the fluorescence obtained by lysing PMNs by 0.2% (w/v) Triton X-100 corresponded to 100% of the pores formation.

For $Ca^{2+}$ experiments, 1.1 mM $CaCl_2$ were added to the PMNs suspension 5 min before measurements in order to obtain 1 mM free $Ca^{2+}$. In some experiments, PMNs were pre-incubated with 500 µM DIDS or 50 µM flufenamic acid during 60 min then, rinsed to avoid fluorescence interferences from these compounds. The auto-fluorescence of PMNs was substracted by the PTI software and the data extracted for transfert to SigmaPlot 4.1 (Jandel, Erckrath, Germany). The experiments described in figures are the most representative of four similar ones.

## 3. Results

### *3.1. HlgA/HlgB Induces $Cl^-$ Fluxes*

Previously, we have demonstrated that staphylococcal leukotoxins did induce the opening of pre-existing $Ca^{2+}$ channels in human neutrophils [7]. Simultaneously, membrane pores were formed which were not permeable to divalent ions. Recently, we have reported that, only in the presence of extracellular $Ca^{2+}$, all leukotoxins pairs tested (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) provoked the opening of $Ca^{2+}$-Release Activated $Ca^{2+}$ (CRAC) channels, as results to the internal $Ca^{2+}$ stores depletion [12]. In this context, we were interested in the present study to investigate an eventual activation of chloride channels by staphylococcal leukotoxins in human neutrophils, as it has previously demonstrated for other agonists [21-22]. In PMNs, the fluorescence intensity of the chloride-sensitive indicator MQAE decreased with increasing of the intracellular $Cl^-$ ions by collisional quenching and was unaffected by pH variations [19, 23]. This property was used to determine the influence of the HlgA/HlgB leukotoxins addition on $Cl^-$ movements in human neutrophils by recordings the fluorescence intensity variations of MQAE as shown in Figure 1.

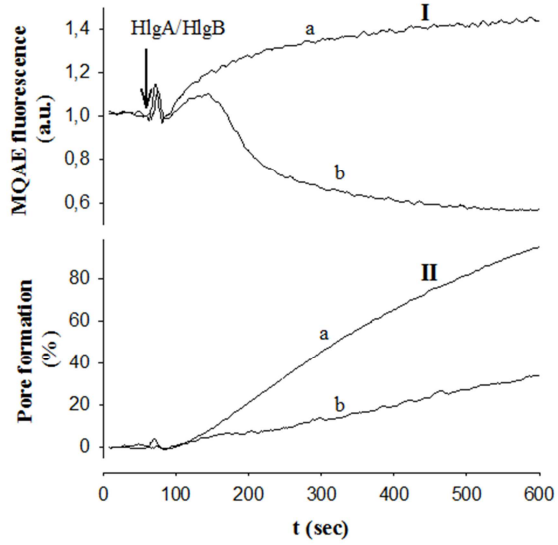



***Figure 1.*** *Effect of the HlgA/HlgB application on (I) MQAE fluorescence variations and (II) membrane pores formation in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular $Ca^{2+}$.*

In the absence of extracellular $Ca^{2+}$, the HlgA/HlgB addition induced a significant increase of the MQAE fluorescence represented as an arbitrary unit (a.u.) (Figure 1 Ia), due to a decrease in intracellular $Cl^-$ level. This event was more likely due to an efflux of $Cl^-$ ions from neutrophils. The simultaneous recording of the ethidium fluorescence intensity indicated an important concomitant pore-formation by leukotoxins (about 95%) (Figure 1IIa). The same experiment was carried out in the presence of 1 mM extracellular $Ca^{2+}$. Under these conditions, the HlgA/HlgB addition resulted in a bi-phasic response. First, an increase of MQAE fluorescence for about 70-80 seconds on average according to the donor, and then decreased during time (Figure 1Ib) as results of an increase in intracellular $Cl^-$ level. This event indicated a dual effect of $Cl^-$ movements, consisting of a small efflux followed by a marked influx of $Cl^-$ ions. Thus, depending on the presence of extracellular $Ca^{2+}$, HlgA/HlgB provoked opposite $Cl^-$ fluxes. In addition, a less important pore-formation (about 35-40%) was observed than, in the absence of extracellular

$Ca^{2+}$ (Figure 1IIb) as described in previous studies [7-8]. However, although in the two $Ca^{2+}$ situations, the pattern of the pore-formation was different, it could not be correlated with $Cl^-$ movements suggesting that, $Cl^-$ ions fluxes probably could involve another conductive chloride pathway.

### *3.2. Impermeability of Pores and Activation of $Cl^-$ Channels*

Previously, it has been confirmed that: *i)* trans-membrane pores formed by staphylococcal leukotoxins were not permeable to divalent ions ($Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$) and *ii)* divalent ions fluxes were driven by the opening of pre-existing $Ca^{2+}$ channels [7]. Similarly, the question arising in the present research was to determine whether the pores formed by leukotoxins were permeable to $Cl^-$ ions or, if $Cl^-$ ions fluxes were driven through pre-existing chloride channels. To answer this question, we attempted to disturb the staphylococcal pore-formation by adding divalent ions ($Ca^{2+}$ and/or $Zn^{2+}$) following the leukotoxins addition as previously described [7]. As shown in Figure 2, in the absence of extracellular $Ca^{2+}$, the HlgA/HlgB addition provoked an important pores formation (about 90%), this event persisted during time (Figure 2Ia).

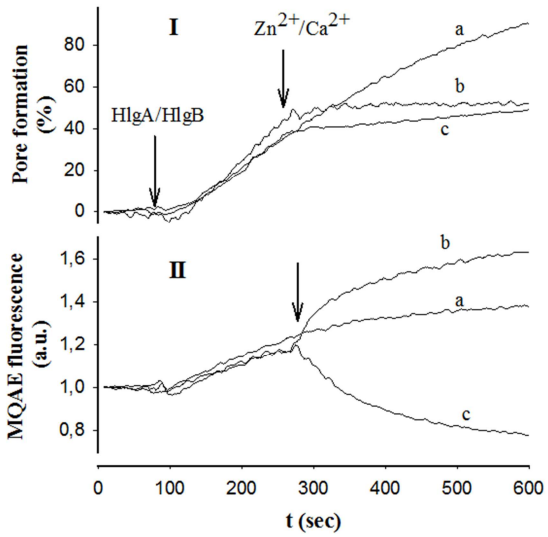



***Figure 2.*** *Effect of 2 mM $Zn^{2+}$ (b) or 1 mM $Ca^{2+}$ (c) addition on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs after the HlgA/HlgB application, in the absence (a) of extracellular $Ca^{2+}$.*

Simultaneously, a significant increase of the MQAE fluorescence was shown (Figure 2IIa), which was likely associated to an efflux of $Cl^-$ ions. Interestingly, the addition of 2 mM $Zn^{2+}$ blocked totally the pore formation (Figure 2Ib) as it has previously shown [7-8], whereas variations in the MQAE fluorescence were still increased and sustained during time (Figure 2 IIb). These results suggested that, the $Cl^-$ ions efflux was not going through the membrane pores, but might use another ions pathway of the PMNs membrane. Furthermore, in another set of experiment, when 1 mM $Ca^{2+}$ was added in the cuvette after the HlgA/HlgB application, the functional pore formation was immediately and significantly reduced (Figure 2Ic). However, conversely to the $Zn^{2+}$ effect, an immediate decrease of MQAE fluorescence was observed (Figure 2IIc), likely due to an activation of new $Cl^-$ ions pathways, which seemed to be a $Ca^{2+}$-dependent process. Our results strongly suggested that, membrane pores formed by HlgA/HlgB were not permeable to $Cl^-$ ions. This was confirmed under conditions where the pores formation was totally abolished by adding 0.1 mM $Ca^{2+}$ and 0.2 mM $Zn^{2+}$ [7]. As shown in Figure 3, although the pores formation was completely inhibited, a significant movement of $Cl^-$ ions was recorded after the ions blockers addition.

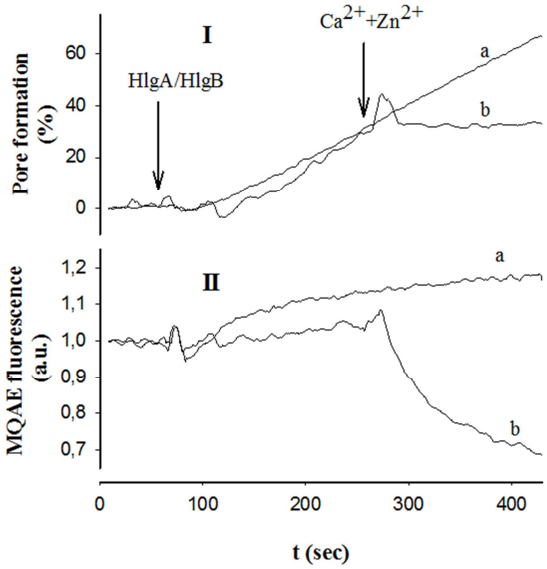



***Figure 3.*** *Effect of 0.1 mM $Ca^{2+}$ and 0.2 mM $Zn^{2+}$ addition (b) on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs after the HlgA/HlgB application, in the absence (a) of extracellular divalent ions.*

These results did confirm two events: *i)* the impermeability of membrane pores to $Cl^-$ ions and *ii)* the $Cl^-$ movement (efflux/influx) did involve at least, two independent pathways, including a $Ca^{2+}$-dependent pathway. It has previously reported that a number of agonists such as ionomycin, could induce the activation of $Ca^{2+}$-dependent $Cl^-$ channels [14, 24]. To further investigate this observation under both conditions, first, we tested the effect of the $Ca^{2+}$ ionophore ionomycin on $Cl^-$ movements in the absence of extracellular $Ca^{2+}$. As observed in Figure 4Ia, under this condition, no chloride movement was recorded, however, when 1 mM $Ca^{2+}$ was injected, a significant decrease of MQAE fluorescence was observed. Thus, activation of PMNs with ionomycin led to an increase in a $Cl^-$ channels activity likely due to a $Ca^{2+}$-dependent $Cl^-$ pathway activation. This later event was abolished (Figure 1Ib) when PMNs were pretreated with a potent inhibitor of chloride channels, DIDS [25]. Similar experiments were performed to verify whether staphylococcal leukotoxins could induce the opening of $Ca^{2+}$-dependent $Cl^-$ channels sensitive to DIDS. Figure 4IIa showed that, in the absence of extracellular $Ca^{2+}$, conversely to the ionomycin, HlgA/HlgB did induce an efflux of $Cl^-$ ions, followed immediately by an influx of $Cl^-$ ions when 1 mM $Ca^{2+}$ was injected. The $Cl^-$ fluxes

(efflux/influx) were totally inhibited when PMNs were pretreated with DIDS, in the absence as well as in the presence of extracellular $Ca^{2+}$ (Figure 4IIb).

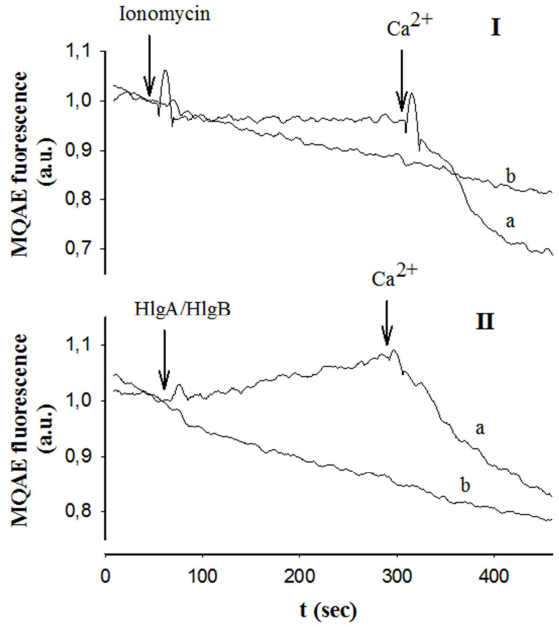
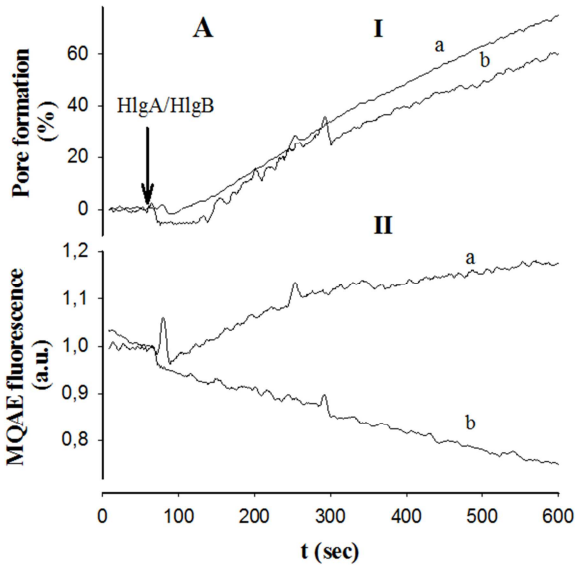



***Figure 4.*** *Effect of the 0.1 µM ionomycin (I) and HlgA/HlgB (II) application on MQAE fluorescence variations in human PMNs, before and after 1 mM $Ca^{2+}$ addition in the absence (a) or presence (b) of DIDS (500 µM, 60 min).*

Consequently, it seemed likely that, the $Cl^-$ movement provoked by HlgA/HlgB in the presence of extracellular $Ca^{2+}$ was likely due to the opening of $Ca^{2+}$-activated $Cl^-$ channels (CaCC). A further evidence was given by Figure 5A, which did show that, in the absence of extracellular $Ca^{2+}$, the $Cl^-$ efflux provoked by HlgA/HlgB was completely inhibited by DIDS but, not by flufenamic acid PMNs pre-treatment, a potent inhibitor of $Ca^{2+}$-activated $Cl^-$ current [26] (Figure 5B) although, the pore-formation was not modified by both inhibitors, ruling out the role of pores in $Cl^-$ ions fluxes.



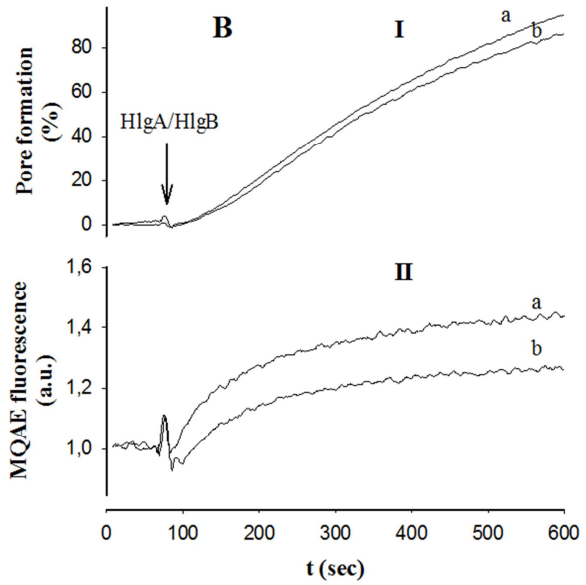




***Figure 5.*** *Effect of the HlgA/HlgB application on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs, in the absence (a) or presence (b) of 500 µM DIDS (A) or 50 µM flufenamic acid (B). [$Ca^{2+}$]=0 mM.*

Again, these observations were further confirmed when another set of experiments were performed on the same donor in the presence of extracellular $Ca^{2+}$. Conversely to in the absence of extracellular $Ca^{2+}$ (Figure 6I), the $Cl^-$ movement induced by HlgA/HlgB in the presence of extracellular $Ca^{2+}$, was completely inhibited by both blockers DIDS and flufenamic acid (Figure 6II). Again, the inhibitory effect of flufenamic acid was not observed in the absence of extracellular $Ca^{2+}$.

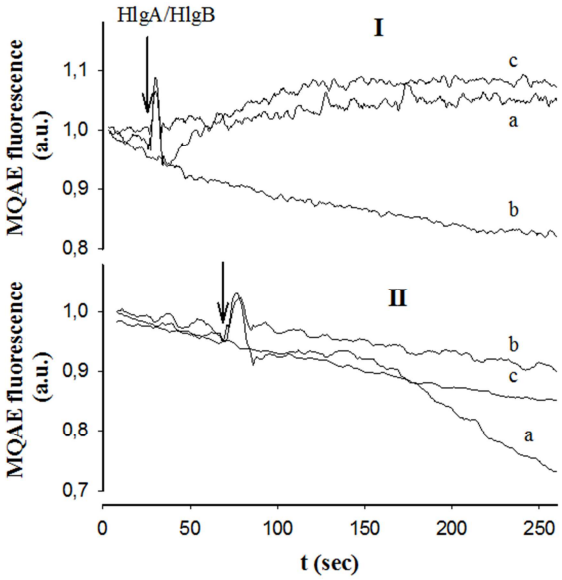



***Figure 6.*** *Effect of the HlgA/HlgB application on MQAE fluorescence variations in human PMNs, in the absence (a) or presence of 500 µM DIDS (b) or 50 µM flufenamic acid (c). Experiments were performed in the absence (I) or presence (II) of 1 mM extracellular $Ca^{2+}$.*

Furthermore, as shown in Figure 7, the $Cl^-$ efflux induced by either LukS-PV/LukF-PV or HlgC/HlgB addition in the

absence of extracellular $Ca^{2+}$, was completely abolished when PMNS were pretreated with the potent $Cl^-$ channels inhibitor, DIDS.

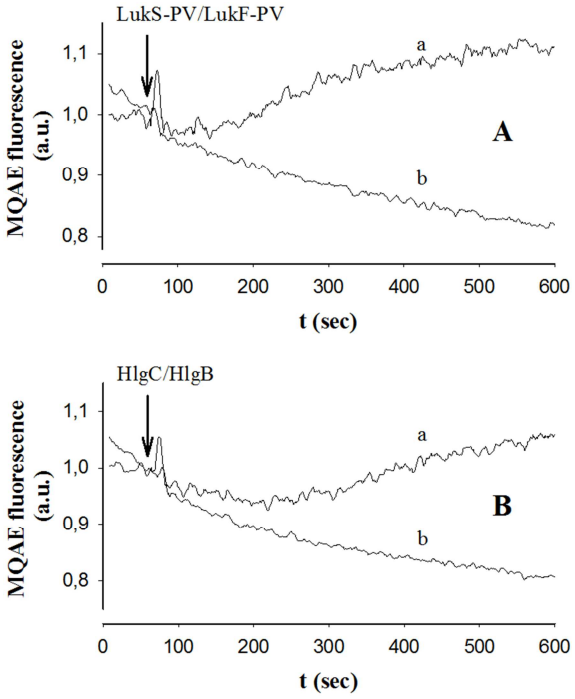



***Figure 7.*** *Effect of the LukS-PV/LukF-PV (A) or HlgC/HlgB (B) application on MQAE fluorescence variations in human PMNs, in the absence (a) or presence of 500 µM DIDS (b). [$Ca^{2+}$]=0 mM.*

The present study strongly suggested that, intracellular variations of $Cl^-$ ions fluxes (efflux/influx) did occur through pre-existing and specific chloride pathways and not through leukotoxins membrane pores. This new evidence was further confirmed by our results obtained with a particular donor in the presence of extracellular $Ca^{2+}$ (Figure 8).

### *3.3. Comparison of HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV Effects*

It was of interest to compare the effect of each pair of staphylococcal leukotoxins on the pore formation and $Cl^-$ movements through the PMNs membrane. As obviously described, in the absence of extracellular $Ca^{2+}$, each pair tested HlgA/HlgB, HlgC/HlgB and LukS-PV/LukF-PV did induce the pore formation. Simultaneously, the same increase in the MQAE fluorescence intensity was recorded during the time, with differences in a time lag after the leukotoxins addition (Figure 7).

As previously confirmed, this event was due to a decrease in intracellular $Cl^-$ level likely associated to an efflux of $Cl^-$ ions from human PMNs. Thus, the three leukotoxins from *S. aureus* tested induced an efflux of $Cl^-$, with different potentialities, under the dependence on extracellular $Ca^{2+}$. In comparison experiments performed in the absence of extracellular $Ca^{2+}$ as shown in Figure 8, either HlgA/HlgB (Figure 8A) or LukS-PV/LukF-PV (Figure 8B) did induce simultaneously, the pores formation and significant efflux of $Cl^-$ ions.

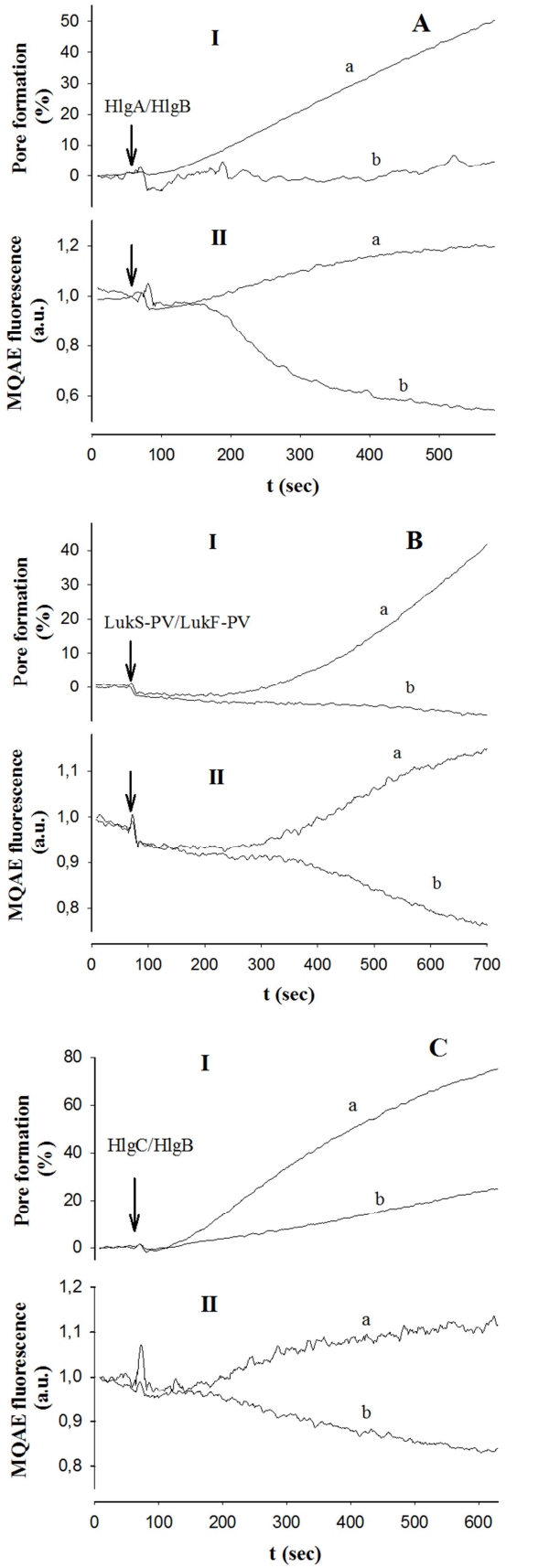



***Figure 8.*** *Effect of the HlgA/HlgB (A), LukS-PV/LukF-PV (B) or HlgC/HlgB (C) application on (I) the membrane pores formation and (II) MQAE fluorescence variations in human PMNs, in the absence (a) or presence (b) of 1 mM extracellular $Ca^{2+}$.*

However, in the presence of extracellular $Ca^{2+}$, an important $Cl^-$ movement was observed although the pores formation by both leukotoxins was abolished in this particular donor. These findings strongly confirmed that, both HlgA/HLgB γ-hemolysin and LukS-PV/LukF-PV leukotoxins did provoke simultaneously two independent cellular events: *1)* the membrane pores formation not permeable to chloride ions and *2)* the opening of specific $Cl^-$ ions pathways, including $Ca^{2+}$-activated $Cl^-$ channels sensitive to both DIDS and flufenamic acid. Nevertheless, we have observed that, in the presence of extracellular $Ca^{2+}$, the HlgC/HlgB combination did not induce an influx of $Cl^-$ ions through $Ca^{2+}$-dependent $Cl^-$ channels, as shown in Figure 8C.

# 4. Discussion

We have previously provided evidence that, bi-component staphylococcal leukotoxins did induce two independent cellular events in human neutrophils: *1)* the opening of pre-existing $Ca^{2+}$ channels and *2)* the formation of trans-membrane pores not permeable to divalent ions ($Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$…) [7]. These membrane pores determined by the ethidium influx seemed to be specific to monovalent ions ($K^+$, $Na^+$) [11]. In a recent report, we have demonstrated that, these pore-forming leukotoxins from *S. aureus* were able to trigger a massive internal $Ca^{2+}$ stores depletion followed by the opening of $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels [12]. Interestingly, staphylococcal leukotoxins were considered as $Ca^{2+}$-channels agonists, they bound to specific membrane receptors [5-6] and as results they were able to activate neutrophils and induced consequently, the oxidative burst activation [13].

It has already been reported in a previous work that, a drastic decrease of intracellular $Cl^-$ level might represent an essential step for the activation of the respiratory burst in human PMNs [22]. This event was thought to play a crucial role in the modulation of several critical neutrophil responses including, activation and up-regulation of adhesion molecules, cell attachment and spreading, cytoplasmic alkalinization and activation of the oxidative burst [27]. Again, the $Cl^-$ efflux has been shown to regulate $\beta_2$ integrin-mediated spreading and activation of the respiratory burst [28]. Since, the chloride ions efflux is an early event, that occurred following the human PMNs activation by several physiological soluble agonists such as, tumor necrosis factor-α (TNF-α) [15, 21] or non-physiological soluble agonists like fMLP, PMA [16], and ionomycin with the concomitant decrease of the intracellular chloride content, we were interested in the present study, to investigate the $Cl^-$ ions movement in staphylococcal bi-component leukotoxins-activated human neutrophils.

A very little was known about an eventual involvement of $Cl^-$ channels in chloride fluxes in the staphylococcal leukotoxins activity. This was explored by using the chloride-sensitive quencher fluorescent indicator MQAE, as a useful tool for determination of the intracellular chloride levels in human neutrophils [29]. First, we tested the effect of HlgA/HlgB γ-hemolysin on the membrane pores formation simultaneously to $Cl^-$ movements in neutrophils. Indeed, we found that, in the absence of extracellular $Ca^{2+}$, a significant increase in the MQAE fluorescence intensity was recorded as results to a sustained $Cl^-$ efflux through the PMNs membrane. This event might lead to a marked decrease in intracellular chloride levels in these phagocytes. As it has previously described that, staphylococcal leukotoxins did not alter the intracellular $Ca^{2+}$ level in the absence of extracellular $Ca^{2+}$ [12], it was expected that in this condition, the $Cl^-$ ions efflux provoked by HlgA/HlgB γ-hemolysin was independent of intracellular $Ca^{2+}$ elevation. Nonetheless, in a previous work, it has been shown that, the $Cl^-$ efflux appeared to be independent on either alterations of $[Ca^{2+}]_i$ or changes in the plasma membrane potential, and was independent on the protein tyrosine phosphorylation and decrease of cAMP levels [28]. Again, the $Cl^-$ efflux triggered by the cross-linking of $\beta_2$-integrin occurred in a $Ca^{2+}$-independent manner. A simultaneous cellular effect of HlgA/HlgB on PMNs was the trans-membrane pores formation (about 90%) which was significantly reduced (about 20%) in the presence of extracellular $Ca^{2+}$ as previously reported [7-8]. However, under these conditions, we have observed a biphasic response of HlgA/HlgB, closely associated to an efflux of $Cl^-$ followed by a sustained influx of chloride ions. This later event seemed to be a $Ca^{2+}$-dependent process. Consequently, we thought that $Cl^-$ fluxes mediated by leukotoxins could be an independent event of the membrane pores formation. We further verified this hypothesis when the formation of pores was altered by adding the ions blockers ($Ca^{2+}$/$Zn^{2+}$) at a given concentration. Interestingly, under these conditions, an opposite $Cl^-$ movement (efflux/influx) was observed, depending on the absence or presence of extracellular $Ca^{2+}$ and conversely to $Zn^{2+}$ ions, only the presence of $Ca^{2+}$ could induce a massive influx of $Cl^-$ ions. This strongly suggested that, membrane pores formed by staphylococcal leukotoxins were not permeable to $Cl^-$ ions. This was again, confirmed by another set of experiment where the formation of membrane pores were completely abolished by 0.1 mM $Ca^{2+}$ and 0.2 mM $Zn^{2+}$. As results, we provided the first evidence that, staphylococcal membrane pores did not drive $Cl^-$ ions. Furthermore, it seemed that, another type of a $Cl^-$ pathway was involved only in the presence of extracellular $Ca^{2+}$, which could be a $Ca^{2+}$-activated $Cl^-$ pathway. Consequently, we proposed that bi-component staphylococcal leukotoxins could induce in addition to $Ca^{2+}$ channels, the opening of specific $Cl^-$ channels in their cellular membrane activity.

The intracellular chloride, as being a major anion in living cells, played a crucial role in maintaining cellular functions in many cell types and all these responses, were inhibited when PMNs were pre-treated with $Cl^-$ channels blockers [25, 30]. For this reason, we tried to test the effect of known potent $Cl^-$ channels inhibitors, DIDS and flufenamic acid on leukotoxin-activated $Cl^-$ fluxes in both conditions. First, we attempted to verify whether the $Ca^{2+}$ ionophore ionomycin could activate the $Cl^-$ flux as it has previously reported [14, 24]. Thus, we found that only in the presence of extracellular $Ca^{2+}$, a significant movement of chloride ions was occurred which

was completely inhibited by the PMNs pretreatment with DIDS, used as a potent $Cl^-$ channels blocker. This effect was compared to the HlgA/HlgB activity. Since both movements (efflux/influx) of chloride ions induced by HlgA/HlgB were totally abolished by DIDS, we proposed that γ-hemolysin was able to activate at least, two types of DIDS-sensitive $Cl^-$ pathways, depending on the absence or presence of extracellular $Ca^{2+}$. Furthermore, these results strongly suggested the opening of $Ca^{2+}$-activated $Cl^-$ channels by staphylococcal leukotoxins, as it has previously reported in neutrophils [14] and which were not found in macrophages.

It is unclear to date whether these pathways correspond to anion exchangers or to $Cl^-$ channels, although the kinetics suggested the opening of $Cl^-$ channels. Moreover, since the anions gradient was the same in every experiment, $Cl^-$ fluxes through an anions exchanger/transporter would be always in the same direction whatever the $Ca^{2+}$ situation. Furthermore, since the cell suspension was rinsed after pre-incubation with DIDS and before the leukotoxins addition, DIDS was only able to block irreversibly pre-existing membrane channels. Additionally, it has been shown that, DIDS was essentially inert towards anion exchange in PMNS and did not alter the $Cl^-$ exchange activity [31-33]. Another type of chloride channels known as VRAC (*volume-regulated anion ($Cl^-$) channel*)-mediated $Cl^-$ currents has been proposed in β-cells [34]. Alternatively, changes in cell volume might be part of the response to the increase in intracellular [$Ca^{2+}$] that could occur during cell activation. Thus, chloride movements across the cell plasma membrane might play a central role in cell volume and pH regulation and maintaining cellular functions in many cells types.

More interestingly, we have demonstrated in another set of experiments that, in the absence of extracellular $Ca^{2+}$, only DIDS could inhibit totally the $Cl^-$ efflux, whereas it had no effect on the pores formation. The flufenamic acid inhibitor did not inhibit leukotoxins-induced $Cl^-$ efflux under this condition. It has previously reported that, flufenamic acid was ineffective or slightly effective on the $Cl^-$ efflux [24]. At a given concentration, the major route of $Cl^-$ entry was inhibited by furosemide, another flufenamic acid analogue, in fibroblasts [35]. Again, it has been shown that, flufenamic acid did inhibit the increases in intracellular free calcium concentration in human neutrophils activated by fMLP. This inhibition was concluded to be due to the $Ca^{2+}$ influx blocking in neutrophils [36]. Previously, it has also been shown [19] by using MQAE as a novel fluorescent indicator that, the initial $Cl^-$ influx rate was reduced after exposure to furosemide in cultured aortic smooth muscle cells.

However, in the presence of extracellular $Ca^{2+}$, a rapid loss of $Cl^-$ channels activity was observed by both inhibitors, DIDS and flufenamic acid, even no inhibitory effect was observed on the membrane pores formation. Our data suggested that, in addition to $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels [12], bi-component leukotoxins from *S. aureus* did induce the opening of $Ca^{2+}$-activated $Cl^-$ channels. These channels were indeed voltage-independent over the voltage range [14].

Previous studies have reported that, several neutrophils agonists did stimulate a $Cl^-$ out flux in human PMNs in the absence of extracellular $Ca^{2+}$ [21]. Under this condition, although staphylococcal leukotoxins did not modify the intracellular $Ca^{2+}$ level [7, 12], an important efflux of $Cl^-$ ions was observed. Furthermore, Shimizu and coworkers [21] have demonstrated that, the intracellular $Ca^{2+}$ elevation was not a pre-requisite for $Cl^-$ fluxes in neutrophils, whereas Meneqazzi *et al*. [28] described $Cl^-$ fluxes in human PMNs independent to the intracellular $Ca^{2+}$ alteration.

Another important result of the present study was that, the combined actions of HlgA/HlgB and $Ca^{2+}$ induced a consequent influx of $Cl^-$. Moreover, if the $Cl^-$ channels opening was only due to the increase of intracellular $Ca^{2+}$, a $Cl^-$ efflux instead of an influx would have been observed as already described [21]. One hypothesis would be that, this $Cl^-$ influx is induced by a signaling transduction pathway activated by the leukotoxins binding and dependent on the extracellular $Ca^{2+}$ since it has been shown that, leukotoxins induced an increase of intracellular $Ca^{2+}$ by the opening of $Ca^{2+}$ channels [7]. Thus, it was proposed that, this $Cl^-$ conductive pathway corresponded to $Ca^{2+}$-activated $Cl^-$ channels. This hypothesis was confirmed by *i)* the absence of the inhibitory effect of flufenamic acid in $Ca^{2+}$ free medium, *ii)* the inhibitory effect of flufenamic acid on the $Cl^-$ movement was only observed in the presence of $Ca^{2+}$. Previously, it has been reported that, an increase of the glucose concentration resulted in an accelerate entry/influx of $Cl^-$ leading to an increase of $Cl^-$ permeability into β-cells. [37]. Consequently, it has been proposed by these authors that, $Ca^{2+}$-activated $Cl^-$ channels were required to a sustain glucose-stimulated membrane potential oscillations and insulin secretion in dispersed β-cells secretory responses. The oscillatory $Ca^{2+}$ signaling event was thought to be critically dependent on trans-membrane $Cl^-$ fluxes. Agonist-stimulated $Cl^-$ movements seems to be a common phenomenon activated by several agonists, which act through different signal transduction pathways. Thus, the $Ca^{2+}$-activated $Cl^-$ channels may play a key role in cell volume homeostasis and/or cell activation.

Finally, the comparative effect of bi-component staphylococcal leukotoxins on the PMNs membrane activity did show that: *i)* in the absence of extracellular $Ca^{2+}$, HlgA/HlgB; HlgC/HlgB and LukS-PV/LukF-PV did provoke a sustained efflux of chloride ($Cl^-$) anions, *ii)* the time course of an agonist-stimulated $Cl^-$ efflux differed, depending on the leukotoxins pair tested and which, could act through different signal transduction pathways (unpublished data) and *iii)* in the presence of extracellular $Ca^{2+}$, only HlgA/HlgB and LukS-PV/LukF-PV were able to induce the opening of $Ca^{2+}$-activated $Cl^-$ channels, allowing a massive influx of $Cl^-$ ions. A previous work [13] did already report that, in contrast to HlgC/HlgB, only HlgA/HlgB and LukS-PV/LukF-PV combinations did induce the $H_2O_2$ production.

# 5. Conclusion

The present study seems to be the first one that analyses

chloride ion movements in human PMNs activated by bi-component leukotoxins from *S. aureus*. We have recently reported that, staphylococcal leukotoxins did trigger the opening of $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels as results of a massive depletion of internal $Ca^{2+}$ stores [12]. This may represent an important event in the signal transduction pathway in activated neutrophils by different combinations of staphylococcal pore-forming leukotoxins. Since the $Cl^-$ efflux was a phenomenon that occurred early in PMNs activated with a variety of agonists, we were interested in the present research to investigate the $Cl^-$ ions movement in leukotoxins-activated human neutrophils.

In this research, we have shown that, bi-component leukotoxins from *S. aureus* did exhibit a massive efflux of $Cl^-$ ions in the absence of extracellular $Ca^{2+}$ sensitive to DIDS, whereas in the presence of extracellular $Ca^{2+}$, an opposite $Cl^-$ movement was recorded due to a sustained influx of $Cl^-$ ions through the PMNs membrane. This was completely inhibited by either DIDS or flufenamic acid. Nevertheless, the role of $Cl^-$ movements in the modulation of PMNs responses is still not yet define and a little is known about these ions channels in staphylococcal leukotoxins-activated PMNs. The mechanism of reuptake remains to be elucidated and, the question as to whether a relationship exists between $Cl^-$ and $Ca^{2+}$ movements deserves further investigations.

All together, these studies provide new insights into the role of $Cl^-$ movements in neutrophils pathophysiology during staphylococcal infections. It has previously reported that, the most well-known serious human disease was cystic fibrosis, due to mutations in chloride channels [38]. In this context, this paper might contribute to highlight the role of $Cl^-$ movements in signal transduction pathways that regulates PMNs responses (degranulation, oxidative burst) when activated by different staphylococcal pore-forming leukotoxins.

In conclusion, the trans-membrane pores formed by bi-component staphylococcal leukotoxins (HlgA/HlgB, HlgC/HlgB, LukS-PV/LukF-PV) do not drive $Cl^-$ ions fluxes. In addition, it is proposed that these leukotoxins induce at least, the opening of two different types of $Cl^-$ channels according to the leukotoxins pair considered. One of these two types seems to correspond to $Ca^{2+}$-activated $Cl^-$ channels. These findings provide new evidence in favor of a close correlation between lowering of $[Cl^-]_i$ and activation of PMNs functions and are in agreement with previous studies. Further studies might be needed to better elucidate the connections between the different signaling pathways mediated by bi-component staphylococcal leukotoxins during infectious diseases.

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The authors declare that they have no competing interests.

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