Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner

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Summary

*Staphylococcus aureus* is a major pathogen responsible for both nosocomial and community-acquired infections. Central to its virulence is its ability to secrete haemolysins, pore-forming toxins and cytolytic peptides. The large number of membrane-damaging toxins and peptides produced during *S. aureus* infections has hindered a precise understanding of their specific roles in diseases. Here, we used comprehensive libraries of recombinant toxins and synthetic cytolytic peptides, of *S. aureus* mutants and clinical strains to investigate the role of these virulence factors in targeting human macrophages and triggering IL-1β release. We found that the Panton Valentine leukocidin (PVL) is the major trigger of IL-1β release and inflammasome activation in primary human macrophages. The cytolytic peptides, δ-haemolysin and PSMα3; the pore-forming toxins, γ-haemolysin and LukDE; and β-haemolysin synergize with PVL to amplify IL-1β release, indicating that these factors cooperate with PVL to trigger inflammation. PVL+ *S. aureus* causes necrotizing pneumonia in children and young adults. The severity of this disease is due to the massive recruitment of neutrophils that cause lung damage. Importantly, we demonstrate that PVL triggers IL-1β release in human alveolar macrophages. Furthermore, IL-1β released by PVL-intoxicated macrophages stimulates the secretion of the neutrophil attracting chemokines, IL-8 and monocyte chemotactic protein-1, by lung epithelial cells. Finally, we show that PVL-induced IL-8/monocyte chemotactic protein-1 release is abolished by the inclusion of IL-1 receptor antagonist (IL-1Ra) in a mixed culture of lung epithelial cells and macrophages. Together, our results identify PVL as the predominant *S. aureus* secreted factor for triggering inflammasome activation in human macrophages and demonstrate how PVL-intoxicated macrophages orchestrate inflammation in the lung. Finally, our work suggests that anakinra, a synthetic IL-1Ra, may be an effective therapeutic agent to reduce the massive neutrophils infiltration observed during necrotizing pneumonia and decrease the resulting host-mediated lung injury.

Introduction

*Staphylococcus aureus* is a Gram-positive bacterium chronically carried by approximately 30% of the human population (Wertheim *et al.*, 2005). Yet, this bacterium is responsible for a wide variety of nosocomial and community-acquired diseases, such as skin and soft tissue infections, endocarditis, osteomyelitis, pneumonia, sepsis and toxic shock syndromes. These diseases differ widely in both their clinical presentations and their severity and each clinical entity requires different therapeutic interventions.
The ability of *S. aureus* to persist and cause disease relies on an artillery of virulence factors that promote nutrients acquisition (Hammer and Skaar, 2011), adherence to host tissues (Foster and Hook, 1998), dissemination within the host (Edwards and Massey, 2011), or resistance to and escape from the immune system (Kraus and Peschel, 2008; Veldkamp and van Strijp, 2009). Particularly, *S. aureus* secretes a large number of proteins and peptides that are able to target the host cell membrane (Table 1). These include alpha, beta, delta and gamma haemolysins, Panton Valentine leukocidin (PVL) (Panton and Valentine, 1932) and other bi-component leukotoxins, such as LukDE (Gravet et al., 1998) and LukGH (also known as LukAB) (Ventura et al., 2010; Dumont et al., 2011), and a family of cytolytic peptides termed the phenol-soluble modulins (PSMs) (Wang et al., 2007). *S. aureus* produces two classes of pore-forming toxins (PFT). α-haemolysin assembles into a homoheptamer to form a transmembrane β-barrel pore in the target plasma membrane (Song et al., 1996). In contrast, bi-component toxins (γ-haemolysin, PVL, LukDE and LukGH) require the hetero-octamerization of class S compounds (e.g. LukS-PV) with class F compounds (e.g. LukF-PV). For example, pores formed by PVL are composed of four molecules of LukS-PV and LukF-PV arranged alternately in a circular pattern (Miles et al., 2002). LukS-PV or LukF-PV alone displays no lytic activity against the target cell (Genestier et al., 2005). Interestingly, two γ-haemolysin class S (HlgA and HlgC) and one γ-haemolysin class F (HlgB) compounds are encoded by the *S. aureus* chromosome. Hence, γ-haemolysin corresponds to two different toxins (HlgA-HlgB and HlgC-HlgB). To further add to the complexity of these virulence factors, class S compounds from one toxin can interact with class F compounds from another, thereby creating a combinatorially diverse array of toxins. In addition to these pore-forming toxins, *S. aureus* secretes numerous peptides with membrane destabilizing activities. δ-haemolysin and PSMs are small (20–40 amino acids), α-helical and amphipathic peptides (Wang et al., 2007). They are thought to create transient pores or act as a detergent depending on their concentration (Verdon et al., 2009). Finally, β-haemolysin is a neutral sphingomyelinase and its lytic activity is highly dependent on the sphingomyelin contents of the target cell plasma membrane (Tibball, 1993). These polypeptides can each cause host cell membrane damage and lysis; however, they do so with varying efficiency that is highly cell type-, species- and concentration-dependent. Thus, their specific or redundant roles in the various human pathologies caused by *S. aureus* have been difficult to decipher.

*Staphylococcus aureus* has acquired a large number of toxins during its co-evolution with its host. The host itself has evolved a number of defence mechanisms to detect membrane lesions and trigger membrane repair mechanisms (Idone et al., 2008) or immune signalling. In particular, macrophages can detect membrane damage and pore formation leading to the assembly of an innate immune signalling platform named the inflammasome (Martinon et al., 2002). Inflammasome activation leads to caspase-1 activation and subsequent caspase-1-mediated release of the inflammatory cytokines IL-1β and IL-18. Following exposure to pore-forming toxins, inflammasome activation relies on the inflammasome receptor NLRP3 and on the adaptor ASC (Mariathasan et al., 2006). Indeed, α-, β-, and γ-haemolysins trigger the NLRP3 inflammasome in murine macrophages (Munoz-Planillo et al., 2009), although the efficacy of α-toxin to activate the NLRP3 inflammasome in murine macrophages is controversial (Craven et al., 2009). Exposure of human monocytes to α-haemolysin has also been shown to trigger IL-1β secretion (Bhadki et al., 1989; Craven et al., 2009). To our knowledge, the ability of the various membrane-damaging toxins and peptides to activate the inflammasome in human macrophages has not been investigated. In tissues, macrophages are the main source of IL-1β and are key to both monitoring infections and rapidly orchestrating immune responses. The ability of macrophages to orchestrate an immune response is particularly important in the lung and in the alveolar

### Table 1. Membrane-damaging toxins and cytolytic peptides secreted by *S. aureus* strains.

| Class     | Name                        | Alternative name | Active form | S compound | F compound |
|-----------|-----------------------------|------------------|-------------|------------|------------|
| BiComp    | Panton Valentine leukocidin | PVL              | (lukS-PV)₄ (lukF-PV)₄ | LukS-PV    | LukF-PV    |
| BiComp    | LukDE                       |                  | (lukD)₄ (lukE)₄ | LukE       | LukD       |
| BiComp    | LukAB                       | LukGH            | (lukG)₄ (lukH)₄ | LukH       | LukG       |
| BiComp/Hly| γ-haemolysin               | γ-toxin          | (HlgA)₄ (HlgB)₄ | HlgA       | HlgB       |
| BiComp/Hly| γ-haemolysin               | γ-toxin          | (HlgC)₄ (HlgB)₄ | HlgC       | HlgB       |
| Hly       | α-haemolysin               | α-toxin          |              |            |            |
| Hly       | β-haemolysin               | β-toxin          |              |            |            |
| Hly/CytoPep| δ-haemolysin              | δ-toxin          |              |            |            |
| CytoPep   | PSM₁₉₋₁₄                  |                  |              |            |            |
| CytoPep   | PSM₁₋₂                   |                  |              |            |            |

BiComp, bi-component toxin; Hly, haemolysin; CytoPep, cytolytic peptides.
space. Inflammasome-dependent IL-1β release is critical for neutrophil recruitment in a mouse model of cutaneous *S. aureus* infection (Miller et al., 2007). Neutrophils are innate immune cells that play a critical role in clearing *S. aureus* infections. However, they have also been shown to contribute to deleterious inflammation in a rabbit model of PVL- mediated necrotizing pneumonia (Diep et al., 2010). It is, thus, of great importance to decipher the pattern of bacterial virulence factors and host responses involved in neutrophil recruitment.

*Staphylococcus aureus* is one of the leading causes of severe pneumonia in hospitals (Park, 2005) and in community settings where co-infections with influenza viruses may occur (Shilo and Quach, 2011). Methicillin resistant *S. aureus* (MRSA) strains emerged more than 50 years ago and community-acquired MRSA are now found worldwide (Vandenesch et al., 2003; Otto, 2010). A better understanding of how *S. aureus* interacts with its host during pneumonia is clearly required to identify new therapeutic targets to limit *S. aureus*-mediated mortality. Mouse models of pneumonia have shown a predominant role for protein A (Gomez et al., 2004) and α-haemolysin in this disease (Bubeck Wardenburg et al., 2007; Inoshima et al., 2011) and, to a lesser extent, a role for β-haemolysin (Hayashida et al., 2009). The role of PVL in this model is highly controversial (Bubeck Wardenburg et al., 2007a; Labandeira-Rey et al., 2007; Vandenesch et al., 2010; Yoong and Pier, 2010). This controversy is likely due to the low sensitivity of murine cells to this toxin (Loffler et al., 2010). In a rabbit model of pneumonia (Diep et al., 2010), PVL has been associated with an increase in IL-8 (CXCL8) and monocyte chemotactic protein 1 (MCP-1/ CCL2) cytokine expression and with an increase in disease severity due to deleterious inflammation. Finally, human clinical studies have indicated an epidemiological association between PVL and very severe necrotizing pneumonia affecting children and young adults (Gillet et al., 2002; Vardakas et al., 2009; Li et al., 2011; Peyrani et al., 2011).

In the present study, we characterized the role of the various *S. aureus*-derived membrane-damaging exotoxins on primary human macrophages including alveolar macrophages. We used a comprehensive library of recombinant *S. aureus* haemolysins, leukotoxins and cytolytic peptides to compare their ability to trigger inflammasome activation in macrophages. Our results demonstrate a major role for recombinant PVL, β- and γ-haemolysin in eliciting IL-1β release upon macrophage intoxication. Using *S. aureus* isogenic mutants, in which the various exotoxin-encoding genes were deleted and a collection of clinical strains, we further confirmed a critical role for PVL in triggering inflammasome activation. Importantly, IL-1β released by PVL-intoxicated macrophages triggers IL-8 and MCP-1 secretion by lung epithelial cells, thereby further amplifying the cytokine/chemokine responses.

Finally, we demonstrated that IL-1 receptor antagonist (IL-1Ra) could impair this immune amplification mechanism and abolish PVL-mediated IL-8 and MCP-1 secretion by lung epithelial cells. Altogether, our data identify PVL as a major inducer of neutrophil-attracting cytokines/chemokines. Furthermore, our work suggests that anakinra, a synthetic IL-1Ra drug, may block the IL-1β/IL-8/ MCP-1 cascade and, thus, reduce the detrimental recruitment of neutrophils during necrotizing pneumonia.

**Results**

*Screening of a library of recombinant membrane-damaging toxins and cytolytic peptides identified γ-haemolysin, PVL and β-haemolysin as the major inflammasome elicitors in human macrophages*

Macrophages are the first line of defence in the lung and act as key sentinels to trigger inflammation and the recruitment of neutrophils. Therefore, we decided to screen an exhaustive library of *S. aureus* recombinant haemolysins, bi-component PFT and lytic peptides for their ability to trigger IL-1β release from primary human monocyte-derived macrophages. IL-1β release depends on both transcriptional control of the proIL-1β precursor and inflammasome-dependent post-translational processing of this precursor. To induce proIL-1β and prime the NLRP3 inflammasome (Bauermeister et al., 2011), we treated macrophages with heat-killed *S. aureus* (HKSA) before adding a panel of *S. aureus* PFT. Two bi-component toxins, γ-haemolysin (HlgBC) and PVL, triggered robust IL-1β release in a dose-dependent manner and at very low concentrations (10 ng ml⁻¹ i.e. < 300 pM) (Fig. 1A). This release was highly specific of active toxins as neither of the class S or class F compounds triggered IL-1β release when added individually (Fig. S1 and data not shown). α-haemolysin treatment did not elicit a strong IL-1β release in human macrophages. This result was not due to an inactive recombinant α-haemolysin because the toxin was cytolytic to human THP-1 monocytes at 1000 ng ml⁻¹ as previously described (Craven et al., 2009) (Fig. S2). Despite often being considered as a single toxin, the two γ-haemolysin toxins (HlgBC and HlgAB) behaved very differently. Indeed, while HlgBC was a very potent inducer of IL-1β from human macrophages, HlgAB displayed no activity towards these cells. Once again, this absence of activity towards human macrophages was not due to an inactive recombinant α-haemolysin because the toxin was cytolytic to primary murine macrophages (Fig. S3). These data indicate that the two Hlg class S subunits (i.e. HlgA or C) confer susceptibility to myeloid cells of different species. This result highlights both the species-specific nature of *S. aureus* virulence factors and the critical need to perform studies on human cells.
IL-1β release was observed from macrophages exposed to very low concentrations of PVL or γ-haemolysin (HlgBC) (10 ng ml⁻¹, i.e. <3 nM). While, to our knowledge, in vivo γ-haemolysin concentrations remain uncharacterized, the effective PVL concentration is consistent with those observed in vivo during lung infections (median 110 ng ml⁻¹ in pulmonary samples) (Badiou et al., 2010; Rouzic et al., 2010). We also
screened recombinant LukDE and LukAB/GH. However, these toxins did not trigger the release of IL-1β by primary macrophages in the concentration range tested (1–1000 ng ml−1), despite the toxin LukAB/GH exhibiting cytolytic activity towards primary human PMNs and PMA-differentiated THP-1 macrophages at higher concentrations (5000 and 1000 ng ml−1 respectively) (data not shown and Fig. S4) consistent with DuMont et al. (Dumont et al., 2011). In addition, LukD and LukA/G were cytotoxic to THP-1 macrophages at 100 ng ml−1 when combined with LukS-PV (Fig. S5). In agreement with previous work by Gravet et al. (Gravet et al., 1998), LukDE was not cytotoxic toward any of the human cells tested (THP-1 monocytes/macrophages or primary macrophages) at concentrations of up to 1000 ng ml−1.

We next screened macrophages exposed to the cytolytic peptides; δ-haemolysin, PSMc1 and PSMc3, two of the most active PSMs against human neutrophils (Wang et al., 2007) for IL-1β production. These peptides did not trigger consistent IL-1β release from human macrophages (Fig. 1B). In contrast, we identified an effect of β-haemolysin even at very low concentrations (1 ng ml−1, i.e. 27 pM) on the level of IL-1β secretion from human macrophages (Fig. 1C). However, the β-haemolysin dose response curve differed from that observed with γ-haemolysin or PVL intoxication because the level of IL-1β released upon β-haemolysin exposure never reached the concentrations measured after PVL or γ-haemolysin intoxication. The ability of these toxins to trigger IL-1β secretion in a dose-dependent manner correlated with their dose-dependent cytotoxicity towards human macrophages. Indeed, both PVL and γ-haemolysin (HlgBC) were highly cytotoxic towards human macrophages, whereas β-haemolysin was cytotoxic at very low concentrations, but the level of cytotoxicity reached a plateau and did not increase with higher concentration (Fig. 1D).

PSMs and LukAB/GH have a synergistic cytolytic activity on human neutrophils when combined with PVL (Wang et al., 2007; Ventura et al., 2010). Therefore, we asked whether haemolysins, PFT and cytolytic peptides could act in synergy with PVL to trigger IL-1β release in human macrophages. Interestingly, we detected a synergistic activity in the triggering of IL-1β release from human macrophages from almost all of the toxins and cytolytic peptides tested when combined with PVL (Fig. 1E). Strong synergy (corresponding to a roughly threefold increase in IL-1β release) was observed with δ-haemolysin (10 ng ml−1), PSMc1 (100 ng ml−1), PSMc3 (10 ng ml−1), γ-haemolysin (1 ng ml−1), LukDE (100 ng ml−1) and β-haemolysin (1 ng ml−1). In contrast, no synergy was observed when PVL was combined with LukAB/GH. These results suggest that while PVL has a powerful intrinsic ability to trigger IL-1β release from human macrophages, several other secreted factors can strongly potentiate this activity.

Overall, these results using recombinant or synthetic toxins and lytic peptides highlight a redundant role of β-, γ-haemolysins and PVL to elicit an IL-1β response from human macrophages. While in vivo concentrations are difficult to determine, these three toxins are active at very low concentrations (nM range), suggesting a physiological role in targeting human macrophages and eliciting IL-1β responses. In contrast to these three toxins, our data do not indicate a major intrinsic role for any of the other toxins in targeting human macrophages. However, in vivo they could synergize with PVL (and potentially with β- and γ-haemolysins) to drive IL-1β release from macrophages higher.

Screening S. aureus mutants in the genes encoding haemolysins, PFT and cytolytic peptides revealed PVL as the main secreted factor triggering IL-1β release from human macrophages

To exclude any bias associated with the use of recombinant proteins, we investigated the ability of naturally secreted haemolysins, bi-component toxins and cytolytic
peptides to trigger IL-1β release. Supernatants from a 16 h culture of various *S. aureus* isogenic mutants and their parental strains (grown in CCY broth) were diluted in culture medium and added onto HKSA-primed primary human macrophages. Strikingly, deletion of PVL-encoding genes in a USA300 MRSA strain (SF8300) drastically reduced the level of IL-1β secretion, while none of the other deletion affected the ability of the corresponding supernatant to trigger IL-1β secretion by macrophages (Fig. 2A). Indeed, we did not detect any difference in the ability of supernatants from α-haemolysin, LukDE, LukAB/GH or α-PSM mutants to elicit IL-1β secretion from human macrophages when compared with the supernatant from their respective parental strains. Surprisingly, IL-1β concentrations did not differ when supernatants from β-haemolysin or γ-haemolysin mutants were used, indicating that under these conditions, secreted β-haemolysin and γ-haemolysin did not significantly trigger IL-1β secretion from macrophages. Importantly, a similar PVL-dependent effect was observed in the supernatants of two other *S. aureus* mutants independently generated in both a Community Acquired-MRSA (CA-MRSA) ST80 and a USA300 MSSA genetic background (Fig. 2B). Finally, we could specifically complement the PVL mutant for its ability to trigger IL-1β secretion from human macrophages (Fig. 2A and C). These results demonstrate that the primary *S. aureus* secreted factor responsible for triggering IL-1β secretion by human macrophages is the PVL.

The presence of PVL in *S. aureus* clinical isolates is associated with higher IL-1β secretion by human macrophages

PSMs, LukAB/GH, α- and γ-haemolysin are expressed by almost all *S. aureus* isolates. In contrast, β-haemolysin, LukDE and PVL are expressed in approximately 10%, 30% and 2–3% of *S. aureus* strains respectively (Hedstrom and Malmqvist, 1982; Gravet et al., 1998; Kuehnert et al., 2006; van Wamel et al., 2006; Wang et al., 2007; Deleo et al., 2010; Otter and French, 2010; Ventura et al., 2010). To evaluate the relative contributions of β-haemolysin, LukDE, PVL and other non-ubiquitous virulence factors to IL-1β production, we used a library of 32 unrelated clinical strains isolated from community-acquired pneumonia patients during a prospective clinical study (Gillet et al., 2002). This collection included 16 PVL+ and 16 PVL− strains. Each strain was

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**Fig. 2.** PVL is the major secreted toxin triggering IL-1β release in human macrophages. The indicated parental and isogenic mutant strains were grown for 16 h in CCY broth. The bacterial supernatant was collected and added at a 1/625 dilution onto HKSA-primed primary human macrophages for 3 h. IL-1β concentrations in the supernatant were determined by ELISA at 3 h post treatment. The mean and standard deviation from biological triplicates are shown. Note that absolute IL-1β values are not comparable from one panel to another due to the different reactivity of the various donors. From left to right, the strains used were as follows: (A) BD0425/SF8300; BD0415; BD0452; BD0423; BD0405; BD0406; BD0546; BD0419; 8325-4; DUS719; (B) HT20020209; LUG1800; ST20090065; LUG1672 (C) LUG855 (WT PVL+); LUG776 (ΔPVL); LUG1560 (ΔPVL + empty vector); LUG862 (ΔPVL + Complementation vector). One experiment representative of three independent experiments performed on primary cells from three different donors is shown. A–C. An unpaired *t*-test was performed to compare untreated macrophages with supernatant-treated macrophages (A), to compare the effect of supernatants from WT or ΔPVL isogenic mutants (B) and to compare the effect of PVL complementation (C). Two-tailed *P*-value is shown.
analysed by StaphyType microarray for the presence or absence of approximately 180 virulence genes (Table S1) and their supernatants were tested for the ability to trigger IL-1β secretion in macrophages. The relative contribution of these 180 virulence factors to IL-1β secretion was analysed in an unbiased way by a multivariate linear regression. Importantly, only two genes encoding the two subunits of PVL, lukS-PV \((P = 0.0053)\) and lukF-PV \((P = 0.0169)\) were statistically identified as contributing to IL-1β secretion. The difference in \(P\)-values between lukS-PV and lukF-PV was due to the presence of three lukF-PV array spots, which were classified by automatic reading as ambiguous. The presence of both lukS-PV and lukF-PV genes in these ‘LukF-PV ambiguous’ strains has been confirmed by PCR-based methods (Gillet et al., 2002). Macrophages exposed to supernatants from PVL+ clinical strains produced significantly more IL-1β than those exposed to supernatants from PVL- strains (Fig. 3). In contrast, we did not observe any significant contribution of β-haemolysin (undisrupted gene observed in 7 strains out of 32) or LukE (observed in 16 strains) on IL-1β release (Fig. 3). Further experiments are required to explore the possible contributions (which fell under the threshold of statistical significance) of other variable factors in IL-1β release (see capsular serotypes 5/8 in Fig. S6). Overall, our results highlight a key and specific role of PVL in triggering IL-1β secretion by human macrophages in both laboratory and clinical \(S.\ aureus\) strains. Therefore, we focused on this virulence factor to better characterize its impact on innate immune functions.

### Recombinant PVL activates the inflammasome in a time and dose-dependent manner in primary human macrophages

Panton Valentine leukocidin has been reported to bind to human neutrophils and monocytes (Gauduchon et al., 2001). To our knowledge, PVL fixation onto macrophages has not been described. LukS-PV is the subunit that confers binding specificity to the PVL toxin (Gauduchon et al., 2001). Hence, to gain a better knowledge of PVL activity towards human macrophages, we characterized LukS-PV fixation onto these cells. We generated a modified LukS-PV-FITC recombinant protein to detect fixation by flow cytometry as previously described (Gauduchon et al., 2001). We could detect fixation at concentrations as low as 10 ng ml\(^{-1}\) LukS-FITC, which correlates with the high sensitivity of human macrophages (Figs 4A and S7). We then performed a kinetic analysis of IL-1β secretion by human macrophages following PVL exposure (Fig. 4B). At low PVL doses, IL-1β concentrations increased from 1 to 6 h. In contrast, at high PVL concentrations, IL-1β concentrations reached a plateau at 1 h post treatment (data not shown). This dose-dependent response correlated with the difference in cytotoxicity between low and high PVL doses (data not shown), indicating that sublytic PVL concentrations triggered sustained IL-1β production.
in macrophages. To confirm that IL-1β secretion in PVL-treated macrophages was associated with caspase-1 activation within the inflammasome and processing of proIL-1β into the mature form of IL-1β, we analysed macrophage supernatant by western blot. As presented in Fig. 4C/D, PVL treatment induced caspase-1 and proIL-1β processing in a dose-dependent and time-dependent manner. Finally, to ensure that IL-1β released upon PVL treatment resulted from inflammasome activation, we independently generated two THP-1 cell lines knocked-down for ASC, the critical inflammasome adaptor used in the response to PFT (Fig. S8). The concentration of IL-1β in the supernatant of THP-1\textsubscript{ASC\textasciidcdbl} cells was highly reduced (Fig. 4E), demonstrating that PVL treatment triggers IL-1β release in an inflammasome-dependent manner. Importantly, PVL-induced cell death was similar in PMA-differentiated THP-1\textasciidcdbl ASC\textasciidcdbl cells and in control THP-1 cells (Fig. 4F). The latter result indicates that the different cell lines are equally susceptible to PVL-mediated cytotoxicity and that cell death proceeds
PVL triggers IL-1β secretion by alveolar macrophages and IL-1β-dependent IL-8 and MCP-1 secretion by lung epithelial cells in a mixed culture with macrophages

Epidemiologically, PVL is associated with a very severe clinical entity affecting children and young adults, namely necrotizing pneumonia (Gillet et al., 2002). In PVL-susceptible species such as rabbits, PVL was demonstrated to play a key role in the detrimental inflammation and host-mediated lung injury associated with severe S. aureus pneumonia (Diep et al., 2010). Therefore, we investigated the ability of PVL to trigger cytokine and chemokine secretion by various human pulmonary cells. We first obtained primary human alveolar macrophages from bronchoalveolar lavages. As previously observed with monocyte-derived macrophages, treatment of alveolar macrophages with recombinant PVL (rPVL) led to IL-1β release in a dose-dependent manner, suggesting that this pathway is relevant for pulmonary infections (Fig. 5A). As expected, based on the specificity of PVL for myeloid cells (Gauduchon et al., 2001; Löffler et al., 2010), A549 cells, a model for type II alveolar epithelial cells (Lieber et al., 1976) were not susceptible [based on lactate dehydrogenase (LDH) release assay] or responsive to PVL (based on IL-1β and IL-8 ELISA) (see below and data not shown). However, epithelial cells can be an important source of chemokines following an interaction with activated macrophages (Thorley et al., 2007). Therefore, we assessed the consequence of PVL intoxication in a mixed culture of A549 cells (10⁵ cells, 99%) and monocyte-derived macrophages (10³ cells, 1%). Treatment of 10⁴ macrophages with PVL led to IL-1β concentrations in the supernatant that were below the level of detection by ELISA (data not shown). When cultured alone, macrophages secreted IL-8 in a constitutive manner and IL-8 secretion slightly increased at sublytic concentrations of PVL (Fig. 5B). When A549 cells were cultured alone, IL-1β could not be detected (not shown) and IL-8 concentrations were low (<300 pg ml⁻¹) irrespective of PVL concentrations. However, in the mixed culture system, the concentration of IL-8 in the supernatant increased by approximately 600% when cells were treated with a minute amount of PVL (10 ng ml⁻¹) (Fig. 5B). As this effect could be recapitulated by treating A549 cells but not macrophages with rIL-1β (Fig. 5C), we hypothesized that A549 cells produced IL-8 in response to IL-1β released by PVL-intoxicated macrophages. Indeed, in a mixed culture of THP-1wt or THP-1ascw with A549 cells, PVL-mediated IL-8 secretion was abolished when THP-1 cells were deficient for inflammasome activation (and thus for IL-1β secretion) (Fig. 5D). Finally, rIL-1 receptor antagonist (IL-1Ra) drastically reduced, in a dose-dependent manner, PVL-induced IL-8 secretion in the mixed culture system (Fig. 5E). Similarly, we observed a PVL-dependent twofold increase in MCP-1 secretion in the same mixed culture setting and PVL-induced MCP-1 secretion was fully inhibited by IL-1Ra (Fig. 5F). These results demonstrate a key role for IL-1 in eliciting IL-8 and MCP-1 secretion by lung epithelial cells upon PVL treatment and highlight how inflammasome activation in PVL-intoxicated macrophage orchestrates the pulmonary immune response.

Discussion

One key challenge in understanding S. aureus infections lies in deciphering the specific and redundant roles of its impressive arsenal of toxins. Furthermore, S. aureus is able to trigger infections in numerous species and some of its virulence factors display high species specificity (Löffler et al., 2010). The low susceptibility of murine cells to PVL has led to controversy regarding its role in murine models of infection (Bubeck Wardenburg et al., 2007a; 2008; Labandeira-Rey et al., 2007; Vandenesch et al., 2010; Zivkovic et al., 2011). Consistent with this species specificity, here we found that one of the two γ-haemolysins (Hlg BC) is highly active on human macrophages, but inactive on murine macrophages, whereas the converse is true for the other γ-haemolysin (Hlg AC). This result highlights the need to characterize these virulence factors on human cells to identify the relevant therapeutic targets. Here, we investigated a comprehensive library of recombinant and synthetic pore-forming toxins, cytolytic peptides and haemolysins for their ability to trigger inflammation following intoxication of primary human macrophages. Surprisingly, characterization of S. aureus virulence factors has seldom been performed on human macrophages. While neutrophils are very rapidly recruited into the lung following infection, macrophages constitute the majority of immune cells residing in tissue and in the alveolar space at steady state. Thus, macrophages act as sentinels and are the first immune cells to detect and react to S. aureus infection. As the first minutes following infections are probably key to determining the outcome of the infection, we reasoned that it would be informative to characterize the interaction of S. aureus virulence factors with human macrophages.

Using recombinant toxins, we observed a specific ability of β-haemolysin, γ-haemolysin (HlgBC) and PVL to elicit IL-1β release from human macrophages at 1−10 ng ml⁻¹ (30−300 pM). We failed to detect any strong IL-1β releasing activity for any of the other considered pore-forming toxins, haemolysins or cytolytic peptides analysed even at concentrations 100-fold higher. One
A

**Alveolar MΦ**

Mixted culture: macrophages (1%) lung epithelial cells (99%) IL-8

B

Mixted culture: macrophages (1%) lung epithelial cells (99%) MCP-1
possibility is that human macrophages are not the main target cells for those factors as was exemplified here with γ-haemolysin (HlgAB), which we found to be mouse-specific. An alternative possibility is that the main function of these factors might not be to damage host cell membranes but to trigger some more subtle signalling events. Indeed, β-haemolysin modulates ceramide signalling and inhibits IL-8 secretion by endothelial cells (Tajima et al., 2009). Similarly, α-haemolysin has been very recently described to activate ADAM10, a metalloprotease leading to tight junction disruption in epithelium (Inoshima et al., 2011).

Our results demonstrate that PVL, γ-haemolysin (HlgBC) and β-haemolysin are potent at very low concentrations to trigger human macrophage cell death and IL-1β secretion. To our knowledge the role of γ-haemolysin in pneumonia has not been evaluated in animal models. γ-haemolysin has been shown to contribute to inflammation in a rabbit model of endophthalmitis (Supersac et al., 1998) and to participate in the severity of septic arthritis in mice in combination with other haemolysins (Nilsson et al., 1999). It is still unclear at this stage what concentrations of γ-haemolysin are reached in vivo during an infection. However, based on the very potent activity of γ-haemolysin, it is likely that this toxin can target myeloid cells and activate the inflammasome pathway during an infection. As this toxin is present in almost every S. aureus strain, it will be interesting to assess its role in pneumonia. β-haemolysin has been shown to trigger neutrophil recruitment in a mouse model of pneumonia (Hayashida et al., 2009). We demonstrate here that recombinant β-haemolysin elicits IL-1β secretion from human macrophages when applied at very low concentrations, which could partially explain its neutrophil-recruiting activity. Interestingly, while IL-1β release upon treatment with either γ-haemolysin or PVL displayed a very good dose–response dependency, culminating with the rapid death of 100% of intoxicated macrophages, β-haemolysin elicited less IL-1β release and was associated with less toxicity. Furthermore, this activity barely increased across a dose curve of β-haemolysin concentrations. β-haemolysin is a sphingomyelinase, which can break sphingomyelin lipids into phosphocholine and ceramide. The absence of an obvious dose-dependent effect might be linked to a limited amount of β-haemolysin-accessible sphingomyelin in the plasma membrane. Alternatively, the NLRP3 inflammasome might detect a sphingomyelinase-mediated increase in intracellular ceramide, as was recently described during metabolic imbalance (Vandemagsar et al., 2011).

We did not detect any effect of β- or γ-haemolysins in the bacterial supernatant. In contrast, we did observe a strong effect of PVL regardless of the genetic background on which the mutation had been performed. This absence of an effect might be partly due to our growth conditions; bacteria were grown in CCY broth, which is a medium known to promote PVL expression (Graves et al., 2010) but also leads to a general increase in staphylococcal exotoxins (Coleman and Abbas-Ali, 1977). Alternatively, it might indicate that under physiological conditions, these factors are secreted at insufficient concentrations to trigger the inflammasome pathway or activate the innate immune system. Importantly, we observed a strong contribution of PVL in IL-1β release across 32 clinical strains isolated from PVL+ and PVL− S. aureus community-acquired pneumonia. However, supernatants from some of the PVL-negative strains did induce a large amount of IL-1β release, indicating the presence of other potent inflammasome-activating molecules in those clinical S. aureus supernatants. However, our data do not suggest any major role for LukAB/GH; LukDE; PSMα1–4 or any of the haemolysins. Thus, the molecules responsible for inflammasome activation in PVL− strain supernatants remain to be identified. Such molecules may include peptidoglycan fragments (Shimada et al., 2010) or capsular polysaccharide ([Soell et al., 1995] and Fig. S6).
While it is clearly established that inflammation is required to clear bacterial infections, inflammation can also be deleterious and lead to lung injury. Indeed, inflammation has been demonstrated to contribute to *S. aureus* pneumonia severity in several animal models (Diep et al., 2010; Kapetanovic et al., 2010). Particularly, deleterious effects of virulence factors secreted by *S. aureus* can synergize with PVL activity to increase IL-1β release and, thus, further amplify the inflammatory process. Therefore, it is likely that PVL cooperates with numerous other secreted toxins and peptides to trigger the massive inflammation observed in patients undergoing necrotizing pneumonia. The relative roles of the various cell types to overall lung inflammation, including PVL-intoxicated macrophages, neutrophils, α-haemolysin-intoxicated and protein A-exposed epithelial cells (Gomez et al., 2004; Inoshima et al., 2011) and endothelial cells (Teijaro et al., 2011), remain to be studied.

Mortality in patients with necrotizing pneumonia has been observed even when appropriate antibiotic treatment was used (Wargo and Eiland, 2005). This observation highlights the critical need to develop new treatments. In patients presenting with an acute lung infection, an attractive possibility is the combined use of antibiotics to target the bacteria and anti-inflammatory molecules to suppress inflammation and neutrophils recruitment. Here, following our observation that PVL activates the inflammasome and promotes IL-1β release in human macrophages, we identified a role for IL-1β in the activation of lung epithelial cells. These epithelial cells, which are otherwise non-responsive to PVL, respond to IL-1β with the production of neutrophil-attracting chemokines, including IL-8 and MCP-1. Similarly, IL-1β amplifies IL-6 responses in a mouse model of cutaneous infection (Hruz et al., 2009). As such, PVL-induced secretion of IL-8 and MCP-1 was completely suppressed by the addition of IL-1Ra to mixed cell cultures of macrophages and lung epithelial cells. Anakinra (Kineret), a drug currently used to treat patients suffering from rheumatoid arthritis, could thus dually target neutrophil recruitment (i) by directly inhibiting IL-1β and its subsequent effects on endothelial cells, an event required to recruit neutrophils from the underlying blood capillaries into the lung, and (ii) by disrupting the IL-8 and MCP-1 chemokine gradients. Hence, our work highlights the possibility that anakinra administration could reduce neutrophil recruitment, inflammation and, thus, host-mediated lung injury in patients presenting with necrotizing pneumonia.

### Experimental procedures

#### Ethics statement

Collection of alveolar macrophages has been conducted according to Declaration of Helsinki principles and internal rules at the Hospices civils de Lyon. Blood packs were obtained anonymously through a convention with Etablissement Français du Sang (EFS, Lyon, France). Patients undergoing bronchoalveolar lavage for diagnostic purposes gave a written informed consent. Extra BAL not used for diagnostic purposes were anonymized before harvesting alveolar macrophages. For the human samples, the study was approved by the local ethics committee (comité de protection des personnes Sud-Est IV) and declared to the Ministry of Research under the agreement n°DC-2008-176. For animal work, this study was performed in strict accordance with the French recommendations in the guide for the ethical evaluation of experiments using laboratory animals (http://gircor.net/qui/ethicalEvaluationGuide4LaboratoryAnimals.pdf) and the European guidelines 86/609/CEE. The protocol was approved by the local ethics committee for animal experimentation CECCAPP under the number #ENS_2009_020.

#### Recombinant toxin production

The *S. aureus* strains listed in Table S2 were used to amplify coding sequences of toxins. *Escherichia coli* M15 (Qiagen) and *E. coli* BL21 pLys (Invitrogen) were used for plasmid amplification and genetic manipulations. Primers were designed following the identification of suitable hybridization sites in the toxin genes (Table S2). Chromosomal DNA of *S. aureus* was extracted and used as a template for PCR amplification, as described previously [23]. The 5′ primers were chosen within the coding sequence of each gene, omitting the region predicted to encode the signal peptide, as determined on the SignalP V3.0 World Wide Web Prediction Server (http://www.cbs.dtu.dk/services/SignalP/). The 3′ primers were chosen to include the stop codon of the toxin genes. Primers are presented in the Table S2. PCR products were codigested with appropriate restriction enzymes (New England Biolabs, Ipswich, USA), purified with the High Pure PCR Product Purification kit (Roche, Meylan, France) and ligated using T4 DNA Ligase (Promega, Madison, WI, USA) into either the pQE-30 (Qiagen, Courtaboeuf, France) or pIVEX2.4d (Roche) expression vectors that had been digested with the restriction enzymes described in Table S1. The resulting pQE plasmids were transformed into *E. coli* strain M15. For toxin expression in pIVEX2.4d, the vector was transformed into *E. coli* strain DH5α (Life Technologies, Saint-Aubin, France) before transformation into *E. coli* strain BL21 pLys. The integrity of the open reading frame was verified by sequencing the junctions between the plasmid and the insert.
**Protein purification**

Transformed *E. coli* cells grown exponentially in Luria–Bertani broth (LB; Conda, Madrid, Spain) medium supplemented with ampicillin 100 mg ml⁻¹ were inoculated into 1 l of fresh LB medium and incubated with continuous rotary shaking for 2–3 h at 37°C until OD₆₀₀ reached 0.5–0.7. Protein expression was induced by adding isopropyl-b-d-thiogalactopyranoside at a final concentration of 1 mM. After 5 h, the cultures were then centrifuged at 4000 g for 20 min at 4°C. The cell pellets were stored overnight at −80°C, thawed for 15 min on ice and resuspended in lysis buffer (Qiagen). The lysates were sonicated on ice after adding lysozyme. Then, the lysates were centrifuged at 10 000 g for 30 min at 4°C and the supernatants were collected. The His-tagged proteins were purified on Ni-ntllotrictiacid acid columns (Qiagen) and dialysed against phosphate-buffered saline (PBS). Contaminating LPS was then eliminated using a Polymixin-B column (Detoxi-Gel Endotoxin Removing gel, ThermoScientific, Rockford, IL, USA) following manufacturer's instructions. Protein concentrations were determined according to the Bradford method. The quality of toxin preparations was assessed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, mass spectrometry (Ultraflex MALDI-ToF; Bruker Daltonics) and a Limulus amebocyte lysate assay (Lonza, Levallois-Perret, France). The endotoxin content of the recombinant protein solutions was determined to be less than 0.004 endotoxin units per µg of protein. 5-haemolysin was a kind gift of J.M. Berjeaud and Y. Hechard (Université de Poitiers, Poitiers, France). PSMα-1 and PSMα-3 have been synthesized by GeneCust (Dudelange, Luxembourg).

**LukS-FITC and flow cytometry binding assay**

LukS-FITC was generated as previously described (Gauduchon et al., 2001). Briefly, purified LukS G10C was coupled to fluorescein-5-maleimide (Life Technologies) provided in five times molar excess overnight at 4°C. The coupling reaction was quenched by adding isopropyl-b-d-thiogalactopyranoside at a final concentration of 1 mM. After 5 h, the cultures were then centrifuged at 4000 g for 20 min at 4°C. The cell pellets were stored overnight at −80°C, thawed for 15 min on ice and resuspended in lysis buffer (Qiagen). The lysates were sonicated on ice after adding lysozyme. Then, the lysates were centrifuged at 10 000 g for 30 min at 4°C and the supernatants were collected. The His-tagged proteins were purified on Ni-ntllotrictiacid acid columns (Qiagen) and dialysed against phosphate-buffered saline (PBS). Contaminating LPS was then eliminated using a Polymixin-B column (Detoxi-Gel Endotoxin Removing gel, ThermoScientific, Rockford, IL, USA) following manufacturer's instructions. Protein concentrations were determined according to the Bradford method. The quality of toxin preparations was assessed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, mass spectrometry (Ultraflex MALDI-ToF; Bruker Daltonics) and a Limulus amebocyte lysate assay (Lonza, Levallois-Perret, France). The endotoxin content of the recombinant protein solutions was determined to be less than 0.004 endotoxin units per µg of protein. 5-haemolysin was a kind gift of J.M. Berjeaud and Y. Hechard (Université de Poitiers, Poitiers, France). PSMα-1 and PSMα-3 have been synthesized by GeneCust (Dudelange, Luxembourg).

**Cells and culture conditions**

Unless otherwise specified, ‘macrophages’ refer to human monocyte-derived macrophages. Leukocytes were purified from blood by centrifugation onto a lymphocyte separation medium gradient (density = 1.077, Eurobio, Courtaboeuf, France). Monocytes were enriched by a selective adhesion step (45 min at 37°C) followed by two washes in DMEM (Life Technologies). Monocytes were cultured in high glucose DMEM medium supplemented with 40 ng ml⁻¹ M-CSF (Abcys, Paris, France), 10% fetal calf serum (FCS, Lonza, Basel, Switzerland), 2 mM glutamine (Life Technologies) and 1 mM sodium pyruvate (Life Technologies) to trigger their differentiation into macrophages. Cells were washed to remove non-adherent cells on day 3 or 4. When applicable, macrophages were treated with HKSA at a multiplicity of 100:1 for 16 h. Cells were washed twice to remove HKSA before toxin or bacterial supernatant addition. Using these conditions, LPS had not impact on toxin-mediated cell death (not shown) or toxin-mediated IL-1β release (Fig. S9).

Alveolar Macrophages were obtained from patients undergoing broncho-alveolar lavage for diagnosis purposes. Extra BAL not used for diagnostic purposes were diluted in PBS, centrifuged at 450 g for 10 min at 4°C, washed twice in medium with antibiotics (penicillin 100 U ml⁻¹, streptomycin 100 µg ml⁻¹ (Life Technologies) and ciprofloxacin at 20 µg ml⁻¹) and plated in high glucose DMEM containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate. Ten hours post plating, cells were thoroughly washed before Pam3CSK4 (Invivogen, Toulouse, France) addition (100 ng ml⁻¹ for 3 h) followed by toxin addition.

THP-1 cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% FCS and 2 mM glutamine. THP-1 monocytes were differentiated into macrophages by the addition of phorbol myristate acetate (PMA, Invivogen, Toulouse, France) (100 ng ml⁻¹) for 48 h. While THP-1 monocytes are resistant to PVL-mediated lysis, PMA-differentiated THP-1 cells have similar IL-1β responses as primary macrophages following incubation with *S. aureus* supernatant (Fig. S10). A549 cells were cultured in high glucose DMEM supplemented with 10% FCS and 2 mM glutamine. For the mixed culture experiment, 10⁵ A549 cells were added to the wells containing 10³ macrophages or PMA-differentiated THP-1 cells 24 h before the addition of PVL.

**Cell death assay**

Cells were plated in high glucose DMEM without phenol red (Life Technologies) and supplemented with 10% FCS, 2 mM glutamine and 1 mM sodium pyruvate. Cell death was assessed by the release of LDH in the supernatant and measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

**ELISA**

Release of IL-1β, IL-8 and MCP-1 into the supernatant was quantified by enzyme linked immunosorbent assay (ELISA) using DuoSet ELISA kits (R&D systems, Lille, France) and TMB substrate from Sigma (Saint-Quentin Fallavier, France). The range of detection and the corresponding coefficients of correlation for each ELISA are as follows: IL-1β, 2–1000 pg ml⁻¹ (R² = 0.99995); IL-8, 4–4000 pg ml⁻¹ (R² = 0.99983); and MCP-1, 2–1000 pg ml⁻¹ (R² = 0.99998).

**Bacterial strains and culture conditions**

Bacterial strains used in this study are listed in Table S3. SF8300 is a minimal-passaged USA300 clinical strain representative of the epidemic clone USA300-0114 (Diep et al., 2008). Its isogenic *pvl* mutant (SF8300Δluk-PV), and the complemented strain (SF8300compΔluk-PV) in which LukS-PV and LukF-PV were reintroduced into their original chromosome sites in the *lukPV* mutant by allelic replacement mutagenesis with plasmid pKOR1 (Baek and Schneewind, 2006) have been described previously (Diep et al.,
2008; 2010). In-frame deletion of the alpha-toxin gene (SF8300.\textit{pha1}), PSM alpha1-4 gene (SF8300.\textit{psm}), gamma toxin genes (SF8300.\textit{hlgABC}), LukED genes (SF8300.\textit{LukED}) and LukGH genes (SF8300.\textit{LukGH}) from the parental SF8300 strain were constructed by the use of a pKOR1 plasmid as described previously (Diep et al., 2010; H.N. Le et al., manuscript in preparation). Strain 8325-4 corresponds to NCTC8325 cured of three prophages (Novick, 1967). DU5719 (8325-4 \textit{hlg::p42E}) was obtained by inactivating the beta-toxin gene (\textit{hlg}) in 8325-4 by lysogenization with a converting bacteriophage (42E) that integrates into the \textit{hlg} gene (Bramley et al., 1989). Strain Newman was isolated in 1952 from a human infection (Duthie and Lorenz, 1952) and has been used extensively in animal models of staphylococcal disease due to its robust virulence phenotypes (Baba et al., 2008). Deletion of the gamma toxin gene in strain Newman (Newman.\textit{hlg}) was obtained by allelic replacement of the \textit{hlg}ABC genes with a tetracycline resistance cassette (Supersac et al., 1998). HT20020209 is a minimal-passaged ST80 SCCmedV PVL + MRSA isolate in France and is representative of the European ST80 CA-MRSA clone (Tristan et al., 2007). ST20090065 is a minimal-passaged ST8 SCCmedV PVL + MSSA isolated in Spain and belongs to the USA300 lineage (Blanco et al., 2011). The isogenic \textit{pvl} mutants (HT20020209.\textit{Luk-PV} and ST20090065.\textit{Luk-PV}) were obtained by allelic replacement mutagenesis in HT20020209 and ST20090065, respectively, using the pMAD plasmid as described previously with primers described in the Table S2 (Labandeira-Rey et al., 2007). \textit{S. aureus} RN6390 derives from 8325-4 and is a standard \textit{agr}+ strain (Peng et al., 1988). The RN6390\textit{SLT} strain (LUG855) was obtained by lysogenization of RN6390 with phiSLT phage carrying luk-PV (Genestier et al., 2005). The LUG855.\textit{Luk-PV} strain (LUG776) was obtained by replacement of \textit{luk-PV} operon in LUG855 by a tetracycline resistance cassette using the pMAD plasmid. LUG862 was constructed by complementing LUG776 with a plasmid carrying the entire \textit{luk-PV} operon (Tristan et al., 2009); and LUG1560 with the same plasmid without insert (Tristan et al., 2009). HKSA was prepared by a 30 min incubation at 95°C of an overnight culture of LUG960 strain (Tristan et al., 2009).

Clinical \textit{S. aureus} strains isolated from patients hospitalized for community-acquired pneumonia were collected during a prospective study that was previously described (Gillet et al., 2002). The genomic content of the clinical isolates was analysed by commercial microarray (StaphyType, Alere Technologies, Jena, Germany) as previously described (Albrecht et al., 2002). The genomic content of the clinical isolates was analysed by commercial microarray (StaphyType, Alere Technologies, Jena, Germany) as previously described (Albrecht et al., 2002). The genomic content of the clinical isolates was analysed by commercial microarray (StaphyType, Alere Technologies, Jena, Germany) as previously described (Albrecht et al., 2002).

### Western blotting

Caspase-1 and IL-1\textit{\beta} processing were monitored in trichloroacetic acid (TCA)-precipitated supernatant. \(10^{6}\) macrophages from a single well of a 6-well plate were washed and the medium was replaced with DMEM High glucose without phenol red and without any additional supplement. At 3 h post treatment, the supernatant was collected and precipitated with 10% TCA. The resulting pellet was washed twice with cold acetone and resuspended in NuPAGE LDS Sample Buffer (4X) (Life Technologies). Western blot analysis was performed following standard methods by loading the TCA-precipitated supernatant from \(5 \times 10^{5}\) cells into each well of the electrophoresis gel. The following antibodies were used for blotting: anti-caspase-1 antibody (sc-515) from Santa Cruz Biotechnology (Heidelberg, Germany) used at 1/10 000 dilution and anti-human cleaved IL-1\textit{\beta} antibody (2021S) (Cell Signaling Technology, Danvers, MA, USA) used at 1/1000 dilution. The rabbit anti-human ASC antibody (ALX210-905-C100, Enzo Life Science, Villeurbanne, France) was used at 1/1000 dilution.

### ASC Knock-down in THP-1

shRNA-mediated silencing of ASC in THP-1 cells was performed using pLVTHM lentiviral vector. Sequences of the shRNA targeting human ASC were previously described (Taxman et al., 2006). THP-1 cells were transduced with empty vector or ASC-shRNA pLVTHM. Cells were sorted according to their GFP expression.

### Statistical analysis

Due to the heterogeneity of the primary macrophage responses from different donors (Fig. S10), one experiment is shown that is representative of at least two independent experiments. Statistically significant differences were determined using paired t-tests. Two-tailed P-values are shown. When working with the THP-1 cell lines, averages and standard deviations from four independent experiments are shown. In the latter case, statistically significant differences were determined using paired t-tests. Two-tailed P-values are shown. A multivariate linear regression was performed to assess the influence of PVL, LukE and intact haemolysin beta locus on IL-1\textit{\beta} secretion. Statistical analyses were performed using Prism 5.0a (GraphPad, La Jolla, CA, USA) or SPSS 10.0 (SPSS, Chicago, IL, USA) software. For all analyses, P-values are shown in the figures using the following convention: * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\). Independently of statistical analyses, the impact of PVL, LukE, an intact haemolysin locus and the capsule serotype on IL-1\textit{\beta} secretion is shown in Figs 3 and S6 as independent groupings for purposes of clarity.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** LukF-PV or LukS-PV alone does not trigger IL-1beta release. Primary human HKSA-primed macrophages were treated with LukS-PV alone, LukF-PV alone or LukS-PV and LukF-PV combined (PVL) at the indicated concentrations. IL-1beta secretion was determined by ELISA on supernatant collected 3 h post treatment. One experiment performed in triplicates and representative of five independent experiments performed on different donors is shown. The mean and standard deviation are shown. Unpaired t-test analysis was performed; two-tailed P-value is shown.

**Fig. S2.** THP-1 monocytes are sensitive to alpha-haemolysin-mediated lysis. THP-1 monocytes were treated with increasing concentrations of PVL (1, 10, 100 ng ml–1) or alpha-haemolysin...
(1, 10, 100, 1000 ng ml\(^{-1}\)). Cell death was determined at 3 h post treatment by LDH assay. In contrast to PMA-treated THP-1 cells or to primary monocytes (not shown), THP-1 monocytes are highly resistant to PVL-mediated cytolysis. In contrast to PMA-treated THP-1 cells or to primary macrophages (not shown), THP-1 monocytes are sensitive to \(\alpha\)-haemolysin-mediated lysis. This difference of susceptibility might be due to a higher expression of \(\alpha\)-haemolysin receptors (ADAM10 and caveolin-1) in THP-1 monocytes as compared with THP-1 macrophages (not shown). One experiment performed in triplicates and representative of two independent experiments is shown. The mean and standard deviation are shown. An unpaired \(t\) test analysis was performed, two tailed \(P\)-value is shown.

**Fig. S3.** HlgAB targets murine macrophages in contrast to HlgBC. HlgB (the F component of \(g\)-haemolysin) was combined with one of the S components of \(\gamma\)-haemolysin (HlgA or HlgC) and used at 10 or 100 ng ml\(^{-1}\) to treat murine bone marrow-derived macrophages. Cell death was assessed at 3 h post treatment by LDH assay. HlgBC is cytolytic towards human macrophages (Fig. 1D) yet inactive against murine macrophages. The converse is true for HlgAB. One experiment performed in triplicates and representative of five independent experiments is shown. The mean and standard deviation are shown. An unpaired \(t\) test analysis was performed; two tailed \(P\)-value is shown.

**Fig. S4.** High concentration of LukAB/GH is cytolytic to THP-1 macrophages. PMA-differentiated THP-1 macrophages were treated with PVL, LukED or LukAB/GH at the indicated concentrations. Cell death was assessed at 3 h post treatment by LDH assay. NT: concentration not tested. One experiment performed in triplicates and representative of two independent experiments is shown. The mean and standard deviation are shown. An unpaired \(t\) test analysis was performed; two tailed \(P\)-value is shown.

**Fig. S5.** LukD and LukE are cytolytic to THP-1 macrophages when combined with LukS-PV. LukS-PV at 100 ng ml\(^{-1}\) was combined with one of the following F components at 100 ng ml\(^{-1}\) lukD, LukG or LukF-PV (PVL). Similarly: LukF-PV at 100 ng ml\(^{-1}\) was combined with one of the following S components at 100 ng ml\(^{-1}\) LukF-PV (PVL), lukE, LukH. Cell death was assessed at 3 h post treatment by LDH assay. One experiment performed in triplicates and representative of two independent experiments is shown. The mean and standard deviation are shown. An unpaired \(t\) test analysis was performed, two tailed \(P\)-value is shown.

**Fig. S6.** A non-significant trend is observed between the presence of capsule serotype 5 and the ability of the corresponding strain supernatant to induce IL-1\(\beta\). All the clinical strains collected were either of capsule serotype 5 or 8, as determined by microarray analysis (Table S1). Supernatant from the various strains was normalized, diluted 1/25 in macrophage medium and added onto human HKSA-primed macrophages. IL-1\(\beta\) levels in the supernatant were determined by ELISA at 3 h post treatment. The contribution of capsule serotype was determined as non-significant by multiple linear regression analysis (\(P = 0.0593\)). One experiment representative of two independent experiments performed on two different donors is shown. Each point corresponds to the IL-1\(\beta\) secreted by macrophages exposed to one clinical strain supernatant. The geometric mean in each group is shown.

**Fig. S7.** Binding of LukS-PV onto human macrophages is highly specific. LukS-FITC binding to primary human macrophages was detected by flow cytometry following a 5 min incubation period on ice. A clear shift in fluorescence was observed between untreated macrophages (grey tinted histogram) and macrophages treated with LukS-FITC at 100 ng ml\(^{-1}\) (blue line histogram). The specificity of the binding is shown by competition with unlabeled LukS-PV (1000 ng ml\(^{-1}\)) (Red line histogram). One experiment representative of three independent experiments performed on three different donors is shown.

**Fig. S8.** ASC was knocked-down in two stable THP-1 cell lines. Knock-down efficiency was verified by Western blot analysis on PMA-differentiated THP-1 macrophages.

**Fig. S9.** LPS does not affect the IL-1\(\beta\) response in HKSA-primed primary human macrophages. HKSA-primed macrophages were intoxicated with PVL at the indicated concentrations in the presence or not of LPS at 100 ng ml\(^{-1}\) for 3 h. IL-1\(\beta\) levels in the supernatant were determined by ELISA. IL-1\(\beta\) levels secreted with or without LPS were not statistically different. The mean and standard deviation are shown. One experiment performed in triplicates is shown. An unpaired \(t\) test analysis was performed. Two-tailed \(P\)-value is shown.

**Fig. S10.** PMA-differentiated THP-1 macrophages behave similarly as primary monocyte-derived macrophages. PMA-differentiated HKSA-primed THP-1 macrophages (grey bars) or HKSA-primed monocyte-derived human macrophages (black bars) were treated with \(S. aureus\) supernatant (dilution 1/625) for 3 h. IL-1\(\beta\) levels in the supernatant were determined by ELISA. The mean and standard deviation are shown. One experiment performed in triplicates is shown. From left to right, strains used were as follows: SF8300; BD0415; BD0542; BD0423; BD0405; BD0406; BD0546; BD0419; HT20020209; LUG1800; ST20090065; LUG1672; HT200330579; LUG776; LUG1560; LUG862. An unpaired \(t\) test analysis was performed. Two-tailed \(P\)-value is shown.

**Fig. S11.** An heterogeneity in the primary macrophage responses to PVL is observed between different donors. HKSA-primed monocyte-derived macrophages from eight different donors were treated with PVL at 10 ng ml\(^{-1}\) for 3 h. IL-1\(\beta\) levels in the supernatant were determined by ELISA. For donor 2, 5, 6 and 8, the average and the standard deviations from 2 to 3 independent experiments are shown (showing the variability between independent experiments). With donor 1, 3, 4, 7, only one experiment was performed; the average from triplicate values from one experiment is shown. Within one experiment and for every donor, IL-1\(\beta\) levels in PVL-treated macrophages were statistically different from IL-1\(\beta\) levels in untreated macrophages as determined by an unpaired \(t\) test analysis.

**Table S1.** StaphyType microarray analysis of 32 clinical strains.

**Table S2.** Bacterial strains and primers used for cloning, mutagenesis and recombinant protein production.

**Table S3.** \(S. aureus\) strains used in this study.

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