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Short Communication

SHORT COMMUNICATION

Correlation between cytotoxicity induced by *Pseudomonas aeruginosa* clinical isolates from acute infections and IL-1β secretion in a model of human THP-1 monocytes

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One sentence summary: This study shows that clinical isolates of *Pseudomonas aeruginosa* can induce in parallel cytotoxicity and modulation of cytokine balance in the host cells, depending on the virulence factors they produce.
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ABSTRACT

Type III secretion system (T3SS) in *Pseudomonas aeruginosa* is associated with poor clinical outcome in acute infections. T3SS allows for injection of bacterial exotoxins (e.g. ExoU or ExoS) into the host cell, causing cytotoxicity. It also activates the cytosolic NLRC4 inflammasome, activating caspase-1, inducing cytotoxicity and release of mature IL-1β, which impairs bacterial clearance. In addition, flagellum-mediated motility has been suggested to also modulate inflammasome response and IL-1β release. Yet the capacity of clinical isolates to induce IL-1β release and its relation with cytotoxicity have never been investigated. Using 20 clinical isolates from acute infections with variable T3SS expression levels and human monocytes, our aim was to correlate IL-1β release with toxin expression, flagellar motility and cytotoxicity. ExoU-producing isolates caused massive cell death but minimal release of IL-1β, while those expressing T3SS but not ExoU (i.e. expressing ExoS or no toxins) induced caspase-1 activation and IL-1β release, the level of which was correlated with cytotoxicity. Both

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effects were prevented by a specific caspase-1 inhibitor. Flagellar motility was not correlated with cytotoxicity or IL-1β release. No apoptosis was detected. Thus, T3SS cytotoxicity is accompanied by a modification in cytokine balance for *P. aeruginosa* clinical isolates that do not express ExoU.

Keywords: type three secretion system; inflammasome; ExoS; ExoU; TNF-alpha; flagellin

Pseudomonas aeruginosa is a major cause of healthcare-associated infections, with severe morbidity and high mortality. Its capacity to develop antibiotic resistance limits therapeutic options, leading to treatment failures. Moreover, the *P. aeruginosa* genome encodes several virulence factors and environmental sensor-regulator systems, allowing it to adapt to hostile environments, induce tissue injury and control inflammatory reactions. Studying cellular responses to the virulence factors expressed by clinical isolates is critical in order to evaluate the usefulness of combining antibiotics with specific inhibitors of these virulence mechanisms or immunomodulators.

Type III secretion system (T3SS) is a major virulence factor in *P. aeruginosa,* associated with poor clinical outcome and high morbidity in acute infections. T3SS enables bacteria to inject exotoxins into the host cell cytoplasm (Sawa 2014). Among them, the phospholipase A2 ExoU causes rapid cell death by disrupting membrane integrity, while ExoS prevents bacterial internalization and induces cell necrosis or apoptosis (Okuda, Hanabusa and Gotoh 2014). Beside these toxin-dependent effects, T3SS also delivers flagellin or T3SS rod proteins into the mammalian cytosol, inducing secretion of IL-1β and IL-18 (Miao *et al.* 2008, 2010). Contrary to TNF-α, IL-1β and IL-18 secretion requires two signals (Miao *et al.* 2008), namely (i) Toll-like receptor (TLR) activation (inducing transcription and translation of the proforms of these cytokines) and (ii) NOD-like receptors (NLR) (activating caspase-1 and inducing cytokine processing and secretion, leading to pyroptotic cell death). *Pseudomonas aeruginosa* T3SS specifically activates NLRC4 (NLR family, CARD domain containing 4; also known as Ipaf) inflammasome. In the case of *P. aeruginosa* infection, IL-1β release seems essentially related to this process, as it is totally inhibited in *nlrc4 -/-* macrophages (Faure *et al.* 2014).

Bacteria-induced inflammasome activation and subsequent IL-1β and IL-18 release have been generally considered as protective (Sutterwala *et al.* 2007; Cai *et al.* 2012). Yet, in the case of *P. aeruginosa* infections, these processes are rather deleterious, contributing to increased tissue injury and impaired bacterial clearance (Schultz *et al.* 2002; Cohen and Prince 2013; Faure *et al.* 2014). Moreover, flagellum–mediated motility has been suggested to also modulate inflammasome response and IL-1β release (Patankar *et al.* 2013). At this stage, however, these concepts have been studied only with reference strains and mutants thereof expressing or not specific proteins from the T3SS or the flagellum.

In this context, this study aimed at (i) examining whether T3SS positive-clinical isolates collected from patients suffering from acute infections are also able to induce IL-1β release and (ii) determining whether the IL-1β production is related to toxin expression or flagellar motility, and correlates with the degree of cytotoxicity induced. TNF-α release was also followed as a control because its production is unrelated to inflammasome activation (Akira and Takeda 2004). Human THP-1 monocytes were used as model of phagocytic cells capable to activate NLRC4 inflammasome in response to T3SS.

Table S1 (Supporting Information) shows the strains under study. They include (i) 20 clinical isolates from acute infections retrospectively collected in four Belgian hospitals, (ii) three reference strains, namely PA103 (expressing ExoU), PAO1 and CHA (expressing ExoS, ExoT, ExoY) and (iii) mutants with deletions in genes coding for T3SS toxins (PA103ΔUT; CHAΔSTY), proteins from the translocation apparatus (PA103ΔpcrV; CHAΔpopB/popD) or T3SS regulon (CHAΔExsA). These strains were characterized for the expression of genes encoding toxins, translocation apparatus or flagellin, and for swarming motility in 0.3% LB agar plate. Their serotype and genetic relatedness were determined in order to check for sufficient diversity within the collection (Table S1 and Fig. S1, Supporting Information).

If excluding negative controls (PA103ΔpcrV, CHAΔpopBD, CHAΔExsA), the other strains could be classified in two groups (Table S1, Supporting Information), namely (i) 8 clinical isolates plus PA103 expressing ExoU, ExoT and the translocation apparatus (T3SS+ ExoU+) and (ii) 12 clinical isolates plus PAO1, CHA, CHAΔSTY and PA103ΔUT expressing the translocation apparatus but not the ExoU toxin (T3SS+ExoU−). ExoS+ strains were thus more represented in this collection than ExoU+ strains, and none of them expressed both toxins, as generally observed (Garey *et al.* 2008; El-Solh *et al.* 2012). No toxin was expressed in PA103ΔUT and CHAΔSTY. *exoU*, *exoS* and *exoT* expression levels were highly variable, ranging from 283 to 1% of the values measured in reference strains. Flagellin expression and swimming motility were variable, with no correlation between flagellin expression and motility (Table S2, Supporting Information).

THP-1 monocytes were then incubated with each of these isolates. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) release in the culture supernatant. IL-1β and TNF-α were quantified in supernatants by ELISA. As IL-1β is released in response to caspase-1 activation, cells were also preincubated with 40 μM of the specific caspase-1 inhibitor Ac-YVAD-cmk (N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone, Sigma-Aldrich, Saint-Louis, MO). Incubation time was set at 5 h based on preliminary experiments demonstrating that cytotoxicity was essentially mediated by T3SS in these conditions (see values in Fig. 1; cytotoxicity high for PA103; moderate for CHA and largest difference with that of CHAΔExsA).

Figure 1 shows data for each individual strain (left) or for T3SS+ strains grouped according to the expression of ExoU (right). Considering reference strains, PA103 induced a massive LDH release but a low release of cytokines, which was not affected by Ac-YVAD-cmk. CHA was less cytotoxic but induced a significantly larger release of IL-1β (reversed by Ac-YVAD-cmk) but not of TNF-α. Remarkably, PA103ΔUT and CHAΔSTY showed a profile similar to that of CHA despite the fact they did not express any toxin but still the translocation apparatus. PA103ΔpcrV, CHAΔpopBD and CHAΔExsA were not cytotoxic and induced minimal IL-1β release. All together, these data confirm the role of T3SS apparatus rather than toxins in this

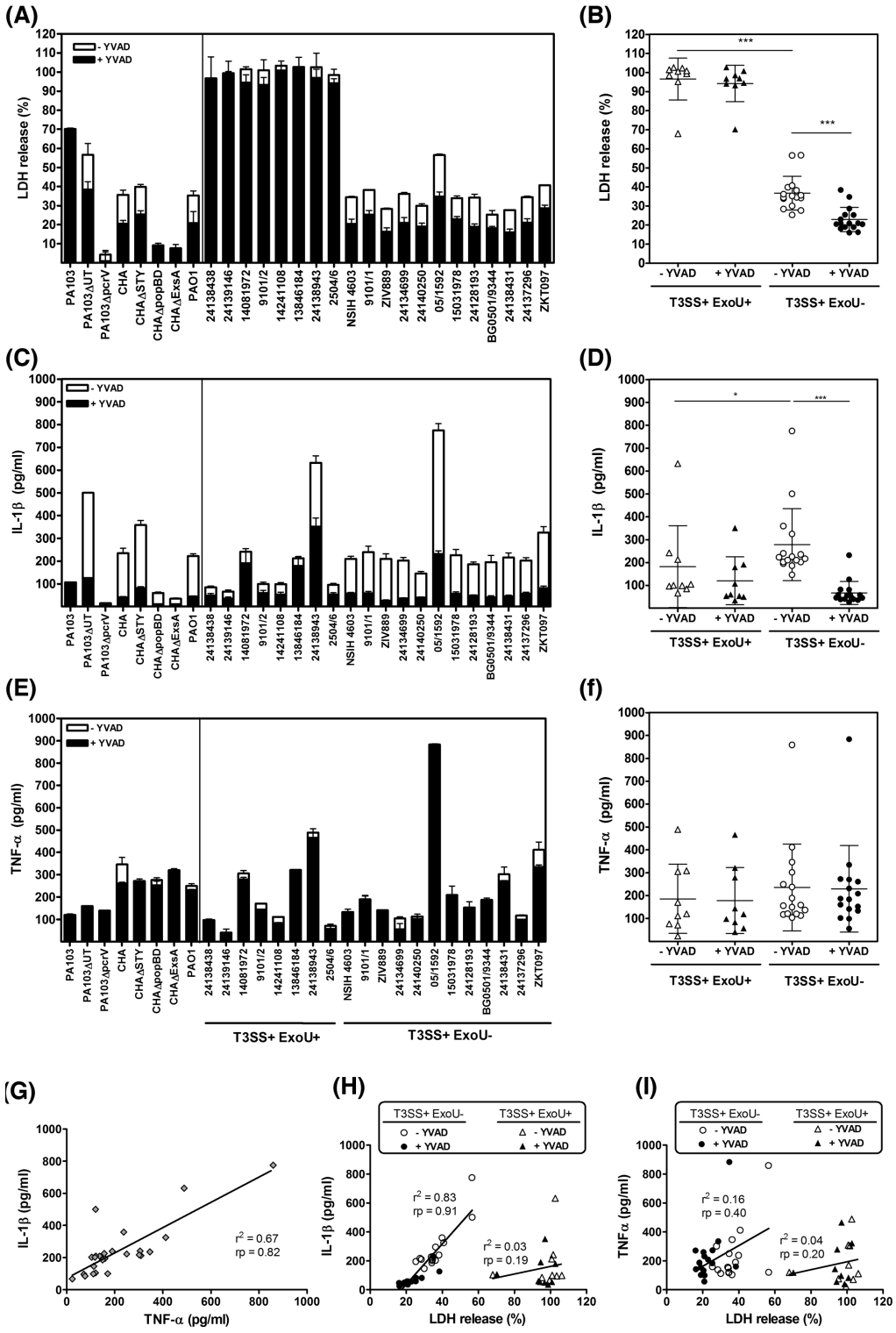

Figure 1. Cytotoxicity and cytokine production induced by *P. aeruginosa* reference strains and clinical isolates. THP-1 cells seeded into 96-well plates (2.5×10^5 cells/mL) were incubated with each of these isolates (10 bacteria/cell) during 5 h. Cytotoxicity was assessed by measuring the release of LDH into the culture supernatant using the cytotoxicity detection kit PLUS (Roche, Basel, Switzerland). IL-1β and TNF-α were quantified in supernatants using a commercially available ELISA kit (R&D systems, Minneapolis, MN and BD Biosciences, San Jose, CA, respectively). Upper panels: percentage of release of LDH (**A** and **B**), IL-1β (**C** and **D**) and TNF-α (**E** and **F**) in the supernatant of THP-1 monocytes exposed during 5 h to clinical isolates (10 bacteria/cell) in the presence of the caspase-1 inhibitor Ac-YVAD-cmk [+YVAD] or in control conditions [−YVAD; DMSO added as the solvent of Ac-YVAD-cmk]. Left panels show the data for individual strains, and right panels for strains grouped according to the expression of ExoU toxin. Values are mean ± SD of representative experiments performed in triplicates and reproduced at three independent occasions at least with similar results. Statistical analyses performed among groups of strains using two-way ANOVA, Bonferroni post-test: *** $P < 0.001$, * $P < 0.05$. Lower panels: correlation between IL-1β and TNF-α release (**G**), IL-1β and LDH release (**H**), and TNF-α and LDH release (**I**) for all strains in control conditions (G) or for strains grouped according to the production of ExoU toxin (H and I) (r2 = coefficient of determination; rp = Pearson correlation coefficient).

process (Miao *et al.* 2008). PA103ΔUT and CHAΔSTY induced a larger IL-1β release than their parental strains, which is compatible with the previously demonstrated inhibitory effect of the toxins on inflammasome activation (Sutterwala *et al.* 2007; Galle *et al.* 2008). Moving to clinical isolates, all T3SS+ExoU+ isolates behaved as PA103, while all T3SS+ExoU− isolates caused only 20–50% LDH release and a commensurate release of IL-1β but not of TNF-α. Again Ac-YVAD-cmk significantly reduced cytotoxicity and IL-1β release for T3SS +ExoU− isolates, with no influence on TNF-α release. Inflammasome activation by T3SS+ ExoU− isolates was confirmed in western blot by the presence of the active form of caspase-1 and of IL-1β in cell culture supernatants, which was not observed for T3SS+ExoU+ isolates (Fig. S2, Supporting Information). As the latter isolates were more cytotoxic, we also checked that inflammasome activation did not occur earlier. To this effect, we followed LDH and IL-1β release over time and showed that they were never inhibited by Ac-YVAD-cmk (Fig. S3, Supporting Information).

In parallel, we looked for the presence of apoptotic cells vs necrotic cells over 5 h of incubation using representative ExoU+ or ExoU− strains and found that cell death was associated with membrane permeabilization (necrosis or pyroptosis) but not apoptosis whatever the incubation time (Fig. S4, Supporting Information), as previously demonstrated for CHA (Dacheux *et al.* 2000).

Potential relationships between toxin or flagellin expression, motility and cytotoxicity or cytokine release were systematically looked for by appropriate statistical analysis of the correlations between pairs of parameters (Table S2, Supporting Information). Globally, a highly significant correlation was observed between IL-1β and TNF-α secretion in the absence of Ac-YVAD-cmk (Fig. 1G). Moreover, the release of IL-1β but not that of TNF-α was correlated with cytotoxicity, whether cells were pretreated or not with Ac-YVAD-cmk, but only for T3SS+ExoU– strains (Fig. 1H/I). Other correlations were not significant.

The main conclusion from this study is that cytotoxicity exerted by T3SS-positive clinical isolates from acute infections is correlated with IL-1β release for strains that do not express ExoU. The high cytotoxicity of T3SS+ExoU+ isolates could rely on the phospholipase activity of the toxin, which may cause cell lysis before any mechanism of cell response could be activated (Sato and Frank 2004). Low IL-1β levels detected after 5 h incubation correspond to its proform released in the medium after cell death, which explains why Ac-YVAD-cmk did not prevent this process. Conversely, cytotoxicity and IL-1β release induced by T3SS+ExoU− strains are largely inhibited by Ac-YVAD-cmk, being consecutive to caspase-1 activation (probably related to NLRC4 inflammasome). Notably also, we did not observe an inverse correlation between toxin expression levels and IL-1β secretion, in spite of the described inhibitory effect of the toxin on inflammasome activation (Sutterwala *et al.* 2007; Galle *et al.* 2008).

We also noticed a correlation between TNF-α and IL-1β release induced by the whole collection. This probably results from a simultaneous production of TNF-α and proIL-1β via TLR activation, because the correlation is lost in the presence of Ac-YVAD-cmk that prevents proIL-1β maturation. We can neither exclude a cytotoxicity related to indirect caspase-1 activation via caspase-11 activation in response to LPS (Kayagaki *et al.* 2013).

On the contrary, we were unable to demonstrate any correlation between flagellin expression level (Miao *et al.* 2008; Cohen and Prince 2013) and flagellar motility (Patankar *et al.* 2013) or between motility and cytotoxicity or cytokine release.

Secretion of ExoU is considered as a marker of high virulence (Schulert *et al.* 2003), but expression of ExoU or ExoS has been both associated with poor clinical outcome (El-Solh *et al.* 2012). We show here that the pattern of cytokines expressed by the host cells in response to T3SS depends, at least *in vitro*, on whether the strain expresses or not ExoU.

Thus, pending for animal studies aiming at confirming these observations and further exploring the consequences of this cytokine dysbalance, our data suggest that inhibiting T3SS functionality by immunotherapy or small compounds (Kline *et al.* 2012) may be more appropriate than blocking specifically toxins. This work opens the door to the study of innovative therapeutic strategy to be combined with antibiotics during acute pseudomonal infections.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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Conflict of interest. None declared.

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Supplementary Table 1 : Characteristics of the strains and of the corresponding patients data

	T3SS status		Strains	Hospital[d]	year of sampling	Serotype [e,f]	Clinical data					mRNA expression							Swimming
							sex	Age (years)	origin	Presenting condition	Underlying condition	T3SS toxins			T3SS translocon			flagellin	(area [cm^2])
												exoU[g]	*exoS*[g]	*exoT*[g]	*popB*[g]	*popD*[g]	*pcrV*[g]	*fliC*[g;h]	
reference strains	PA103 bacground	T3SS+ ExoU+	**PA103** [a]			O : 11						17.5	0.0	2.8	4.8	27.4	1.8	0.0	1.0 ± 0.1
		T3SS+ ExoU-	**PA103ΔUT** [a]			O : 11						0.0	0.0	0.0	3.6	20.8	1.1	0.0	1.0 ± 0.1
		T3SS- ExoU-	**PA103ΔpcrV**[ab]			O : 11						15.8	0.0	1.4	3.4	23.2	0.0	0.0	1.0 ± 0.1
	CHA bacground	T3SS+ ExoU-	**CHA**			O : 6						0.0	57.3	27.2	18.9	96.1	7.6	2.4 (a)	21.7 ± 1.2
			CHA ΔSTY[c]			O : 6						0.0	0.0	0.0	10.4	45.7	8.1	2.1 (a)	18.0 ± 1.2
		T3SS- ExoU-	**CHA ΔpopBD**[c]			O : 6						0.0	17.2	6.8	0.0	0.0	2.1	2.6 (a)	20.3 ± 1.1
			CHA ΔExsA[c]			O : 6						0.0	0.0	0.0	0.0	0.0	0.0	2.9 (a)	18.0 ± 0.8
	T3SS+ ExoU-		**PAO1**			O : 5						0.0	12.5	7.3	6.9	18.5	2.9	8.2 (b)	32.3 ± 3.1
clinical isolates	T3SS+ ExoU+		**24138438**	SL	2014	O : 10	M	0.06	pus	medium otitis	No	28.3	0.0	1.9	2.9	42.9	0.9	1.5 (b)	3.1 ± 0.5
			24139146	SL	2014	O : 8	F	43	wound punction	surgical wound	pseudoarthrosis tibia	15.0	0.0	1.5	1.5	16.8	0.4	0.8 (b)	102.9 ± 1.5
			14081972	SL	2006	O : 11	M	79	bronchial aspirate	pneumonia	Peritonitis	8.7	0.0	3.7	2.1	8.1	1.1	1.9 (a)	4.2 ± 0.7
			9101/2	DG	2013	O : 11	M	67	broncho-alveolar lavage (BAL)	tracheal prosthesis, endobronchial stenosis (colonization)	bi-lung transplantation	7.2	0.0	4.1	3.9	14.2	2.4	0.9 (a)	2.5 ± 0.1
			14241108	SL	2006	O : 11	F	51	wound	surgical wound (tracheostomy)	cranial trauma. Coma.	6.4	0.0	2.6	3.7	12.3	1.4	2.5 (a)	6.0 ± 0.9

		13846184	SL	2006	PA	F	78	bronchial aspirate	pneumonia	diverticulitis; peritonitis with septic shock	1.5	0.0	6.1	2.7	14.2	1.8	3.6 (a)	0.4 ± 0.1
		24138943	SL	2014	PA	M	67	wound swabs	surgical wound	secondary peritonitis with laparotomy	1.2	0.0	0.3	0.2	0.6	0.4	1.9 (a)	11.1 ± 0.6
		2504/6	DG	2010	O : 11	M	75	urine	urinary catheter (colonization)	prostatectomy, complicated urinary tract infection	0.6	0.0	2.4	5.4	11.4	0.4	3.8 (a)	2.2 ± 0.3
	T3SS+ ExoU-	**NSIH4603**	DG	2013	O : 3	M	69	blood	post-operative surgical wound abscess; septicemia	kidney transplantation, femoral endarderiectomy	0.0	162.0	23.8	17.2	149.2	12.3	23.9 (b)	54.3 ± 1.4
		9101/1	DG	2013	O : 6	M	64	abdominal collection	post-operative abdominal collection	acute pancreatitis with septic shock, chronic inflammatory bowel disease	0.0	49.1	18.2	19.4	93.6	7.4	0.3 (a)	2.5 ± 0.2
		ZIV889	ER	2007	O : 6	M	47	ENT-eye swabs	pneumonia	No	0.0	24.0	6.7	6.3	16.4	1.1	2.3 (a)	17.5 ± 2.2
		24134699	SL	2014	O : 1	M	69	wound swabs	wound	peripheral vascular disease	0.0	18.0	8.6	5.9	69.4	0.9	4.5 (a)	6.3 ± 0.1
		24140250	SL	2014	O : 6	M	67	wound swabs	wound (colonization)	liver cirrhosis. liver cancer	0.0	10.8	6 .4	2.4	29.6	0.5	2.9 (a)	18.7 ± 1.3
		05/1592	UZ	2006	O : 2	M	53	blood	septicemia of urologic origin	diabetic nephropathy - kidney transplantation	0.0	10.5	2.5	1.4	8.1	1.4	10.1 (b)	2.2 ± 0.1
		15031978	SL	2007	O : 4	M	54	bronchial aspirate	pneumonia	liver cirrhosis, peritonitis	0.0	10.1	4.5	1.8	6.5	1.5	7.1 (b)	56.6 ± 2.3
		24128193	SL	2014	O : 15	M	9	blood	sepsis	febrile neutropenia. Sarcome Ewing	0.0	7.8	5.6	2.5	19.4	0.3	0.9 (b)	12.9 ± 0.4
		BG0501/9344	ER	2005	O : 16	M	72	bronchial aspirate	bronchitis	COPD, pulmonary arterial hypertension	0.0	3.6	0.8	1.1	3.6	0.5	0.1 (a)	5.5 ± 0.1
		24138431	SL	2014	O : 16	M	25	ear swabs	otitis	tympanoplasty cholesteatoma	0.0	2.1	1.0	0.7	4.2	0.3	7.1 (a)	18.5 ± 0.4

		24137296	SL	2014	O : 3	M	0.6	Urine	viral gastroenteritis	No	0.0	1.6	1.0	0.5	2.6	0.2	3.1 (b)	15.8± 2.8
		ZKT097	ER	2007	AA	M	69	Urine	colonization	Diabetes	0.0	0.7	0.4	0.6	0.5	0.4	7.2 (b)	12.6 ± 0.9

[a] provided by Pr. B. Kazmierczak (Yale University, School of Medicine). Ref : Sutterwala FS, Mijares LA, Li L *et al*, *J Exp Med* 2007; **204**:3235-45

[b] original name: PA103 mutant 1. Ref: Hauser AR, Fleiszig S, Kang PJ *et al*, Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. *Infect. Immun.* 1998; **66**:1413–20

[c] constructed by *cre-lox* recycling antibiotic marker system. Ref : Faure E, Mear JB, Faure K *et al*, Pseudomonas aeruginosa type-3 secretion system dampens host defense by exploiting the NLRC4 coupled inflammasome. *Am J Respir Crit Care Med* 2014 ; **189**:799-811

[d] SL : Cliniques Universitaires Saint Luc, Université Catholique de Louvain, Brussels, DG : CHU Dinant-Godinne UCL Namur, Yvoir ; ER : Hôpital Erasme, Université libre de Bruxelles, Brussels ; UZ : Universitair Ziekenhuis Bussel, Vrije Universiteit Brussel, Brussels, Belgium.

[e] PA: polyagglutination ; AA: autoagglutination

[f] determined by slide agglutination according to the International Antigenic Typing Scheme, using a panel of 16 type O monovalent antisera (Bio-Rad). Reference: Liu et al, Survey of heat-stable major somatic antigens of *Pseudomonas aeruginosa*. Int J Syst Bacteriol 1983; 33:256-264.

[g] Relative quantification of mRNA levels (ratio between the number of copies for the gene of interest and for *rpsL* as housekeeping gene). Bacteria were resuspended in LB medium containing 5 mM EDTA and 20 mM $MgCl_2$ and grown from OD_{620nm} 0.1 to 0.8 with aeration and constant shaking (300 rpm). Total RNA was extracted using the InviTrap® Spin Cell RNA Mini Kit (Stratec Molecular, Berlin, Germany) and reverse transcribed using the Transcriptor first strand kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. RNA purity was checked for the absence of contaminating DNA prior to reverse transcription by PCR amplification of a fragment of the *rpsL* gene. Primers were designed based on the published genome sequences of PAO1 or of *P. aeruginosa* NCGM2.S1 for *fliC-a* (not expressed by PAO1). Amplification reactions were performed in the presence of Sybr Green IQ supermix (Bio-Rad Laboratories, Hercules, CA), using a iCycler iQ Single-Color Real-Time-PCR Detection System (MyiQ™ real time PCR software; Bio-Rad), using *rpsL* to normalize transcript levels.

[h] (a) expression of *a-FliC*; (b): expression of *b-FliC*

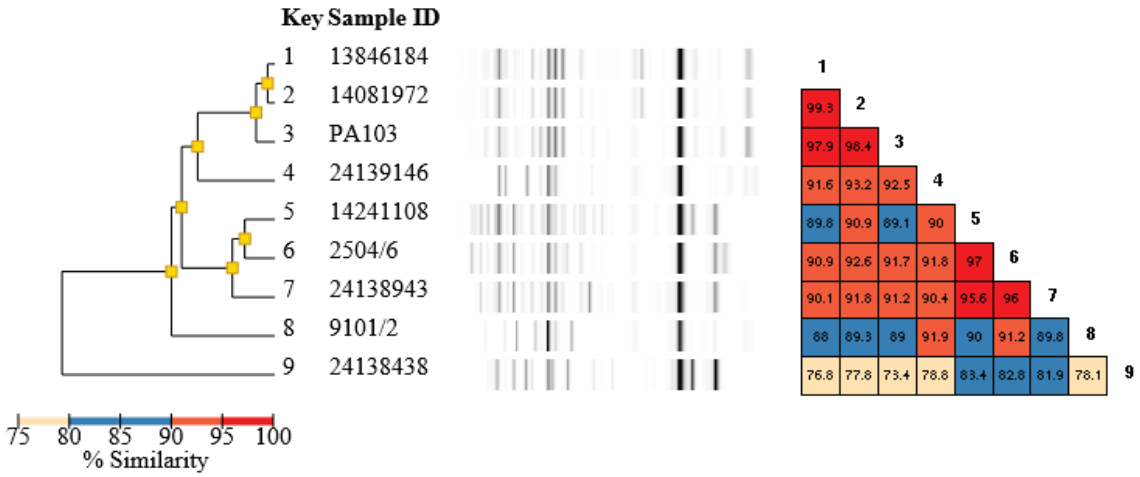
Supplementary Table 2 Correlation between the different parameters for all strains, or within a group of strains (T3SS+ ExoU+ or T3SS+ ExoU-). All data points (cells incubated or not with Ac-YVAD-cmk) were used for these analyses.

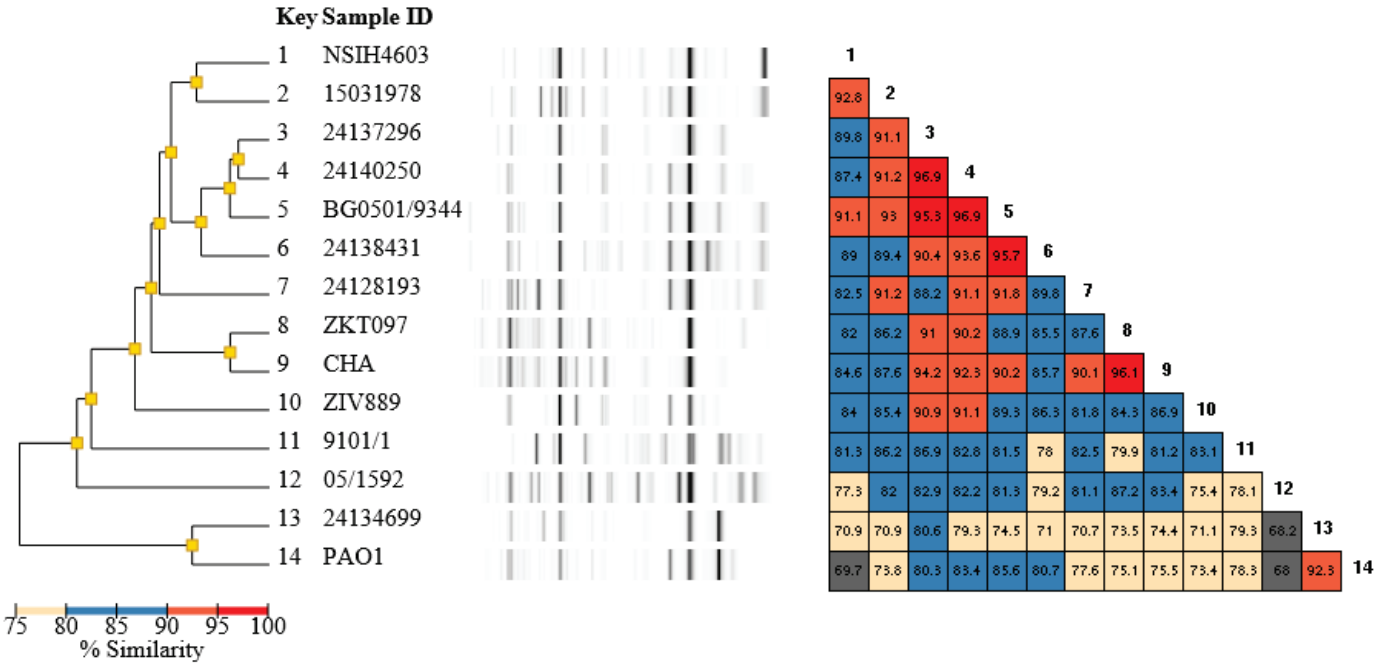
For each combination of parameters, the coefficient of determination r^2 and the Pearson correlation coefficient (rp) were calculated. Values in red point to correlations that are significant; those in italics, to correlations considering both cells pretreated or not with the caspase-1 inhibitor Ac-YVAD-cmk. NA: not applicable.

	all strains	T3SS + ExoU+		T3SS + ExoU-	
fliC(a) expression vs swimming	rp = 0.27 ns	rp = -0.39 ns		rp = 0.43 ns	
	r^2 = 0.07	r^2 = 0.15		r^2 = 0.18	
fliC(a) expression vs IL-1β release	rp = -0.16 ns	rp = -0.20 ns		rp = -0.12 ns	
	r^2 = 0.02	r^2 = 0.04		r^2 = 0.016	
fliC(a) expression vs TNF-α release	rp = 0.00 ns	rp = -0.15 ns		rp = -0.34 ns	
	r^2 = 0.00	r^2 = 0.02		r^2 = 0.12	
fliC(a) expression vs LDH release	rp = -0.11 ns	rp = 0.19 ns		rp = -0.15 ns	
	r^2 = 0.01	r^2 = 0.04		r^2 = 0.02	
fliC(b) expression vs swimming	rp = 0.09 ns	NA		rp = 0.55 ns	
	r^2 = 0.00	NA		r^2 = 0.30	
fliC(b) expression vs IL-1β release	rp = 0.30 ns	NA		rp = 0.10 ns	
	r^2 = 0.09	NA		r^2 = 0.01	
fliC(b) expression vs TNF-α release	rp = 0.21 ns	NA		rp = 0.03 ns	
	r^2 = 0.04	NA		r^2 = 0.00	
fliC(b) expression vs LDH release	rp = -0.42 ns	NA		rp = 0.08 ns	
	r^2 = 0.18	NA		r^2 = 0.01	
exoS expression vs LDH release	NA	NA		rp = 0.00 ns	
	NA	NA		r^2 = 0.00	
exoS expresion vs IL-1β release	NA	NA		rp = -0.10 ns	
	NA	NA		r^2 = 0.01	
exoS expression vs TNF-α release	NA	NA		rp = -0.16 ns	
	NA	NA		r^2 = 0.03	
exoS expression vs swimming	NA	NA		rp = 0.50 ns	
	NA	NA		r^2 = 0.25	
exoU expression vs LDH release	NA	rp = -0.30		NA	
	NA	r^2 = 0.09		NA	
exoU expression vs IL-1β release	NA	rp = -0.12		NA	
	NA	r^2 = 0.03		NA	
exoU expression vs TNF-α release	NA	rp = 0.08		NA	
	NA	r^2 = 0.00		NA	
exoU expression vs swimming	NA	rp = 0.19		NA	
	NA	r^2 = 0.04		NA	
swimming vs IL-1β release	rp = -0.24 ns	rp = -0.24 ns		rp = -0.36 ns	
	r^2 = 0.06	r^2 = 0.06		r^2 = 0.13	
swimming vs TNF-α release	rp = -0.27 ns	rp = -0.43 ns		rp = -0.24 ns	
	r^2 = 0.08	r^2 = 0.19		r^2 = 0.06	
swimming vs LDH release	rp = -0.09 ns	rp = 0.09 ns		rp = -0.34 ns	
	r^2 = 0.00	r^2 = 0.00		r^2 = 0.11	
TNFα release vs IL-1β release	rp = 0.82 p<0.0001	rp = 0.92 p<0.001	*rp = 0.89 p<0.001	rp = 0.78 p<0.001	*rp = 0.54 p<0.01
	r^2 = 0.67	r^2 = 0.84	*r^2 = 0.80	r^2 = 0.62	*r^2 = 0.29
TNFα release vs LDH release	rp = -0.05 ns	rp = 0.20 ns	*rp = 0.20 ns	rp = 0.54 p<0.05	*rp = 0.40 p<0.01
	r^2 = 0.00	r^2 = 0.04	*r^2 = 0.04	r^2 = 0.29	*r^2 = 0.16
IL-1β release vs LDH release	rp = 0.04 ns	rp = 0.30 ns	*rp = 0.30 ns	rp = 0.89 p<0.001	*rp = 0.91 p<0.001
	r^2 = 0.00	r^2 = 0.09	*r^2 = 0.09	r^2 = 0.78	*r^2 = 0.83

Supplementary Figure 1

DNA fingerprint pattern of *P. aeruginosa* ExoU+ (top) and ExoU- (bottom) strains, as evaluated by repetitive extragenic palindromic–polymerase chain reaction exactly as previously described by Riou *et al*, 2010 (In vivo development of antimicrobial resistance in *Pseudomonas aeruginosa* strains isolated from the lower respiratory tract of Intensive Care Unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. *Int J Antimicrob Agents* 36: 513-522).
The figures show the percentage identity among strains. A threshold criterion of 95% similarity was used, corresponding to two or less peak differences in the whole electrophoresis pattern.



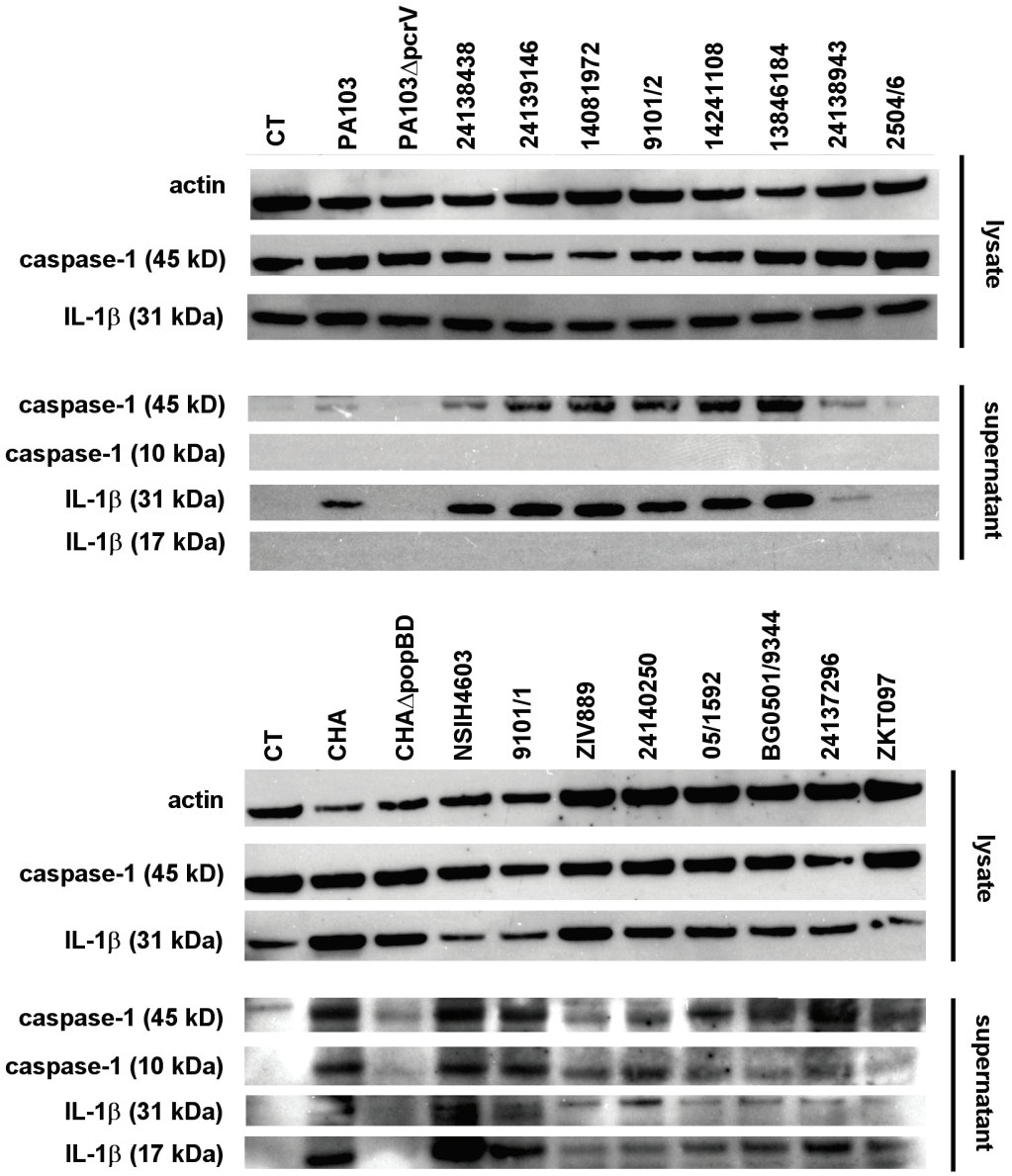


Supplementary Figure 2:
Influence of ExoU+ and ExoU- strains on caspase-1 activation and on IL-1β release and maturation

THP-1 monocytes were incubated during 2 h (T3SS+ExoU+ strains; too high toxicity at 5 h) or 5 h (T3SS+ExoU- strains) in control conditions (CT) or with *P. aeruginosa* strains (10 bacteria/cell). Upper panel: PA103, its deletion mutant PA103ΔpcrV and 8 ExoU+ clinical isolates. Lower panel: CHA, its deletion mutant CHAΔpopBD and 8 selected ExoU- clinical isolates.

Cell lysates were collected in ice-cold PBS and pelleted by low speed centrifugation, resuspended in RIPA buffer (Radio-ImmunoPrecipitation Assay buffer : Tris HCl 25mM pH 7.6, NaCl 150 mM, NP-40 1%, SDS 0.1%, Sodium deoxycholate 1% and a cocktail of protease phosphatase inhibitors diluted according to the manufacturer's instructions [Sigma-Aldrich;product reference P8340]) and subjected to sonication, with gross debris eliminated by centrifugation for 15 min at 14,000rpm (20,000×g). Supernatants (5 mL) were concentrated by centrifugation (10 min; 4900 rpm) through Amicon Ultra 4 centrifugal filters (Merck Millipore, Merck KGaA, Darmstadt, Germany) in order to obtain a final volume of 200 µL. The protein content of both cell lysates and supernatants was measured using the bicinchoninic acid protein assay (Bradford assay; Pierce BCA Reagents, Pierce, Rockford, IL). Appropriate quantities of proteins were mixed to 4X NuPAGE LDS Sample buffer and 10X NuPAGE reducing agent and heated for 10 min at 70°C. Samples were loaded on acrylamide gels (NuPAGE 12% Bis-Tris Gel and 50 mM MES [2-(N-morpholino)ethanesulfonic acid] 50 mM Tris base, 0.1 % SDS, 1 mM EDTA buffer at pH 7.3, Invitrogen, Carlsbad, CA). Proteins were electro-transferred after migration onto a PVDF membrane which was blocked by a 1 h incubation with 5% defatted milk in Tris–buffered saline (TBS; 20 mM Tris–HCl, 500 mM NaCl pH 7.5) containing 0.1% tween-20. Membranes were then incubated overnight with (i) anti-caspase-1 antibody (SantaCruz Biotechnology, Santa Cruz, CA; 1/200 dilution) to detect uncleaved proform (45 kDa) and biologically active cleaved form (10 KDa) of caspase-1 or (ii) anti-IL-1β antibody (R&D systems, Minneapolis, MN; 1/1000 dilution) to detect uncleaved proform (31 kDa) and mature active form (17 KDa), or (iii) anti-actin (Sigma–Aldrich) polyclonal antibody, and then with an appropriate horseradish peroxidase-coupled secondary antibody for 1 h. Blots were revealed by chemiluminescence (SuperSignal West Pico, Pierce, ThermoFisher Scientific Inc.,Rockford, IL).

PA103 and T3SS+ExoU+ clinical isolates induced the release of the proforms of caspase-1 and IL-1β in the supernatant, probably as a consequence of ExoU-induced membrane permeabilization, but not the activation of these 2 proteins, excluding inflammasome activation as a mechanism of cell death induced by these strains. CHA and T3SS+ExoU- clinical isolates induced not only both the release of the proforms of caspase-1 and IL-1β in the supernatant, but also of their active forms, strongly suggesting that cell death occurs for these strains by pyroptosis as a consequence of inflammasome activation.
Note that the release of both pro- and mature forms of these proteins was very low for PA103ΔpcrV or CHAΔpopBD, demonstrating the role of the functionality of T3SS in inflammasome activation and subsequent cytotoxicity.

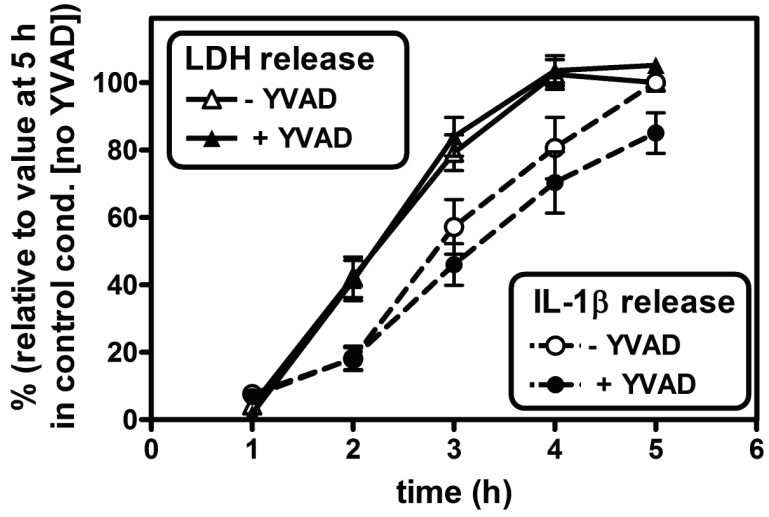


Supplementary Figure S3:
Kinetics of cytotoxicity and IL-1β release induced by ExoU+ strains.

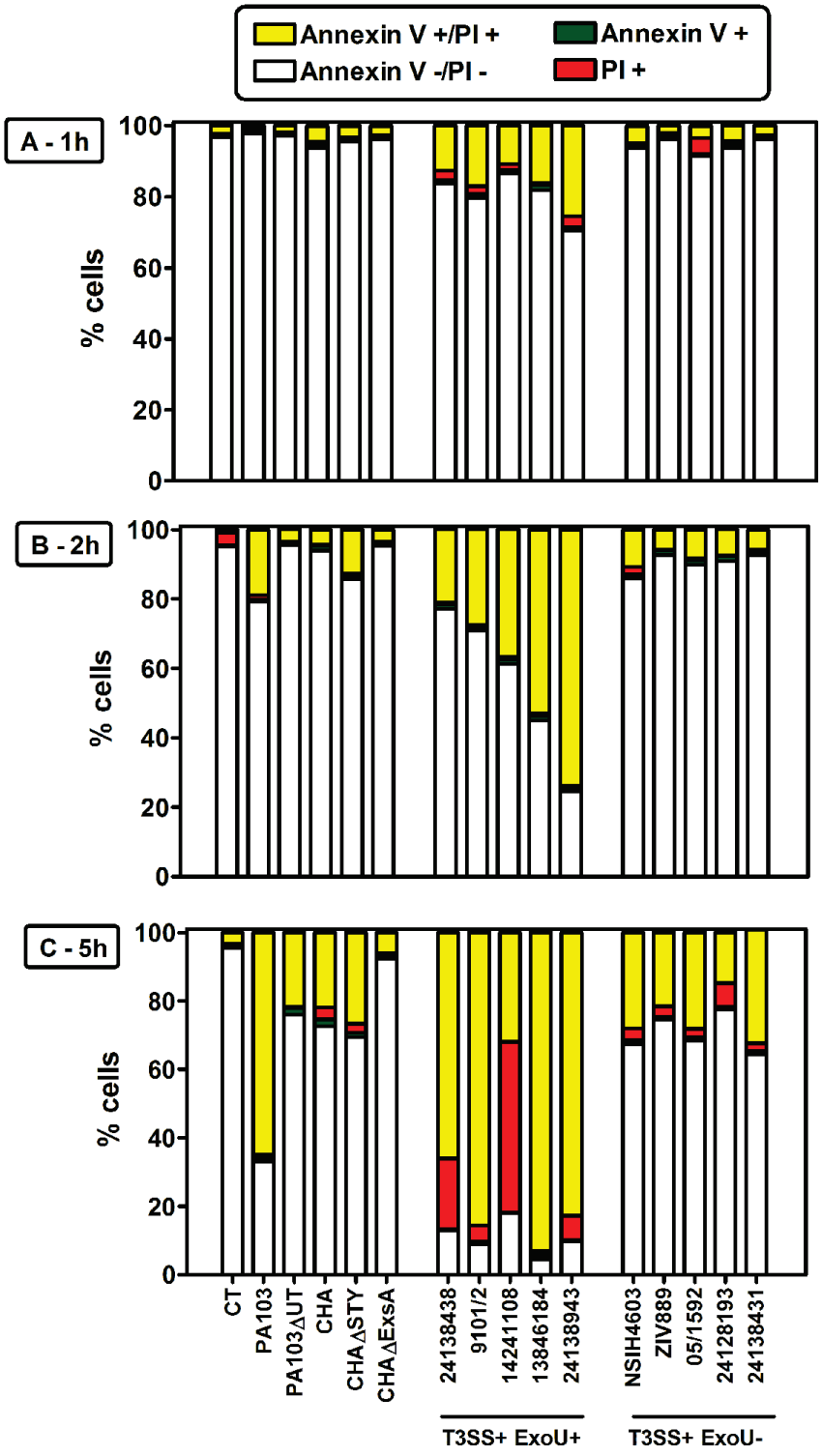
Evolution of cell viability [plain lines] and IL-1β release [dotted lines] over time in cells pre-incubated 1 h in the presence of 40 μM of the caspase-1 inhibitor Ac-YVAD-cmk [+YVAD; closed symbols] or in control conditions [-YVAD (DMSO added as the solvent of Ac-YVAD-cmk); open symbols] and then incubated with PA103 or clinical isolates (n=8) expressing ExoU at an inoculum of 10 bacteria/cell.

Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) into the culture supernatant using the cytotoxicity detection kit PLUS (Roche, Basel, Switzerland). IL-1β was quantified in supernatants using a commercially available ELISA kit (R&D systems, Minneapolis, MN).
Values are means ± SD of two experiments performed in triplicates and expressed as the percentage of LDH release or IL-1β release for the nine strains compared to the maximal value recorded at 5 h in the absence of capsase-1 inhibitor. Statistical analyses: $p > 0.05$ when comparing data obtained in the absence of in the presence of inhibitor over time; two-way ANOVA with Bonferroni post-test.

Cytotoxicity and Il-1β released increased over time but were not reduced by the caspase-1 inhibitor Ac-YVAD-cmk, suggesting that inflammasome activation is not a cause of cell death induced by these strains, even for short incubation times were cytotoxicity is moderate.



Supplementary Figure S4
Type of cell death induced by T3SS+ *Pseudomonas aeruginosa* strains.



THP-1 monocytes at a density of 2.5 x 10^5 cells/mL were incubated with *P. aeruginosa* strains (10 bacteria/cell) for 1h (A), 2h (B) or 5h (C).

The percentage of apoptosis and necrosis was determined by Annexin V and Propidium iodide staining and analyzed by Fluo Cytometry using the FITC Annexin V Apoptois Dectection Kit I (BD Biosciences, San Jose, CA) according to the manufacturer instructions.

Apoptotic cells: Annexin V positive cells (green portion of the bar)
Necrosis or pyroptosis: Propidium iodide positive cells (red portion of the bar) or Propidium iodide and Annexin V positive cells (yellow portion of the bars)
Live cells: Propidium iodide and Annexin V negative cells (white portion of the bar)

All strains caused time-dependent necrosis/pyroptosis but not apoptosis; toxicity was higher and occurred earlier for T3SS+ExoU+ strains than for T3SS+ExoU- strains; it was minimal for T3SS-CHAΔExsA.