The role of Panton-Valentine Leukocidin during lung inflammation

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

Submitted by

Mag.[a] Ana Zivkovic

Supervisor: A.o. Prof. Dr. PhD Sylvia Knapp

Medical University of Vienna, Department of Medicine 1, Division of Infectious Diseases and Tropical Medicine, Währinger Gürtel 18-20, 1090 Vienna, Austria

Center for Molecular Medicine (Ce-M-M-) of the Austrian Academy of Sciences

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1. ABSTRACT

Panton-Valentine Leukocidin (PVL) is a β-barrel pore-forming toxin by *Staphylococcus aureus* (*S. aureus*). PVL is composed of two subunits, called LukF and LukS, which act together and assemble a pore in host cells, thus causing apoptotic or necrotic cell death. The presence of PVL in community-acquired staphylococcal infections is associated with the development of highly lethal necrotizing pneumonia in young immunocompetent individuals. While previous studies revealed that PVL preferably acts on neutrophils, the detailed molecular mechanisms of PVL's biological properties were poorly understood when this work was started.

Therefore, during my PhD studies I aimed to reveal the molecular mechanisms underlying PVL's role during lung inflammation. In the work presented here I show that PVL, independent of its pore forming properties, has the ability to induce inflammation. Strong activation of NF-κB and induction of a small subset of proinflammatory genes suggested that PVL induced Toll-like receptor (TLR) mediated responses. Subsequent investigations revealed that PVL's subunit LukS directly binds to TLR2 and to trigger lung inflammation via involvement of CD14.

The second part of this thesis focuses on the involvement of respiratory epithelial cells, where we discovered that PVL also induces inflammation in a TLR2 dependent fashion. Because intranasal administration of PVL only induced modest inflammation, we hypothesized that other staphylococcal virulence factors might contribute to lung inflammation. We therefore investigated the interaction of PVL with staphylococcal protein A, which was known to be upregulated in the presence of PVL. Our data suggest that protein A synergistically enhanced the inflammatory response by PVL and that this effect depended on the presence of TNF-R1. Further experiments revealed that TNF-α is a key regulator of PVL-induced lung inflammation, as the induction of this cytokine further augments PVL-associated effects via an autocrine and paracrine mechanism.

2. ZUSAMMENFASSUNG

Panton-Valentine Leucocidin (PVL) ist eine Poren-bildenden Toxin das von bestimmten *Staphylokokkus aureus* (*S. aureus*) Stämmen produziert wird. PVL besteht aus zwei Untereinheiten, LukF und LukS, welche gemeinsam in die Membran bestimmter Zellen inserieren können, um hierbei eine Pore zu bilden welche, je nach Konzentration, zum Zelltod führen kann. Die Bedeutung von PVL wurde durch die Beobachtung schwerer nekrotisierender Pneumonien mit oft tödlichem Ausgang bei jungen Patienten, welche durch PVL-sezernierende *S. aureus* infiziert wurden, evident. Während man aus früheren Studien wusste, dass PVL vorallem neutrophile Granulozyten angreift waren die genauen molekularen Mechanismen, sowie die potentielle Beeinflussung anderer Zellen zu Beginn meiner Arbeit schlecht verstanden.

Meine Dissertation beschäftigt sich deshalb mit den genauen molekularen Wirkmechanismen von PVL bei Lungenentzündungen. In der vorliegenden Arbeit wird gezeigt, dass PVL, neben seiner porenbildenden Eigenschaften die Fähigkeit besitzt eine Entzündungreaktion von Alveolarmakrophagen und Epithelzellen zu induzieren. Die starke Aktivierung von NF-κB und spezifische Induktion proinflammatorischer Gene durch PVL legte die Involvierung von Toll-like Rezeptoren nahe. In detaillierten Untersuchungen konnten wir zeigen, dass die PVL Untereinheit LukS direkt an TLR2 bindet und mit Hilfe von CD14 eine Entzündungsreaktion von Alveolarmakrophagen sowohl *in vitro* als auch während einer Lungenentzündung *in vivo* auslöst.

Nachdem wir uns im erste Teil der Arbeit vorallem auf die Bedeutung von Alveolarmakrophagen konzentriert haben, gelang es uns im zweiten Teil dieser Dissertation die Bedeutung von Lungenepithelzellen zu klären. Auch hier zeigte sich, dass PVL, bzw. die Untereinheit LukS, in Epithelzellen eine Porenbildungs-unabhängige Entzündungsreaktion via TLR2 auslösen kann. Im Gegensatz zu myeloischen Zellen ist PVL nicht fähig Epithelzellen zu töten. Im weiteren Verlauf der Studien konnten wir schliesslich zeigen, dass die gleichzeitige Anwesenheit anderer *S. aureus* Virulenzfaktoren, wie Protein A, die Entzündungsreaktion synergistisch verstärken kann. Dieser Synergismus verläuft in Abhängigkeit von TNF-Rezeptor 1, welcher die Anwesenheit von Protein A in der Lunge teilweise mediert. Last but not least entdeckten wir, dass das Zytokin TNF-α einen der wesentlichsten Mediatoren der PVL-induzierten Lungenentzündung darstellt, indem TNF-α die durch PVL induzierte Entzündung sowohl parakrin als auch autokrin verstärkt.

3. AIMS

The general aim of my study was to investigate the molecular mechanisms which are triggered upon PVL challenge and how this may be related to the development of severe pulmonary inflammation.

Aim 1: Firstly, I aimed to study the activation of innate immune responses in the model of PVL induced lung inflammation (role of CD14 and TLR2).

Aim 2: Secondly, I aimed to investigate the role of separate PVL-subunits in inducing an inflammatory response by macrophages.

Aim 3: Finally, I aimed at determining the role of other receptor and virulence factors in aggravating lung inflammation upon PVL challenge and to understand the role of epithelial cells herein (TNFR1).

4. INTRODUCTION

Inflammation is the host response to infection and tissue damage. This well coordinated process leads to the destruction of microbes and tissue repair [1]. Revealing the molecular mechanisms behind this process is one of the most important steps in controlling disease progression and in developing novel treatments. The inflammatory response is divided into four separate phases: 1) recognition of pathogens, 2) recruitment of cells to the site of infection, 3) elimination of microbes and 4) resolution of inflammation and return to homeostasis [2, 3]. In the recognition phase, information is gathered via innate receptors of resident phagocytic cells, such as macrophages. Upon recognition of microbes, these cells become activated and release proinflammatory mediators. Secretion of proinflammatory mediators will lead to the recruitment phase, which is characterized by the migration of leukocytes to the site of infection. Newly arrived leukocytes, together with resident macrophages phagocytose microorganism after which neutrophils undergo apoptotic cell death. Uncontrolled inflammation can damage host tissues, and further progress to cause sepsis, multiple organ failure and death. This is why the rapid and effective inflammatory response is considered the ideal reaction to infection. In the resolution phase, macrophages secrete anti-inflammatory mediators and eliminate necrotic cells, thereby restoring tissue homeostasis. This shift from the inflammatory to the anti-inflammatory phase is currently not fully understood and an active area of research [4].

In higher vertebrates there are two immune systems present that employ different mechanisms and receptors. Both systems are temporally and spatially divided, connected at many levels and have the same aim - defending against invading microorganisms. **Innate immunity** is considered a non-specific defense system, which acts first and is evolutionary older. The evolutionary younger but more specific immune system is called **adaptive immunity.** B- and T-lymphocytes, which are the main effector cells of the adaptive immune system, are stimulated in response to specific antigens derived from pathogens. B-lymphocytes produce antibodies against specific epitopes, while T-lymphocytes have the ability to secrete proinflammatory mediators or have cytotoxic effects against pathogens. In contrast to innate immune cells, lymphocytes have the capacity of memory, which means that specific lymphocytes can be reactivated upon re-encounter of specific antigens, providing a long-lasting defense mechanism [2, 5].

4.1 PULMONARY HOST DEFENSE

Human lungs inhale 10,000 liters of air daily and are continuously exposed to air-borne microorganisms [6]. The inner lung membrane (around 160m^2) is the largest epithelial surface in the body, which is constantly in contact with the outer environment. Small particles or microorganisms that arrive in the lungs are being sensed by alveolar macrophages (AMs), which account for around 95% of airspace leukocytes, while lymphocytes and neutrophils can be found in less than 1% [7]. One of the main roles of AMs is to keep alveoli intact to allow proper gas-exchange. When bacteria are recognized as potentially dangerous, they are recognized via a set of pattern recognition receptors (PRRs), which in turn promotes macrophages to secret proinflammatory cytokines and chemokines and leads to the recruitment of neutrophils and monocytes from the capillary spaces into the alveolar compartments [8]. Proinflammatory molecules secreted from AMs that assist in the recruitment of neutrophils are macrophage inflammatory protein 2 (MIP-2) and monocyte chemoatractant protein 1 (MCP-1). Microbes are additionally recognized via airway epithelium cells that also express PRRs and are capable of secreting proinflammatory mediators. Considering the fact that the lungs are constantly exposed to the outside world with all its microorganisms, respiratory diseases are a relatively rare event, thus illustrating the efficacy of pulmonary host defense pathways.

4.2 CELLS CONTRIBUTING TO INNATE IMMUNITY

Innate immune cells in the lungs are diverse and include structural cells, like epithelial cells and fibroblasts and classical immune cells of myeloid origin, like neutrophils, monocytes and macrophages. Moreover, dendritic cells and mast cells help in the organization of immune responses within the lungs [9]. Cells of the innate immune system have a central role in combating pathogens directly by sensing, opsonizing, phagocytosing and eliminating them and indirectly by secreting different antimicrobial molecules and modulating inflammatory responses [10].

4.2.1 Immune cells

Polymorphonuclear leukocytes (neutrophils or PMNs) are essential for the innate host defense against invading microorganisms. During pulmonary infections, neutrophils migrate from pulmonary capillaries into the air space [11] where they encounter and ingest pathogens. One of the main properties of PMNs is to generate reactive oxygen species (ROS), proteases and

cationic peptides, which are stored in their granules ready to merge with phagosomes and used to eradicate pathogens [12]. Recently, an additional pathway against microbes has been identified and was termed neutrophil extracellular traps (NETs). In this case, PMNs dispose a mixture of chromatin and antimicrobial peptides, which entrap and destroy bacteria in the extracellular space [13]. After fulfilling their tasks, PMNs undergo apoptosis followed by engulfment by surrounding macrophages [14]. While exaggerated and uncontrolled accumulation of PMNs contributes to tissue injury and can cause chronic inflammation, deficits in the number of neutrophils (neutropenia) or their function (chronic granulomatous disease) are predisposing patients for opportunistic lung infections [8]. This is why a better understanding of the molecular mechanisms that induce neutrophil accumulation and neutrophil apoptosis can help to improve treatment of inflammatory diseases.

Alveolar macrophages (AMs) constitute the main population of myeloid cells in the alveolar compartment of lungs [15]. AMs are derived from myeloid precursors in the bone-marrow and have a unique feature. Placed in the alveolar surfactant film between air and lung tissue, they are the only type of macrophages directly exposed to air, where the environment will greatly influence their gene expression pattern and function. Based on their strategic localtion, AMs represent the first line of defense in the lower airway as they recognize the presence of microbes and eliminate inhaled microbes or trigger an inflammatory response upon encounter of virulent bacteria [16]. AM are capable of phagocytosis and intracellular killing of bacteria by oxidative burst [17]. Beside their potent role in mediating inflammation, AM are furthermore important in the resolution phase of inflammation as these cells remove apoptotic PMNs. This anti-inflammatory function of AM was illustrated in a model of pneumococcal pneumonia, where mice without AMs suffered from prolonged inflammation due to an accumulation of apoptotic and necrotic PMNs [18].

Dendritic cells (DCs) play a role as conductors of the immune response. Upon encounter with an antigen, DCs internalize and cleave the antigen. During this process, DCs migrate to the regional lymph node where the antigenic peptides will be presented to lymphocytes together with co-stimulatory molecules leading to the activation of adaptive immunity [19]. Several types of DCs exist in the murine lung. Based on the expression pattern of CD11c, conventional (cDCs, $CD11c^{high}$) and plasmocytoid (pDCs, $CD11c^{dim}$) can be distinguished. In the lungs of resting

mice several distinct DCs subsets were identified, based on the presence of additional surface markers such as CD11b, as well as their anatomical position [20]. During steady state, there are two populations of resident cDCs, intraepithelial and alveolar cDCs, which act as an early warning system for inhaled antigens. Both types can show high or low expression of CD11b ($CD11b^+$ and $CD11b^-$). Intraepithelial cells are easier to study than alveolar, since a pure population of alveolar DCs is extremely hard to isolate due to the predominant presence of AMs. Additionally, another population of pDCs ($CD11b^-$) was isolated in resting lungs, which can migrate to the lymph node and most likely plays a role in immunogenic tolerance [20]. During inflammation, two more populations of DCs are present in lungs: cDCs ($CD11b^+$), which play a role in primary and secondary immune responses and non-migratory pDCs ($CD11b^+$), which participate in the presentation of viral antigens to $CD8^+$ lymphocytes [20, 21].

Mast cells are traditionally associated with allergic reactions but can be activated during the primary immune response. Their strategic position near the epithelial surface or blood vessels, which are common doorways for invading pathogens, makes mast cells suitable for the rapid detection of infection. After recognition of pathogens, mast cells become activated and produce inflammatory mediators which affect vascular permeability of surrounding tissue, induce the expression of adhesion molecules and act chemotacticly on effector cells [22].

Eosinophils were mostly studied in parasite infections and allergic reactions. However, currently these cells are considered to have multifunctional properties and are involved in the initiation and propagation of inflammatory responses. In response to different stimuli, eosinophils are recruitet from the circulation to the site of inflammation by a chemokine mainly secreted from epithelial cells, called eotaxin [23, 24]. Eotaxin is recognized by CCR3 receptors that are highly expressed on eosinophils [25]. Eosinophils constitutively express various PRRs [26] and their activation leads to the release of toxic products from cytosolic granules and the secretion of chemokines and cytokines. Further, eosinophils can either participate indirectly (via inflammatory mediator secretion) in the activation of mast cells or directly by interacting with T cells [27].

4.2.2 Lung cells

Lung epithelium is comprised of epithelial cells (EC) type I and type II, where type I cells cover 97% of the lungs and are involved in conducting airways, while type II cells

(remaining 3%) represent the progenitor cells in the lungs capable to proliferate and replace epithelium (type I cells) and act as secretory cells. Airway ECs play an important role in innate immunity, as they create a monolayer structural barrier and secrete soluble proteins which act against microbes [28]. Through apically exposed PRRs, ECs recognize pathogens and initiate appropriate signaling before immune cells are recruited to the airways [6].

Airway **endothelial cells** are also organized in monolayers, preventing thereby passive movement of small particles and exerting an active role in the inflammatory response. Like other immune cells, endothelial cells also express PRRs, secrete numerous mediators that regulate immune responses and most importantly participate dynamically in leukocyte transmigration [29]. Transmigration of PMNs is a three step process that involves rolling on the endothelium, attachment to the endothelium and transmigration trough endothelial cells. Leukocyte migration begins with tethering and rolling, described as low-affinity interaction of two receptors – leukocytic selectins and endothelial addressins [30]. This event stimulates the expression of receptors called integrins (leukocytic intracellular adhesion molecule-1 (ICAM-1) and endothelial vascular cell adhesion molecule-1 (VCAM-1)), which will mediate a high-affinity bond between endothelial cells and PMNs. Tight binding between leukocytes and endothelium leads to the arrest of leukocytes, which stimulates endothelial cells to firstly produce chemokines to induce leukocyte chemotaxis and to secondly activate protein kinase A (PKA), which will cause cytoskeletal changes in endothelial cells that eventually enable PMNs to pass and migrate to the infected tissue [31].

Fibroblasts (FBs) are the most abundant lung stroma cells, which are responsible for maintaining the lung architectural integrity trough extracellular matrix protein secretion. Secreting different growth factors trough which they interact with epithelial and endothelial cells, FBs also play an important role in processes following inflammation and injury. Secreting cytokines and chemokines , FBs regulate leukocyte transmigration and inflammation [32].

4. 3 INNATE IMMUNE RECEPTORS

To sense endangering molecules, the presence of germline encoded PRR is a prerequisite. PRRs recognize a variety of different pathogen and danger associated molecular patterns (PAMPs and DAMPs). Recently it was proposed to consider pathogen associated molecular patterns (PAMPs) and endogenous molecules, alarmins as a subfamily of DAMPs [33]. PAMPs

are microbial molecules with several important features: they are produced only by microbial pathogens; they are conserved patterns essential for the survival of microbes, often shared by large groups of microorganisms and represent a molecular signature of a whole class of pathogens [34]. These characteristics formulate them as ideal target for the recognition by the innate immune system. Alarmins are equivalent to PAMPs but are secreted from host effector cells and released in (sterile) tissue inflammation, for instance from damaged cells. In the optimal recognition of microbial products there are several families of PRRs described: Toll like receptors, C-type lectin receptors, RIG-I helicases, NOD-like receptors and cytosolic DNA sensors. Below I describe some of these PRRs, with special emphasis on those relevant to this study.

The **Toll-like receptor** (TLR) family is an evolutionary conserved and the best characterized family of PRRs [35]. In the last decade, these type-1 integral transmembrane glycoprotein receptors have been shown to be crucial receptors of PAMPs, sensing diverse molecules, from lipids and lipoproteins to proteins and nucleic acids. TLRs are expressed on various immune cells including macrophages, dendritic cells, B cells, specific subtypes of T cells and even non immune cells like fibroblasts and epithelial cells, where the levels of expression are predominantly determined by environmental factors, pathogens and stress. TLRs consist of an extracellular horseshoe domain, a transmembrane and an intracellular domain that transmit signals to the cytosol. To date, 10 human members and 13 mouse members of the TLR family have been identified in mammals and were subdivided into 6 families based on their sequence similarity, nature of the ligands or subcellular localization [36]. Based on the localization TLRs can be divided into extracellular molecules, like TLR1, 2, 4, 5 and 6, located on the surface of the cell and mainly involved in the recognition of extracellular pathogen, or endosomal receptors, like TLR3, 7, 8 and 9, located endosomally and predominantly recognizing nucleic acids. For all of them, except TLR10, ligands have been identified. TLRs are quite unusual in that some of these TLRs can recognize divergent sets of molecules. For instance, TLR2 is specially studied in response to Gram-positive bacterial infections. In a complex with TLR1 or TLR6 it recognizes triacylated lipopeptides, like Pam_3CSK_4 [37] or diacylated lipopeptides and lipids, like MALP from Mycoplasma or lipoteichoic acid (LTA) [38] and peptidoglycan (PGN) from *S. aureus* [39]. Beside this, TLR2 was shown to play an important role in the recognition of V-antigens from *Yersinia*, haemaglutinin, a protein from measles virus, glycolypids lipoproteins

from *E. coli*, porin from *Neisseria meningitidis* and polysaccharides from *S. cervisiae,* as well as some viral proteins, bacterial toxins or other lipid structures [2]. In viral infections TLR3 was discovered to play a vital role, since it is involved in the recognition of double-stranded RNA, produced by most of the viruses [40].

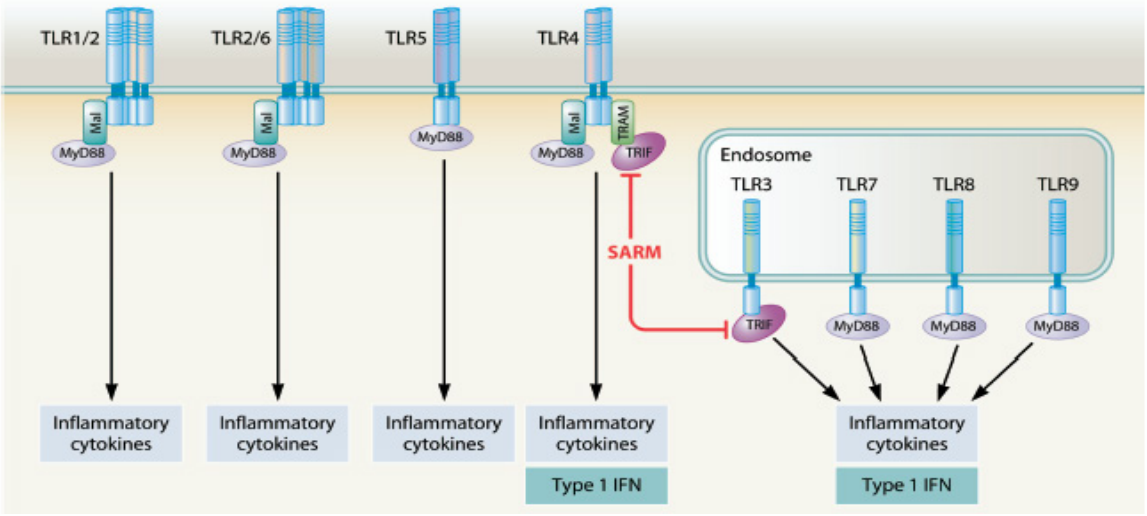


Figure 1. TLRs and adaptor proteins [41].

One of the best studied TLRs in bacterial infections is TLR4 which recognizes lipopolysaharide (LPS) from Gram-negative bacteria in a complex with CD14 and MD-2 [42], several DAMPs [43] and many other structures [2]. TLR5, highly expressed on DCs recognizes bacterial flagellin [44]. TLR7 recognizes imidazoquinoline-like molecules, single stranded RNA from human immunodeficiency virus type 1 (HIV-1), influenza virus and some types of siRNA [45, 46]. Mouse TLR8 is highly homolog to TLR7 but found to be non-functional, while human TLR8 mediates imidazoquinoline and ssRNA recognition [45, 47]. TLR9 recognizes unmethylated CpG DNA motifs [48]. Although the function of TLR10 is not clear, it is believed that because of its structural similarity with TLR1 and TLR6, it probably has a role in the recognition of lipopetides [49]. TLR11, TLR12 and TLR13, have been recently discovered and are believed to be membrane allocated. TLR11 and TLR12 are not precisely distinguished, thought to be the same [49] and shown, in case of TLR11 to be important in the recognition of uropathogenic bacteria and molecules from *Toxoplasma gondii* [50], while TLR13 seems to play a role in viral recognition [51].

Receptor	Cellular localization	Microbial component(s)	Origin(s)
TLRs			
TLR1/TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/TLR6	Cell surface	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	Gram-positive bacteria
TLR2	Cell surface	Lipoproteins	Various pathogens
		Peptidoglycan	Gram-positive and -negative bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	*Neisseria*
		Envelope glycoproteins	Viruses (e.g., measles virus, HSV, cytomegalovirus)
		GPI-mucin	Protozoa
		Phospholipomannan	*Candida*
		Zymosan	Fungi
		β-Glycan	Fungi
TLR3	Cell surface/endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria
		Envelope glycoproteins	Viruses (e.g., RSV)
		Glycoinositolphospholipids	Protozoa
		Mannan	*Candida*
		HSP70	Host
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7/8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria, protozoa
RLRs			
RIG-I	Cytoplasm	dsRNA (short), 5′-triphosphate RNA	Viruses (e.g., influenza A virus, HCV, RSV)
MDA5	Cytoplasm	dsRNA (long)	Viruses (picorna- and noroviruses)
NLRs			
NOD1	Cytoplasm	Diaminopimelic acid	Gram-negative bacteria
NOD2	Cytoplasm	MDP	Gram-positive and -negative bacteria
NALP1	Cytoplasm	MDP	Gram-positive and -negative bacteria
NALP3	Cytoplasm	ATP, uric acid crystals, RNA, DNA, MDP	Viruses, bacteria, and host
Miscellaneous			
DAI	Cytoplasm	DNA	DNA viruses, intracellular bacteria
AIM2	Cytoplasm	DNA	DNA viruses
PKR	Cytoplasm	dsRNA, 5′-triphosphate RNA	Viruses

Table 1. TLRs, RLRs, NLRs and their ligands [41].

The best studied TLR response is the one by LPS. To activate TLR4, LPS has to be in a complex with several proteins, which bind LPS, like LPS binding protein (LBP), CD14, and MD-2. Interaction of these proteins with TLR4 induces TLR4 homo-dimerisation, resulting in the recruitment of adaptor proteins to the cytosolic Toll/IL-1R (TIR) domain. Different TLRs use different adaptor proteins, of which five are known until today to interact with a TIR domain: MyD88, TIR-domain associated protein (TIRAP)/MyD88 adaptor like (MAL), TIR-domain containing adaptor protein inducing IFN-β (TRIF), TRIF related adaptor molecule (TRAM) and sterile alpha and HEAT/Armadillo motif (SARM). TLR4 is the only TLR that recruits both MyD88 and TRIF, while all other TLRs recruit either MyD88 or TRIF. Upon LPS challenge the

MyD88-dependent activation of signaling cascades results in the activation of IRAK4, and degradation of IRAK1 kinase. These events prime TNF receptor associated factor 6 (TRAF6) to create a complex with ubiqutin-ligated enzymes and transforming growth factor β activated kinase 1 (TAK1) activation which will trigger IKK complex and MAPK to control gene regulation [52]. TLR signaling pathways are very complex and tightly controlled. One way to manage this complexity is to regulate proteins involved in recognition and their adaptor proteins on a posttranslational level, like phosphorylation and ubiquitination [53].

Retinoic acid inducible gene I (RIG-I) like receptors (RLR) are cytoplasmic proteins that recognize dsRNA, that are e.g. produced during viral replication. The RLR family consists of RIG-I and MDA-5, and it is believed that RIG-I recognizes shorter, while MDA-5 recognizes longer (>2kb) dsRNA molecules [54]. Cells lacking RIG-I or MDA-5 are not able to recognize viruses like Sendai or influenza virus [2, 55]. RLRs can recognize the dsRNA analogue poly [56] [56], whereas RIG-I signals the presence of 5'-triphosphate (5'3P) end, which is in case of host RNA. RLRs consist of a N-terminal caspase recruitment domain [57], a helicase domain and a C-terminal repressor domain. Upon activation of RLR, CARD associates with CARD-containing adaptor protein MAVS, which is localized in mitochondrial membranes, ultimately resulting in TBK1, IKKi and IRF triggered induction of interferon production. Beside this, RLRs can also trigger NF-κB by activating Fas-associated death domain (FADD) protein and caspase 8 and 10 [58].

Nucleotide binding oligomerisation domain (NOD) like receptors (NLR) are cytoplasmic receptors, which recognize intracellular microbial PAMPs and endogenous danger signals. Members of this family, coded by 22 human and even more mouse genes, share a central nucleotide binding and oligomerisation domain (NACHT), C-terminal leucine-rich repeats (LRR) and N-terminal caspase recruitment or pyrin (PYD) domains [59]. The NACHT domain is of a similar structure for all members of NLR family and enables activation of the signaling. LRRs are important in ligand recognition, similar to TLRs, and can even synergize with TLRs to increase cytokine production [60]. Recognition of ligands via NOD1 and NOD2 leads to the activation of NF-κB. Certain NLR family members can form cytosolic macromolecular complex, called the inflammasome. The inflammasome is involved in caspase-1 activation, which is required for cleavage and activation of the inactive forms of IL-1β, IL-18 and IL-33 [61]. Up to

date, four cytoplasmic receptors have been discovered to take a part in inflammasome formation: NLR pyrin containing 1 (NLRP1, also called NALP1), NLRP3 (also called NALP3), NLR CARD 4 (NLRC4, also called IPAF) and absent in melanoma 2 (AIM2), where the latter doesn't belong to the NLR family [62, 63]. One of the best studied inflammasomes is NLRP3. It can be activated by variety of molecules, like microbes, crystallized or aggregated substances pore forming toxins, necrotic cells or extracellular ATP. It seems that low intracellular potassium concentration can trigger the activation of NLPR3, upon which NLPR3 assembles with caspase-1 and apoptosis spec protein with CARD domain (ASC).

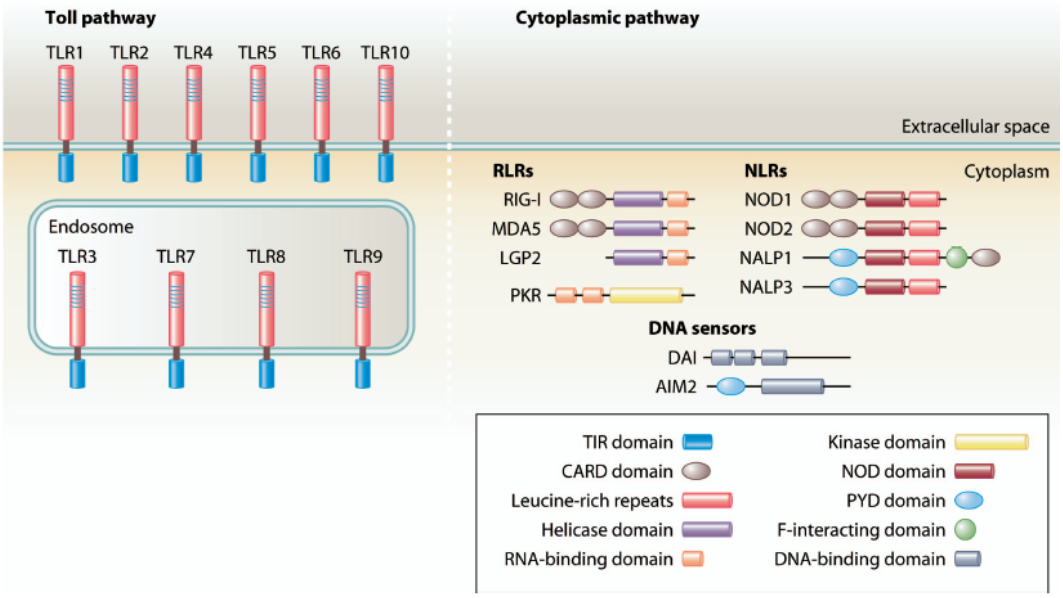


Figure 2. An overview of cellular PRRs [41].

It is understood that IL-1 induction involves the activation by two signals: one, caspase-1 activation and second, TLR, NLR or cytokine receptor activation [62]. In the case of bacterial pore forming toxins, the following scenario is likely to occur: pore formation on a membrane can induce changes in intracellular potassium concentration, which is being sensed by NLRP3, while other bacterial products or toxins themselves can activate TLRs, leading to the activation of inflammasome.

4. 4 INFLAMMATORY MEDIATORS

Inflammatory mediators are small diffusible molecules that act locally at the site of inflammation and at more distant sites. They are usually released from the host cells upon

recognition of microorganisms, thereby controlling accumulation and activation of other cells. Depending on when the mediators are secreted during inflammation, there can be early (6-12h) or late (>12h) phase mediators. Beside this, inflammatory mediators can be already present at the site of infection in an inactive form (i.e. components of complement, coagulation and kinin systems).

Cytokines are a large heterogeneous group of protein mediators that play a vital role in almost any response to invading microorganisms, tissue damage as well as repair mechanisms. Many cytokines have overlapping and pleiotropic biological roles, which mean they can have the same functional effects and can compensate each other's functions. Beside this, cytokines function in cascades and have different effects depending on the concentration. Once synthesized, they are rapidly secreted, since mRNA coding for the cytokine is unstable. Nevertheless, their production can be additionally regulated by post-transcriptional mechanisms [64]. Most cytokines execute their function close to the site they are produced at (autocrine action), on a nearby cell (paracrine action) or if entering the circulation, away from the site of the production (systemic action). Cytokines bind to their receptors with high affinity, which is why small quantities are sufficient to elicit biological effects. Some of the most important cytokines in the early phase of inflammatory response are TNF-α, IL-1β and IL-6. They share many functions and act on monocytes, epithelial cells or others, respectively, in order to activate these cells to eradicate microorganisms.

TNF-α is the main mediator of the acute inflammatory response to bacteria.The principle biological role of TNF-α is to stimulate the recruitment of neutrophils and monocytes and to activate the antimicrobial properties of these cells. Produced in a large amount during severe infections, TNF is responsible for multiple clinical symptoms. Mononuclear phagocytes are the main source of TNF-α, where this molecule is produced as a membrane protein that is later cleaved and released as a 17kDa polypeptide. These 17kDa polypeptides form triple chain resulting in 51kDa soluble TNF-α. The TNF-α receptor for this soluble form is TNF-R1, while the membrane form of TNF-α is recognized by TNF-R2. Cytokine binding to the receptor leads to the recruitment of the proteins called TNF receptors associated factors (TRAFs), which activate transcription factor NF-κB and AP-1. Mice lacking TNF-R1 show impaired survival in

many animal models of bacterial infections, while TNF-R2 deletion mutants have less severe phenotypes.

IL-1 has similar functions as TNF-α, with whom it acts synergistically. Biological effects depend on the cytokine concentration. At lower concentration IL-1 functions as a mediator of inflammation on endothelial cells and induces increased expression of surface molecules that mediate leukocyte adhesion. At higher concentrations IL-1 causes fever, induces the secretion of acute phase proteins from the liver and is involved in metabolic wasting [65]. There are two forms, IL-1-α and IL-1ß, which have less than 30% homology. Both cytokines are secreted in an inactive, pro-IL-1 form. Pro-IL-1α mostly remains bound to the membrane of cells and acts as an autocrine growth factor, while IL-1b is largely found in the circulation, after it was activated by IL-1β converting enzyme (ICE, caspase-1). Several mechanisms are responsible for release of IL-1β, but the most important one is the activation of the inflammasome, an intracellular protein complex that can activate caspase-1, which – as described above in more detail - regulates the release of IL-1β, together with other cytokines like IL-18 and IL-33 [66]. Circulating IL-1β binds to IL-1R I and IL-1R II. IL-1R I is biologically active, and triggers the activation of JNK/SAPK and p38, and subsequently NF-κB and AP-1 gene transcription. On the other hand, IL-1R II has a short intracellular domain and is biologically inert, mainly acting as a competitor for IL-1R I [67].

One of the initially discovered cytokines is **IL-6**. Almost any cell can produce IL-6, the most prominent sources are macrophages, monocytes, and fibroblasts [68], IL-6 has a major role in several acute and chronic diseases. During acute inflammatory response, IL-6 is secreted together with other proinflammatory cytokines, TNF-α and IL-1β and participates in the control of proinflammatory cytokine secretion and upregulation of antiinflammatory molecules which interfere with TNF-α and IL-1β signaling. Furthermore, a complex of IL-6 and its receptor IL-6R was shown to play an important role in the recruitment of leukocytes, where it activates endothelial cells to secrete interleukin 8 (IL-8) and monocytes chemoattractant protein *(*MCP-1) [69]. IL-6 is one of the most important inducers of acute phase proteins. More recently IL-6 was identified as a critical mediator in inducing Th17 cells and preventing differentiation into T_{reg} cells [70, 71]. Th17 are important effector cells that mediate leukocyte recruitment and are importantly involved in several autoimmune diseases.

Chemokines are chemoattract molecules that comprise almost 50 family members with low molecular weight (up to 11kDa) and secreted in nanomolar range. Their main role is to recruit leukocytes to the site of infection. Migration of cells is synchronized according to the chemical gradient of chemokines, which stimulates polymerization and depolymerization of actin filaments. Traditionally chemokines are classified based on the number and location of N-terminal cysteine residues in two bigger families CXC and CC chemokines and two smaller families CX3CL and XCL chemokines. The most critical chemokines for neutrophil and monocyte recruitment are MIP-2, MCP-1 and IL-8.

MCP-1 (CCL2, A2) belongs to the CC family of chemokines and attracts monocytes and PMNs. MCP-1 is primarily produced by epithelial cells in NF-κB regulated manner and binds to CCR2 and CCR4 receptors [72, 73]. MCP-1 is involved in various inflammatory diseases like rheumatoid arthritis and arthrosclerosis [69, 74, 75], but also lung inflammation where it plays an important role in the resolution and repair processes [73, 76].

MIP-2 (CXCL2) was discovered as a 6kDa chemokine, secreted by macrophages treated with LPS [77]. It strongly attracts PMNs trough the interaction with CXCR2, which is mainly expressed on granulocytes [78].

IL-8 (named KC in mice) is primarily produced by mononuclear phagocytic cells, epithelial and fibroblasts [79]. IL-8 binds with low-affinity to CXCR1, a receptor which is highly specific for IL-8, and with high-affinity to CXCR2, which also binds other CXC class chemokines. Binding to receptors is determined by the concentration IL-8: at high concentration IL-8 preferentially binds to CXCR1, while at low concentration CXCR2 is the binding partner of IL-8 [80].

4.5 TRANSCRIPTIONAL REGULATION OF INFLAMMATION

Upon stimulation of cells with bacterial ligands, specific pathways are being triggered, directing toward the activation of different transcriptional factors (TFs) which will cause changes in gene transcription and secretion of different products that mediate host defense mechanisms. The best explored TF is nuclear factor κB (**NF-κB***),* a central regulator of inflammation that is typically activated by TLRs. The mammalian NF-κB family comprises five Rel proteins: RelA (p65), RelB (p100), c-Rel, p50 and p52, which can form homo- and heterodimers depending on

the stimuli. In resting cells the NF-κB dimer (RelA/p50) is bound to IκB-α and kept inactive. Upon activation via e.g. TLRs the IκB kinase (IKK) complex gets activated, leading to the phosphorylation of IκB-α, and subsequent ubiquitination and proteosomal degradation of IκB. This in turn allows RelA/p50 to be released and able to translocate to the nucleus, where it can bind to respective DNA sites to induce gene expression of cytokines and chemokines [81].

Beside NF-κB, other TFs, like activating protein-1 (AP-1) and interferon regulatory factors (IRFs) also play an important role in response to PRR activation.

AP-1 is a dimeric protein complex that acts as a TF and bind to the DNA recognition site, called phorbol 12-O-tetradecanoate-13-acetate (TPA) response element (TRE) *t*hat participates in processes like cellular growth, differentiation and death [82]. *A*ctivation of AP-1 occurs via *t*wo mitogen activated protein kinases (MAPKs), JNK/SAPK and p38 [83] whose phosphorylation leads to the phosphorylation of AP-1 proteins, transcription of inflammatory genes that, depending on the stimuli, act synergistically with NF-κB to enhance the proinflammatory response [54].

IFN regulated factors (IRFs) are involved in host defenses against viruses and pathogens, but also play a role in cell differentiation and tumor development. There are nine IRF family members in mammals (IRF1-9), which share similar structures and two domains, DNA- and IFN-binding domain [84]. IRFs were identified as regulators of IFN production, yet they have much broader role. The classical activation of IRFs occurs via ligands which are recognized by RIG-I/MDA-5 cytosolic receptors that activate TBK1 and IKKε, subsequently leading to the dimerization and phosphorylation of IRF3, IRF5 or IRF7 [85]. Additionally, TLR3 and TLR4 signaling via TRIF are well studied pathways that phosphorylate IRF3 and 7, thus leading to the type I IFN production [86]. Furthermore, TLR7 and TLR9 can utilize the MyD88-dependent pathway to induce IRF7 activation [85, 87].

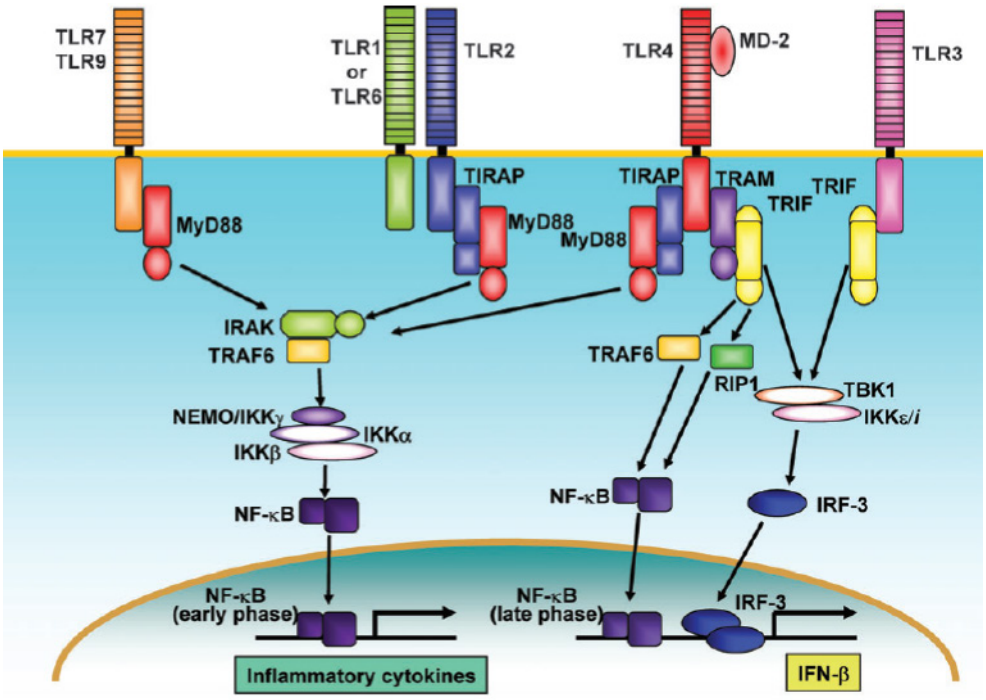


Figure 3. NF-κB, AP-1 and IRF3 activation pathways upon TLR stimulation [36]**.**

5. STAPHYLOCOCCUS AUREUS

Staphylococcus aureus (*S. aureus*) was discovered more than a century ago. It is distinguished from the other staphylococcal species on the basis of its gold pigmentation as well as positive tests for coagulase, mannitol fermentation and deoxyribonuclease tests [88]. Beside structural genes, the staphylococcal circular chromosome carries genes for virulence and resistance to antibiotics. *S. aureus* is a prevalent bacterium carried by humans and can cause numerous diseases, from mild skin infections to serious diseases including boils, abscesses, food poisoning, wound infections, toxic shock syndrome, endocarditis, osteomyelitis and pneumonia. It presents one of the leading causes of human bacterial infections worldwide and is the most frequently isolated bacterium in the hospital setting [89, 90]. Although 20% of people are persistently colonized and around 60 % are intermittent carriers [91], *S. aureus* is considered to be a potentially dangerous pathogen in humans. The primary site of infection is often via the skin or mucus membranes that may lead to mild skin and wound infection but can also infect deeper tissues of the human body causing osteomyelitis, endocarditis or pneumonia [88].

5.1 ANTIBIOTIC RESISTANCE IN S. AUREUS STRAINS

The emergence of antibiotic-resistant bacterial strains became an important problem in human health [92, 93]. Trough evolution bacteria became capable to defend themselves from naturally occurring antibiotics by acquiring resistance either from *de novo* mutation or trough the exchange of genetic material with other bacteria [94, 95]. However, in the last couple of decades, the frequency of antibiotic resistant bacteria increased and multi-drug-resistant strains have emerged in several species that cause infections in humans. It is assumed that around 95% of *S. aureus* worldwide is resistant to penicillin, and 60% to its derivative methicillin [96] and that the origin of new resistant determinants can likely be from other human cultures of *S. aureus* rather than animal reservoirs [97]. One of the most important causes of nosocomial infections in public health today is methicillin-resistant *S. aureus* (MRSA), first isolated in 1961 in the United Kingdom [98]. Resistance of *S. aureus* to methicillin developed shortly after its introduction into clinical practice [99]. Opposite to plasmid-encoded penicillinase, the methicillin resistance gene, *mec*, is encoded by the chromosome. Gene expression analysis showed that the *mec* gene has integrated into several different chromosomal backgrounds, methicillin susceptible *S. aureus* (MSSA), [100] and is very polymorphic with so far five clones of staphylococcal cassette chromosome *mec* (SCCmec) I-V, present in the population [101].

MRSA was first discovered in hospital-acquired (HA) infections (HA-MRSA). Only recently, community acquired MRSA (CA-MRSA) strains emerged that were later recognized as a main cause of community-acquired pneumonia [102], especially in the US, while in Europe the frequency of CA-MRSA is currently relatively low [103]. New CA-MRSA strains behave differently to HA-MRSA strains; they are more virulent and can cause skin and soft tissue infections with unusually severe pathology and life-threatening invasive infections including necrotizing pneumonia [104] . The origin of CA-MRSA is a subject of a debate: either from hospital or community isolates or as a consequence of horizontal transfer of the methicillin-resistance determinant. To explain the global distribution of CA-MRSA, extensive studies have been conducted, especially in association with virulence factors known to be related with CA-MRSA [105-108].

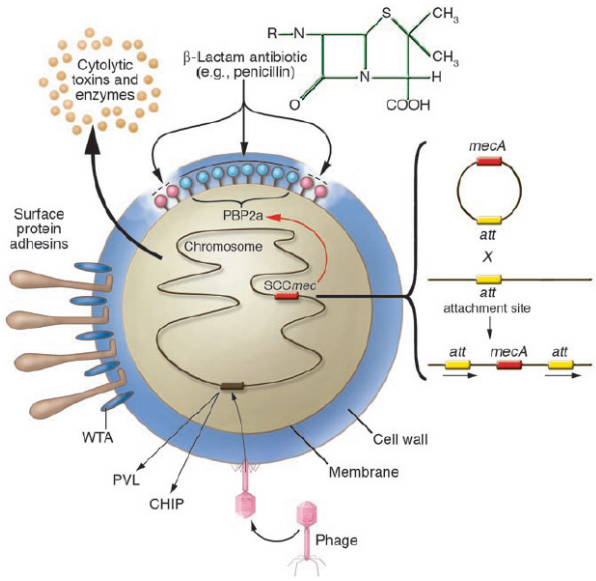


Figure 4. Schematic illustration of *S. aureus* methicillin resistance acquirement and expression of virulence factors [109]. Abbreviations: PVL, Panton-Valentine Leukocidin, CHIP, chemotaxis inhibitory protein; WTA, wall-associated teichoic acids.

The most prevalent stains, determined by multilocus sequence typing (MLST) are ST80 in Europe, ST8 (USA300) and ST1 (USA400) in the US and ST30 in Southwest pacific region (Figure 4). The worldwide dissemination and increasing prevalence of CA-MRSA clones suggest that these strains are easier for transmission and will probably remain a problem in the next decade.

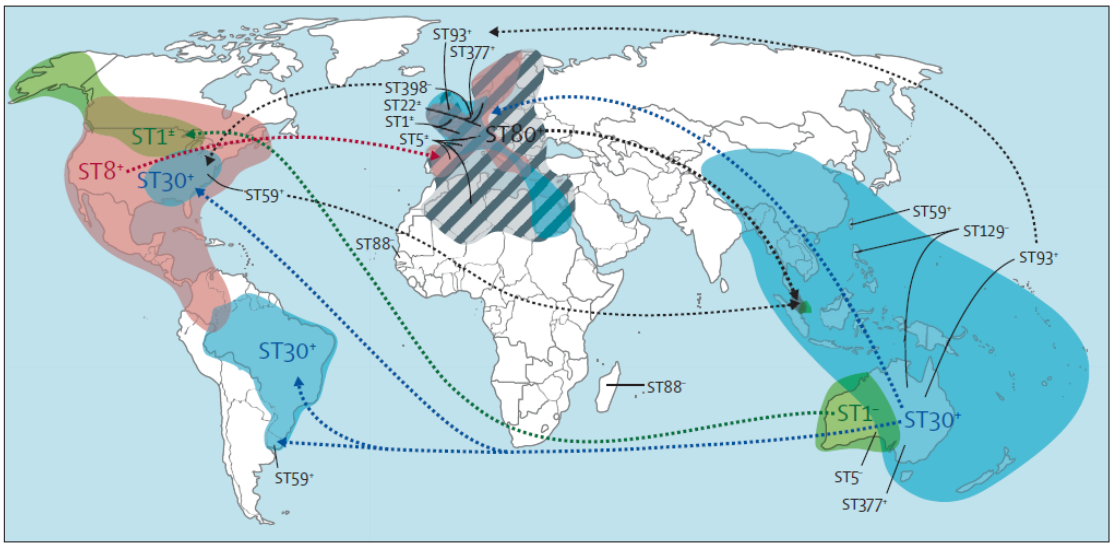


Figure 5. Global distribution of CA-MRSA clones as determined by MLST. Estimated areas of infections with main strains -ST80 (gray hatched), ST8 (red), ST1 (green) and ST30 (blue) and PVL gene distribution- + (PVL positive), - (PVL negative) and ± (PVL positive and negative strains). Dotted lines present possible paths of dissemination [107].

5.2 PATHOGENICITY OF S. AUREUS

Pathogenic microorganisms possess an astonishing armory of virulence factors that facilitate host evasion. Virulence factors are particular gene products that enable microorganism to establish and enhance its potential to cause disease. In case of *S. aureus*, these are **surface proteins** that promote adhesion to damaged tissue and to the surface of host cells [110]; extracellular proteins like **enzymes,** which can facilitate tissue damage and spreading of the pathogen; **membrane-damaging toxins,** which can cause host cell cytolysis; and **superantigens,** which can contribute to the symptoms of shock [88, 111]. Spatial, temporal and combinatorial expression of virulence factors may increase the selective advantage of bacteria over the host organism. One of the main regulators of virulence factor expression is the **accessory gene regulatory (*agr*)** locus**.** Since most of the virulence factors are secreted in the post-exponential phase of bacterial growth, the principal role of the *agr* locus is to control protein production of bacteria, which is done via growth-sensing octapeptides [112]. To shift from the exponential phase, rich in cell wall proteins, protein A and cellular binding protein secretion, to the stationary phase, rich in expression of RNA III and up-regulation of exoprotein production, it is important for the bacteria to change the strategy: colonization to local invasion [113]. This means that surface proteins are predominantly synthesized during the exponential growth phase and secreted proteins are synthesized during stationary phase. It is not clear how *agr* emerged but it is believed that it arose due to environmental adaptation [114-116]. Depending on the strain, *S. aureus* can secrete various virulence factors. The most interesting virulence factors that have been studied in relation with pulmonary diseases are listed beneath.

5.2.1 Surface Proteins - Staphylococcal Protein A

Staphylococcal protein A (spa) is a major surface protein of *S. aureus*, covalently anchored to peptidoglycans. It is a vital virulence factor and belongs to the cell wall-associated virulence factors. Protein A possesses five domains homologous to IgG that can interact with the Fc portion of host IgG [117]. This enables bacteria to coat the host cell surface with IgGs in false orientation, illustrating the anti-phagocytic effect of protein A. Purified protein A elicits secretion of various pro-inflammatory cytokines (IL-1β, IL-8, IL-4, IL-6, IFN gamma and TNF-α from monocytes and fibroblasts) [118]. Intravenous administration of protein A deficient *S. aureus* causes lower mortality than WT strains. Bacterial isolates from patients with pulmonary infections show an increase in protein A expression [119] suggesting that this virulence factor

might play an important role in the pathophysiology of pneumonia. Protein A expression is firmly regulated by the ***spa* locus**. Recently, there has been a new regulator discovered, a DNA binding protein, XdrA that is conserved in all *S. aureus* genomes, which by directly acting on the spa promoter appears to be its major activator [120]. A receptor for protein A is not known but it was reported that, beside activation of pathways downstream of epidermal growth factor receptor (EGFR) [121], parts of protein A can bind to TNF-R1 expressed on respiratory epithelial cells [122]**.** Activation of TNF-R1 significantly contributes to it's mobilization and shedding, leading to IL-8 induction, recruitment of PMNs into airways and finally to the induction of pneumonia. Staphylococcal protein A mutants or mice lacking TNF-R1 do not develop pneumonia when treated with *S. aureus* intranasally [123].

5.2.2 Membrane-damaging Toxins

S. aureus produces many cytotoxic molecules which can be divided into two classes: hemolysins and leukocidins.

Alpha hemolysin is the best investigated *S. aureus* cytotoxin made by most of the strains and lethal for a variety of mammalian cells, especially rabbit erythrocytes [124]. Alpha hemolysin is found to be responsible for pneumonia, sepsis, septic arthritis, brain abscess and corneal infections [125, 126]. Secreted in a monomeric form, alpha hemolysin integrates in the membrane of the target cell and creates a cylindrical homoheptamer, which will develop into a 1-2nm pore [127]. The binding of monomers occur in two ways depending on the concentration: at low concentration it binds to a specific receptor while higher concentrations lead to unspecific adherence to the cell membrane, penetrating the lipid bilayer. In certain cells, this toxin can cause specific gene activation, apoptotic death or pro-inflammatory cytokine synthesis and secretion due to alterations of ionic balances [128].

Beta hemolysin is produced by many *S. aureus* strains and was shown to have hemolytic and cytolytic activity [129] and is mostly produced by animal *S. aureus* strains. It is also called 'hot hemolysin' since its activity is enhanced when preincubated on 37°C [130].

Delta hemolysin is encoded by the *hld* gene and produced by almost 97% of *S. aureus* strains. It has a dermonecrotic activity and the potential to disrupt cellular membranes. Transcription of delta hemolysin is activated at the end of the exponential phase by an *agr*-

dependent mechanism. Inactivation of this toxin has an effect on the production of other toxins, suggesting that delta toxin is a part of the *agr*-dependent *S. aureus* virulence regulation, since RNA III, transcribed from *agr* locus and involved in regulation of *agr* operon, contains genes for the delta toxin [131].

Panton-Valentine Leukocidin (PVL) is a two-component pore forming toxin, constituted of LukS-PV and LukF-PV subunits. Beside pore forming property, it was observed that PVL has the potential to induce the release of inflammatory mediators in myeloid cells [132]. This toxin was also found associated with the development of necrotizing pneumonia in CA-MRSA infections and therefore thought to be one of the main *S. aureus* virulence factors.

Gamma hemolysin is produced by virtually every strain of *S. aureus*. Two proteins constitute this toxin, which can create a pore on erythrocyte or leukocyte membranes, yet a binding receptor is still not discovered [133, 134]. Depending on the strain, subunits of gamma hemolysin are made of HlgA and HlgB or HlgC (ATCC 31899) or Hlg1 and Hlg2 (other strains). Gamma hemolysin is very similar to PVL. A subunit HlgB/Hlg1 is highly similar to LukF-PV subunit, while subunits HlgA/Hlg2 are in some extend similar to LukS-PV [135] and belong to the same class.

5.2.3 New MRSA-Associated Virulence Factors

Phenyl soluble modulins (PSMs) are small amphipatic and α-helical peptide toxins that attract and activate neutrophils. They are encoded by the bacterial core genome and have the capacity to lyse PMNs. Highly virulent strains of *S. aureus* produce strongly cytolytic PSM peptides which induce IL-8 release and lysis of neutrophils and erythrocytes. Therefore PSMs are considered to be important tools in the host evasion by *S. aureus* [136]. PSMs deficient *S. aureus* has a dramatically decreased capacity to lyse neutrophils, cause skin infections and bacteremia indicating their crucial role in the pathogenesis of *S. aureus* infection [137]. PSM peptides are under control of the *agr* quorum sensing system. Recently it has been discovered that they are recognized by human formyl peptide receptor 2 [138].

During sequencing of the most common CA-MRSA strain, USA 300, [139] an **arginine catabolic mobile element** (ACME) was discovered and it was proposed that this locus could play an important role in the emerging dominance of this strain. The whole locus contains 33

genes, but several of them important in the arginine catabolism, are contained in a cluster called arcABCD, that play a role in facilitating bacterial survival in acidic environment. ACME also contains other genes of which the majority is still not characterized. However, an experimental model of rat necrotizing pneumonia or skin infection could not show that ACME is related with survival and severity of lung pathology [140].

5.3 PANTON VALENTINE LEUKOCIDIN

PVL belongs to the group of ß-barrel pore forming toxins. It has been discovered in 1932 by Panton and his student Valentine [141]. PVL is a bi-component toxin, comprised of two subunits called LukF (LukF-PV) and LukS (LukS-PV). LukF and LukS differ in their sizes, 34kDa and 33kDa, respectively and according to the rate they elute from an ion exchange chromatography got appropriate names-slow (LukS) and fast (LukF) component. Both proteins act together synergistically against the membrane of the target cell, which classifies PVL as a synergohymenotropic (SH) pore forming toxin. The genes for these two components are placed one after another in an operon called *luk-PV*; with only one base pair distance and are regulated by the same transcription factors and promoter. Analysis of genetic and peptide sequence of LukS and LukF showed around 30% similarity [133]. Pore formation by PVL is commonly described as a four step process (Figure 6). The first step is presented by (a) water soluble monomers, LukF and LukS, which separately interact with a membrane surface. In the next phase, the membrane bound monomers oligomerize into heterodimer (b), which is followed by the assembly into an oligomeric disc formation (c) and an octameric pre-pore formation, where LukF and LukS, contained in equimolar ration (1:1) were found to be packed in 200kDa molecular complex (d).

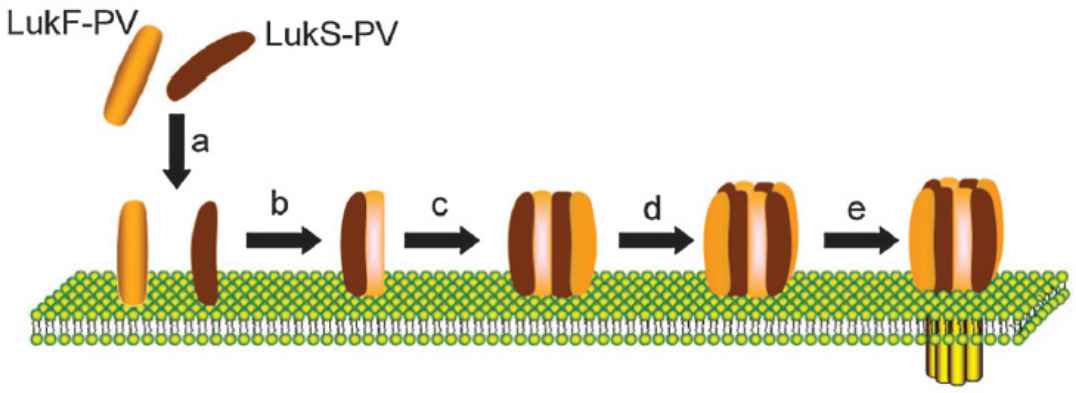


Figure 6. Mechanism of PVL binding to the membrane and pore formation [142].

Finally, the pre-pore undergoes structural changes (alternating arrangement about the central axis of symmetry) resulting in the formation of a transmembrane pore (e), able to transduct ions leading to cell death [142, 143]. On the membrane of human PMNs treated with PVL, electron microscopy showed the presence of a ring-shaped structure with diameters of 3 and 9nm (inner and outer membrane) [144]. Recently, both protein structures have been solved [142, 145, 146].

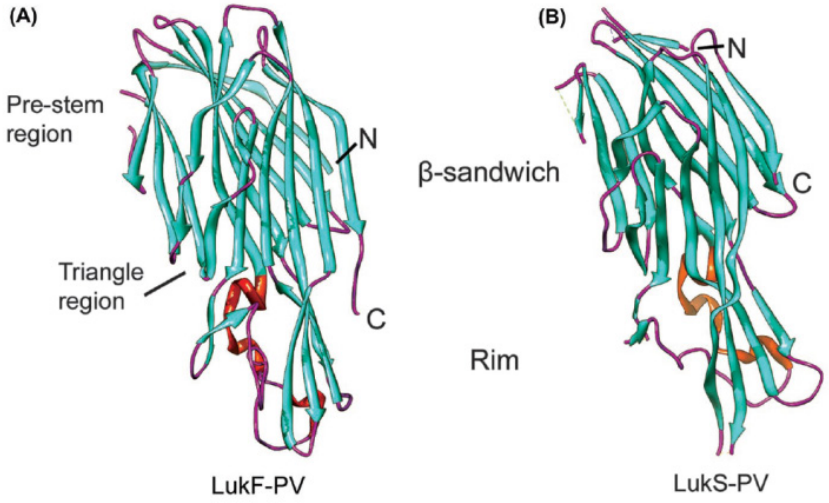
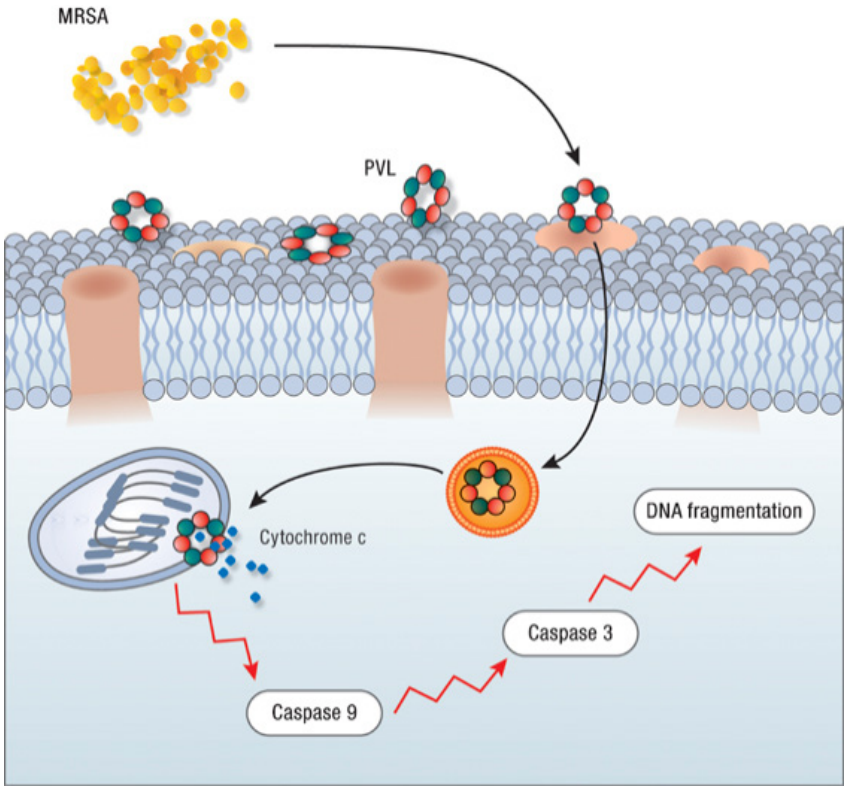


Figure 7. Crystal structures of LukF (A) and LukS (B) [142].

Pathophysiological investigation of PVL properties showed that it has the ability to create a pore on targeted cells (PMNs), resulting in cell permeabilisation and cell lysis. Employment of both subunits in a higher dose had the ability to sufficiently kill neutrophils by inducing necrotic alterations [147]. Further investigations revealed that lower concentration of PVL induces permeabilisation of mitochondrial membrane and release of cytochrome c which subsequently led to the activation of caspase cascade (caspase 9 and caspase 3) and resulted in apoptotic death of PMNs [147].

(A)



(B)

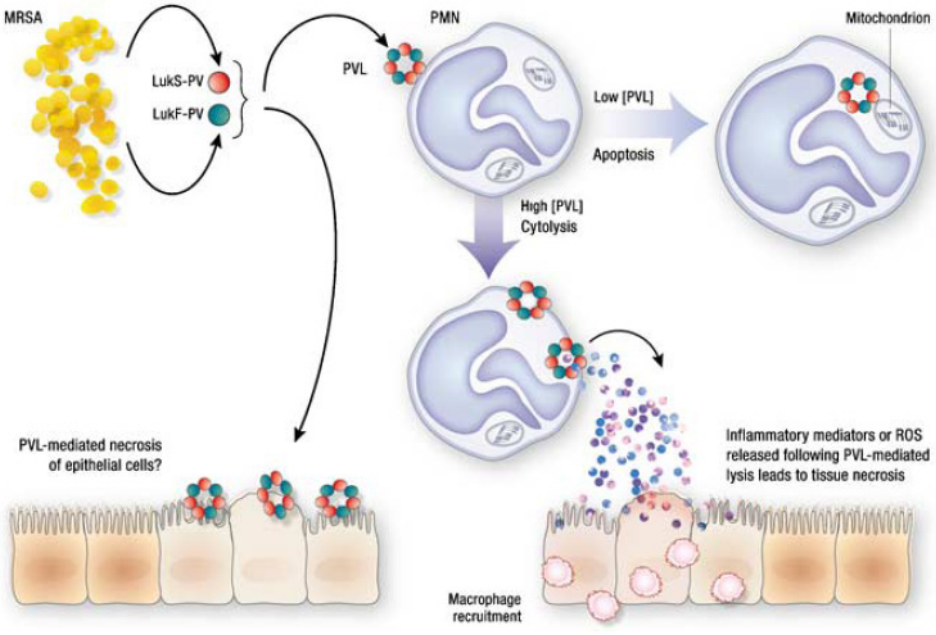


Figure 8. (A) Mechanism of PVL induced apoptosis in PMNs. (B) Mechanism of PVL induced inflammation in the lung [148].

5.4 HOST DEFENSE TO S. AUREUS

S. aureus has evolved and developed many abilities to escape the innate immune system, like factors which inhibit complement activation, toxins which lyse neutrophils or neutralization of host's defensins. Despite innate immune actions against *S. aureus*, like antimicrobial peptides, complement and phagocytic killing, these bacteria still manage to survive and become a leading cause of tissue, bloodstream and lower respiratory tract infections.

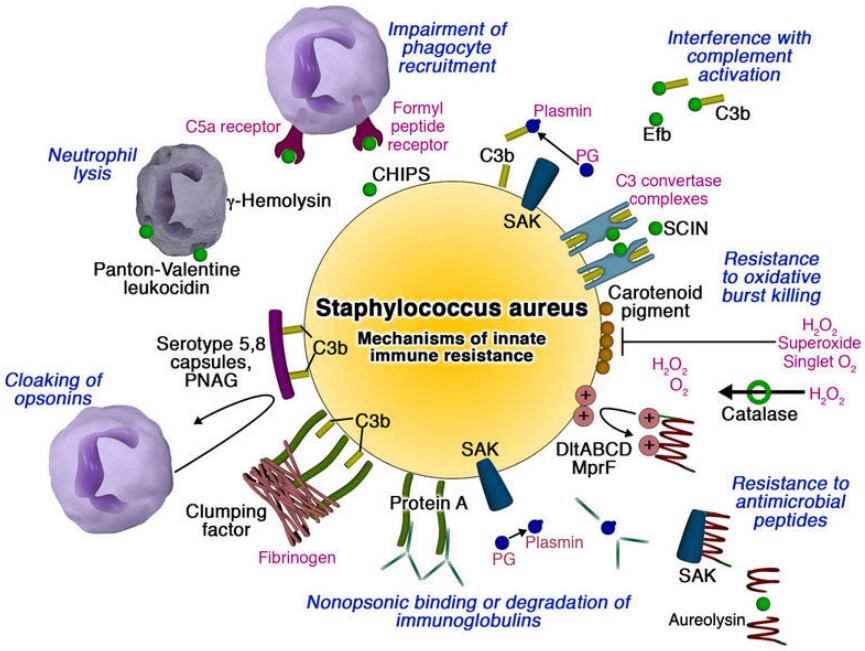


Figure 9. Mechanisms of *S. aureus* host defense [149].

5.4.1 Role of TLR2 and TNFR1 in a model of staphylococcal infections

TLR2 is the essential receptor for the recognition of staphylococcal components. It has been reported to be involved in the recognition of staphylococcal PGN and LTA where its lack increases the susceptibility of the host to staphylococcal infections [150-152]. TLR2 is broadly expressed on a variety of cells and has the ability to detect miscellaneous microbial molecules. One of the explanations for such prominent capabilities is the cooperation with other receptors. As a complex with TLR1 or TLR6, TLR2 can recognize bacterial triacyl and diacyl lipids, respectively [153]. CD14 was revealed to be a crucial component for the recognition of PGN and LTA *in vitro*, although an *in vivo* LTA lung inflammation model showed CD14 to have only minor effects [154]. Another receptor, scavenger receptor CD36 was shown to be indispensible in a model of *S. aureus* infection *in vivo*, where cytokine production upon LTA stimulation was

shown to depend on the presence of CD36, as CD36 was identified as a co-receptor of TLR2 [151].

Besides TLR2 and CD36 signaling, TNF-R1 plays a noteworthy role, since it can recognize parts of spa, a potent inducer of *S. aureus* induced inflammation in the lungs [123]. TNF-R1 is expressed on almost all cell types and its activation can trigger a signaling cascade that leads either to apoptosis or inflammation [155, 156].

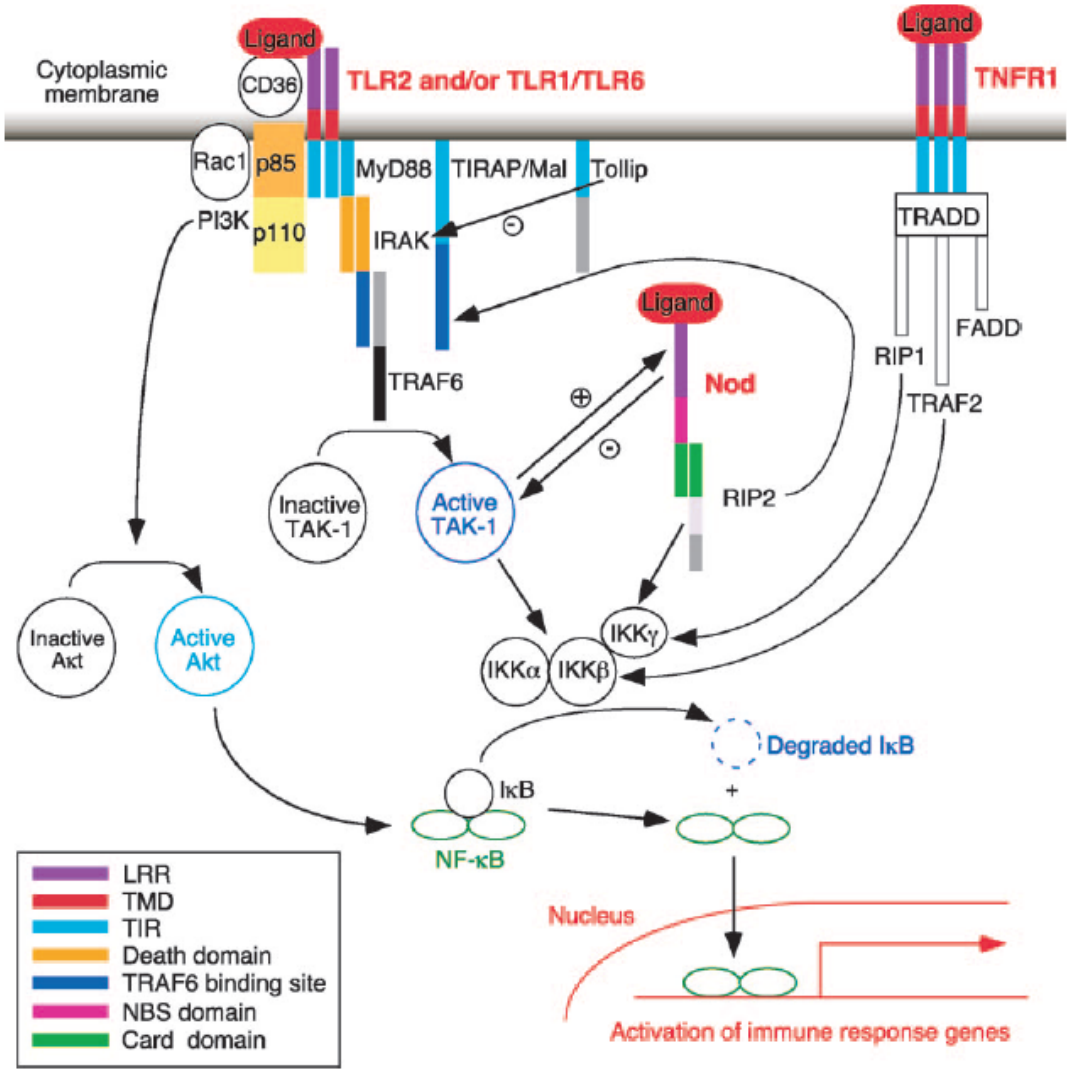


Figure 10. Signaling pathways activated in response to TLR2 and TNFR1 ligand binding [157].

5.5 S. AUREUS LUNG INFECTIONS

Until recently, staphylococcal pneumonia was considered as an infection occurring primarily in patients, which previously suffered from an existing lung disorder such as influenza infection. However, in the past couple of decades there have been important changes. Several medical centers in the US have seen a dramatic increase in *S. aureus* lung infections, which accounted for 20-40% of all hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) cases [158]. In the last several years CA-MRSA emerged, causing a special type of pneumonia associated with influenza-like syndromes, often occurring in young immunocompetent individuals. Highly virulent CA-MRSA strains carry the SCCmec type IV cassette [159], and have the ability to spread rapidly, explaining why CA-MRSA is present in the majority of countries to date. The first infections appeared in Western Australia during the 1990s [160] but the first well documented cases were recorded in 1997-99 in mid-western regions of the US, where four otherwise healthy children died from sepsis or necrotizing pneumonia [161]. These infections were not caused by HA-MRSA and children had no risk-factors for MRSA infection. The strain which caused this outbreak was noticed as MW2, later known as USA400 (ST1). An analysis of the MW2 genome showed that the patients with a lung disorder infected with *S. aureus* in most of the cases carried two genes – a methicillin resistance gene and the gene for PVL. These two genes then became markers for CA-MRSA worldwide. From 2000 on, there were numerous reports on outbreaks all over the world and a new strain unrelated to USA400 has been reported,USA300, spreading rapidly over the US and indicating the replacement of USA400, making it the most prominent cause of the community associated infections in the US today. Soon after that, an emergence of CA-MRSA was noted in Europe, Asia and Australia.

In 2002 an interesting epidemiological study in France described 16 cases of community-acquired pneumonia [102] caused by *S. aureus* (CA-SA) carrying the gene coding for PVL [162]. These patients had influenza-like syndromes, a rapid course of disease and very high lethality rates. Isolates from these patients surprisingly showed that most of the bacteria did not carry the mec gene, suggesting that life threatening necrotizing pneumonia was associated with PVL. Since then PVL has been extensively studied and until today PVL remained the only locus coding for virulence factors epidemiologically associated with CA-MRSA or CA-MSSA infections.

The exact mechanism as to how PVL contributes to necrotizing pneumonia is still unknown but a current model of PVL induced lung injury and inflammation strongly suggests that PVL contributes to the establishment of infection as it directly activates and damages PMNs and contributes to the release of proinflammatory mediators from AMs [163]. However, there is quite some controversy as to the importance of PVL in necrotizing pneumonia. Knowledge of the mechanisms underlying these processes would possibly assist in identifying patients at risk and possibly even identify novel targets to prevent necrotizing pneumonia in humans.

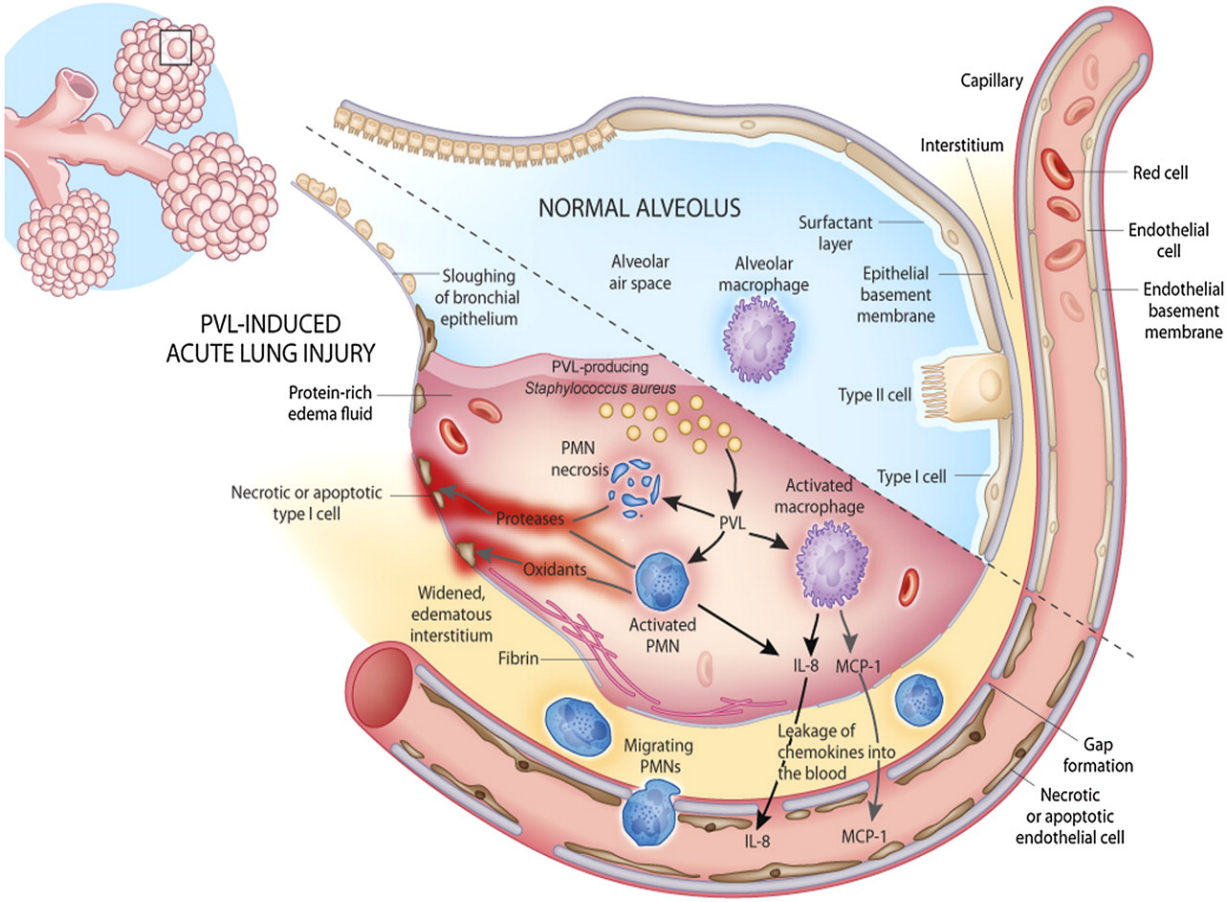


Figure 11. A scheme of PVL induced lung injury and inflammation [163].

6. RESULTS

6.1 TLR 2 and CD14 Mediate Innate Immunity and Lung Inflammation to Staphylococcal Panton -Valentine Leukocidin *in vivo*

Toll-like receptor 2 and CD14 mediate innate immunity and lung inflammation to Staphylococcal Panton Valentine Leukocidin in vivo.

Ana Zivkovic*†, Omar Sharif*†, Karin Stich†, Bianca Doninger*†, Mario Biaggio*†, Jacques Colinge*, Martin Bilban‡, Ildiko Mesteri§, Parastoo Hazemi¶, Rosa Lemmens-Gruber¶ and Sylvia Knapp*†

*Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, Vienna, 1090; Austria; †Department of Medicine I, Div. of Infectious Diseases and Tropical Medicine, Medical University Vienna, Vienna, 1090; Austria; ‡Department of Laboratory Medicine, Medical University of Vienna, Vienna, 1090; Austria; §Department of Pathology, Medical University of Vienna, Vienna, 1090; Austria; ¶Department of Pharmacology and Toxicology, University of Vienna, Vienna, 1090; Austria

Running Title: PVL inflames the lung via TLR2, CD14 and NF-κB

Corresponding author:

Sylvia Knapp M.D., Ph.D, Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, and Department of Medicine I, Div. of Infectious Diseases and Tropical Medicine, Medical University Vienna, Waehringer Guertel 18-20; 1090 Vienna, Austria, Phone: +43-1-40400-5139; Fax: +43-1-40400-5167, e-mail: sylvia.knapp@meduniwien.ac.at

Key words: inflammation, macrophage, lung, bacterial, murine.

Abstract

The pore forming toxin Panton-Valentine Leukocidin (PVL) is carried by community-acquired methicillin-resistant *Staphylococcus aureus* and associated with necrotizing pneumonia together with poor prognosis of infected patients. Although the cell-death inducing properties of PVL have previously been examined, the pulmonary immune response to PVL is largely unknown. Using an unbiased transcriptional profiling approach, we show that PVL induces only 29 genes in mouse alveolar macrophages, which are associated with TLR signaling. Further studies indicate that PVL directly binds to TLR2 and induces immune responses via NF-κB in a TLR2, CD14, MyD88, IRAK-1 and TNFR associated factor-6 dependent manner. PVL mediated inflammation is independent of pore formation but strongly depends on the LukS subunit and is suppressed in CD14/TLR2$^{-/-}$ cells. In vivo PVL or LukS induced a robust inflammatory response in lungs which was diminished in CD14/TLR2$^{-/-}$ mice. These results highlight the proinflammatory properties of PVL and identify CD14/TLR2 as an essential receptor complex for PVL induced lung inflammation.

Introduction

Panton-Valentine Leukocidin (PVL) is a β-barrel pore forming toxin secreted from *Staphylococcus aureus* (*S. aureus*) [1], which is the leading cause of bacterial infections in developed countries [2]. As of lately, PVL gained enormous attention since epidemiological and clinical studies linked the presence of PVL to serious skin infections and life threatening necrotizing pneumonia [3,4]. Although *S. aureus* strains carrying the gene for PVL used to be rare [5], the recent emergence and rapid global spread of community-acquired methicillin resistant *S. aureus* (CA-MRSA) clones not only alerted us of the imminent threat of drug resistant bacteria outside of hospitals but also raised awareness of PVL [6]. In sharp contrast to healthcare-associated MRSA (HA-MRSA) and methicillin-sensitive *S. aureus* (MSSA), the majority of CA-MRSA strains carry genes encoding PVL [6,7]. Moreover, the emergence of CA-MRSA increased the overall rate of staphylococcal infections, which range from skin to blood-stream infections and fatal necrotizing pneumonia in previously healthy people [8].

PVL consists of two subunits, LukS-PV and LukF-PV, which in an equimolar ratio shape the octamer structure that is essential for pore formation on host cells [9,10]. Previous studies show that human and rabbit neutrophils are highly sensitive to the pore-forming properties of PVL and rapidly undergo cell death [11,12]. It is generally accepted that myeloid cells are the prime target of PVL and that low concentrations of the toxin cause apoptosis, while higher amounts induce lysis of neutrophils [13]. This way PVL is considered a virulence factor that mediates an important immune escape mechanism and thus contributes to CA-MRSA pathogenesis.

Within the respiratory tract, alveolar macrophages (AM) are considered to represent the first line of defense and express a plethora of pattern recognition receptors**,** including TLRs, which recognize pattern associated molecular patterns (PAMPs) [14]. Considering the constant exposure to inhaled bacteria, the fast and accurate recognition of PAMPs is an extremely important defense mechanism. TLR activation results in downstream signaling pathways such as activation of MAPK and the transcription factor NF-κB. These pathways then modulate inflammatory gene expression, which is crucial in shaping the innate immune response within the respiratory tract [15].

While the death inducing role of PVL in neutrophils has been previously examined [13], PVL's role within the lung is somewhat controversial and the specific responsiveness of AMs to this toxin is unknown. We therefore explored the detailed function of PVL on AMs and, using a

microarray profiling approach, now show that PVL induces a highly specific inflammatory transcriptional response in AMs. Further biochemical and genetic studies indicate that this inflammatory response is independent from PVLs pore forming ability and is mediated via NF-κB, through a CD14-TLR2 dependent mechanism.

Materials and Methods

Protein production and purification.

To generate PVL we amplified the sequence from *S. aureus V8* (ATCC 49775) using the following primers, close to the coding region of LukF-PV and LukS-PV, as described earlier [13]: *LukF-FW* (5'-caccGCTCAACATAT CACACCTGTAAg3'); *LukF-RV* (5'-TTAGCTCATAGGATTTTTTTCCTTAGATTg-3') and *LukS-FW* (5'-caccGAATCTAAAGCTGATAACAATATTGAGAATATTg-3'); *LukS-RV* (5'-TCAATTATGTCCT TTCACTTTAATTTCA TGAg-3'). PCR products were digested with XhoI and NcoI and ligated into pETM11 [16]. Respective LukF-pETM11 and LukS-pETM11 constructs were transformed into competent *E. coli* DH5-α TM cells (Invitrogen) and clones were confirmed by sequencing. BL21 (DE3) pLys competent cells (Invitrogen) were used for expression of pETM11 plasmids for 6h following induction with 0.05mM IPTG (Promega). Cells were lysed using Emulsiflex-C3 (Avestin Europe Inc.); His-tagged proteins were isolated using Ni-NTA resin (Qiagen) and desalted using ZEBA columns (Thermo Scientific). Finally, proteins were subjected to LPS removal using DetoxiGel columns (Thermo Scientific) until a final LPS concentration of <0.02 EU/ml was ensured (Charles River Analytics). Homogeneity of the final products (LukF and LukS) was checked by silver stains, which showed single protein bands (Supplemental Fig. S1A). LukS and LukF were aliquoted and stored at -20°C until use, when both subunits were mixed at equimolar ratios immediately before added to cells or mice, respectively.

Polymorphonuclear (PMN) cell isolation

Human PMN were isolated from peripheral blood of healthy volunteers using gradient centrifugation (Histopaque) according to the manufacturer's instruction (Sigma). Mouse PMNs were isolated from bone marrow of WT mice by flushing femurs and tibias with HBSS (Sigma) supplemented with 0.5% FCS. Cell suspensions were passed through a 70µm filter, washed and separated using a 62.5% and 81% Percoll gradient (Sigma). After centrifugation PMNs were found in the 81%-62.5% interface. Cell viability was assessed using Trypan Blue exclusion and was greater than 90%.

Cell culture and treatments

MH-S cells (ATCC) were cultured in RPMI 1640 containing 50mM β-mercaptoethanol, 1% penicillin, streptomycin and 10% FCS at 37°C. For generation of MH-S cells stably expressing dominant negative TLR2, $2x10^6$/ml cells were transfected with 1µg of purified pZERO dTIR-TLR2 plasmid (Eubio) according to the suppliers' instructions (Amaxa) and were selected using 10µg/ml puromycin and then grown in the presence of 5µg/ml puromycin. Human embryonic kidney cells 293 (HEK 293) stably transfected with CD14, TLR2+/-CD14, TLR4+/-CD14, or mock transfected cells were kindly provided by Dr. Golenbock (University of Massachusetts Medical School, Worcester, MA) and maintained in DMEM (Gibco) supplemented with 10% FCS, 1% penicillin-streptomycin in the presence of 50µg/ml G418 or puromycin, respectively [17]. Supernatant from HEK293 MD2 cells was filtered and added (1:2) to HEK 293 CD14+/-TLR4 cells. For purity tests, LukS, LukF, PVL, or Pam_3CSK_4 were pre-treated with 1µg/µl proteinase K at 37°C for 30min followed by heat-inactivation of the enzyme at 65°C; alkalizing the compounds was carried out with 0.2M NaOH at RT for 2h, and subsequent neutralization with HCl. Primary AMs were obtained from C57BL/6, $TLR2^{-/-}$, $CD14^{-/-}$ and CD14/TLR2 DKO mice by broncho-alveolar lavage (BAL) and cultured in RPMI 1640. BMDMs (bone marrow derived macrophages) were retrieved from tibia and femur of mice and differentiated in RPMI supplemented with 10% L929-conditioned medium for 7 days as described [18]. Bronchoscopically retrieved BAL fluid from intubated patients without underlying lung disease was used to isolate primary human AM. This procedure was approved by the local Ethical review Board of the Medical University Vienna. Lipoteichoic acid (LTA) was kindly provided by S. von Aulock, University of Konstanz, Germany and Pam_3CSK_4 was purchased from GMC microcollection, Tuebingen, Germany.

Cell death and apoptosis assays.

Cell death was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instruction. For this purpose 10^5/ml cells were incubated with indicated amounts of PVL and lactate dehydrogenase (LDH) release was determined at indicated times at OD450nm. Apoptotic laddering was assayed using a commercially available kit (Roche Diagnostics). $2x10^6$/ml MH-S cells were treated with 280nM PVL or 1µM staurosporine [19] for indicated time points, after which total DNA was isolated as suggested by the manufacturer

(Roche Diagnostics). Two μg of fragmented DNA was run on a 1% agarose gel and visualized by ethidium-bromide staining.

Gene Expression Profiling

Isolated total RNA was purified using the RNeasy kit per manufacturer's instructions (Qiagen). Total RNA (200ng) was then used for GeneChip analysis. Preparation of terminal-labeled cDNA, hybridization to genome-wide murine GeneLevel 1.0 ST GeneChips (Affymetrix) and scanning of the arrays were carried out according to manufacturer's protocols. Affymetrix microarray CEL files were combined and expression normalized with the RMA algorithm [20] to obtain an expression matrix. Identification of regulated genes, comparing control and 1h conditions, was performed with significance analysis of microarrays (SAM) as described [21]; a false discovery rate (FDR) of 1% was imposed. All data are deposited in the ArrayExpress database at www.ebi.ac.uk/arrayexpress, the accession number is E-MEXP-2513. Functional clustering of annotated genes was performed using DAVID bioinformatical tools [22,23].

RT-PCR

Total lung RNA was isolated and RT-PCR performed as previously described [24]. Mouse gene specific primer sequences used are shown in Supplementary Table S1

Western blotting

Western blotting was conducted as previously described [24]. Antibodies specific for phospho-IKK (IkappaB kinase) α/β (S177/181), phospho-IκBα (S32/36), IκB-α, (RnD Systems) phospho-p38 (T180/Y182), p38 and phospho-SAPK/JNK (T183/Y185) (RnD Systems), were used at dilutions of 1:1000; β-actin (Sigma) was used as a loading control at a dilution 1:500. All primary antibodies were obtained from Cell Signaling, unless otherwise indicated.

EMSA

Nuclear extracts were isolated from MH-S or BMDM cells of WT and TLR2$^{-/-}$ mice as previously described [25] and mixed with fluorescently labeled oligonucleotides containing the NF-κB consensus binding site (5'-AGTTGAGGGGACTTTCCCAGGC-3'; 3'-TCAACTCCCCTGAAAGGGTCCG-5'; underlined nucleotides represent the binding site). Mixtures were run on a 5% TGE native gel and visualized using the LI-COR Odyssey Imaging System.

Cytokine and chemokine measurements

Cytokines and chemokines (mouse TNF-α, KC (keratinocyte derived chemokine), IL-1β and MIP-2; human IL-8) were measured using specific ELISAs (RnD Systems) as described [24,26], according to the manufacturers' instructions.

Patch Clamp Measurements

Currents were recorded with the patch clamp technique in whole cell and inside out mode [27]. MH-S cells were plated on glass cover slips 12 to 24 h before experiments were performed. Electrodes pulled of borosilicate capillaries had a tip resistance of 5-6 MΩ for whole cell recording and 8-10 MΩ for single channel recording. After patch formation an equilibrium period of 5min followed, which was succeeded by control recordings at holding potentials ranging from –80 mV to +80 mV with 20 mV increments. During the control period no electrical activity could be observed. After adding single components (LukF-PV or LukS-PV), or both components (PVL) to the bathing solution (140 to 280nM) first channel openings were detected after approximately 1min. Electrophysiological measurements were carried out with an Axopatch-1D patch clamp amplifier (Axon Instruments).

Gene reporter assays

HEK 293 cells were seeded at $1.5x10^5$cells/ml and transiently transfected with 3.6µg of NF-κB, AP-1, CRE reporter vectors or empty backbone (Panomics), respectively, and 0.36µg pRenilla luciferase gene vector (Promega). In the second set of experiments HEK293 cells were transfected with 1µg of msc-pDENY negative control or dominant negative MyD88, TIRAP (Toll-interleukin 1 receptor domain containing adaptor protein), IRAK1 (Interleukin-1 receptor-associated kinase 1) or TRAF6 (TNF Receptor Associated Factor 6) pDENY plasmids (Invivogen), respectively, together with 2.5µg NF-κB reporter vector and 0.25µg of pRenilla luciferase gene vector. 24h post expression, cells were treated with PVL for 16h, lysed and luciferase activity was measured by luminometry according to the manufacturer's instructions.

TLR2 binding assays

PVL, LukF, LukS, Pam_3CSK_4 or KDO (3-Deoxy-D-manno-oct-2-ulosonic acid)-lipid A was immobilized on high binding 96-well plates by overnight incubation at 4°C. After washing steps, plates were blocked using Superblock (Thermo Scientific) for 1h followed by incubation with TLR2-Fc or triggering receptor expressed on myeloid cells (TREM-1)-Fc fusion proteins (2µg/ml) (RnD Systems), respectively, at room temperature for 2h. Specific binding was quantified using anti-Fc IgG–biotin (Sigma), followed by streptavidin-HRP and TMB (3,3',5,5'-tetramethylbenzidine) solution and OD was measured on a spectrophotometer (OD450nm).

In vivo experimental procedures

Age and sex-matched, pathogen-free 7 to 9 week-old C57BL/6, $TLR2^{-/-}$, $CD14^{-/-}$ and CD14/TLR2 DKO mice were used in all experiments. The Animal Care and Use Committee of the Medical University of Vienna approved all experiments. $TLR2^{-/-}$ mice were kindly provided by Dr. Shizuo Akira, University Osaka, Japan [28], $CD14^{-/-}$ mice were obtained from Jackson Laboratories, and CD14/TLR2 DKO were generated at the Medical University Vienna animal facility. Lung inflammation was induced as described [29,30]. Briefly, mice were anesthetized by inhalation of Isoflurane (Baxter) and 1µg/g of PVL was administered intranasally. At indicated times mice were sacrificed and cell counts enumerated from broncho-alveolar lavage fluid (BALF). Cytokine levels were determined in BALF and lung homogenates.

Lung histology

Lungs for histology were harvested 24h after induction of lung inflammation, fixed in 10% formalin and embedded in paraffin. Four-µm sections were stained with H&E, and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endothelitis, bronchitis, pleuritis and thrombi formation. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: moderately severe and 4: severe. The "inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24. Immunohistochemistry: following antigen retrieval and blocking, lung sections were incubated with rat anti-mouse Ly-6G FITC (BD) or isotype control Ab (Cemfret Analytics) at room temperature. After rinsing, rabbit anti-FITC Ab (ZYMED) was added, followed by

incubation with powervision poly HRP-anti-rabbit (ImmunoLogic), and visualization with 3, 3-diaminobenzidin-tetra-hydrochloride (Vector Lab). Nuclear counterstaining was done with Mayer's hemalaun solution.

Statistical analysis

Differences between groups were analyzed using unpaired t test or one-way ANOVA followed by Bonferroni posthoc analyses, where appropriate, using GraphPad Prism software. Time course experiments were calculated using two-way ANOVA. Values are expressed as mean ± SD. A p-value < 0.05 was considered statistically significant. ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

Results

PVL activates transcription of a small subset of NF-κB regulated genes in alveolar macrophages, associated with TLR signaling.

To understand the impact of PVL on lung-specific immune cells we generated recombinant PVL and conducted a microarray profiling of mouse AMs. Considering primary cells are rather heterogeneous and to avoid potential donor effects, we used MH-S cells, which are a routinely used immortalized mouse AM cell line, for our microarray studies. Before conducting this, we determined that PVL is functional and capable of inducing cell death in both human and mouse neutrophils (Fig. 1A) at a concentration similar to previously described [13]. PVL also caused cell death in MH-S cells, albeit much later than in neutrophils (starting at 10h), as determined by a LDH assay (Fig. 1B) and DNA laddering, which is a hallmark of apoptosis (Fig. 1C). In addition, PVL mediated cell death occurred in a dose dependent manner (Fig. 1D) and it relies on both the native confirmation of PVL as well as the presence of both subunits (Fig. 1E). Furthermore, primary AM behaved similar to MH-S cells, since PVL-induced cell death of primary murine and human AM occurred only after prolonged incubation (Fig. 1F). These data show that PVL was biologically active and gave us the confidence to treat MH-S cells with PVL for 1h, (i.e. at a time when no cell death was observed) and to conduct microarrays.

Genome-wide analysis surprisingly showed that PVL induced the expression of only 29 genes (Fig. 2A and B and Supplemental Table S2), suggesting that a 1h treatment of AMs with PVL produces a highly specific response, where only a limited set of transcription factors maybe induced. Importantly, gene ontology analysis suggested that TLR as well as MAPK signaling pathways were activated with the greatest likelihood (Supplemental Table S3). We noticed that out of our 29 genes, many of them were previously identified as being regulated by NF-κB or to negatively regulate the NF-κB pathway such as *MIP-2*, *NF-κB-INS*, *TNF-α, ZPF36* and I*κB-α* [25,31], suggesting that PVL had the ability of inducing NF-κB in AMs. Finally, to verify if the microarray results were accurate we performed RT-PCR and could show that all tested genes were indeed bona-fide transcriptional targets of PVL (Fig. 2C). Therefore, microarray profiling suggested that PVL had the ability of inducing NF-κB as well as MAPK pathways in MH-S cells possibly via a TLR.

The canonical activation of NF-κB is typified by IκB-α phosphorylation at Ser32 and Ser36 and subsequent ubiquitin-induced degradation of IκB-α by the 26S proteasome and translocation of NF-κB into the nucleus [15]. To verify if PVL is activating NF-κB in a canonical way, we treated MH-S cells with PVL for 15, 30 or 60 min and could show that PVL resulted in phosphorylation of IκB-α at 15 min, at a time when it was degraded and was synthesized 60 min post treatment (Fig. 3A). Furthermore, PVL treatment also resulted in phosphorylation of p38 MAP kinase as well as JNK at 30 and 60 min (Fig. 3A). Degradation of IκB-α 15 min post PVL treatment resulted in increased nuclear NF-κB, showing that NF-κB was being activated in a canonical manner in response to PVL (Fig. 3B). Supershift analysis verified that the NF-κB consisted of transcriptionally competent RelA/p50 heterodimers (data not shown). As microarray profiling had uncovered that PVL had the ability to induce pro-inflammatory cytokines such as TNF-α (Fig. 2C), we next addressed whether p38 and/or NF-κB signaling were contributing to transcription of pro-inflammatory genes in MH-S cells in response to PVL using inhibitors of these pathways. SB203580 acts as a potent inhibitor of ATP binding and inhibits phosphorylation of p38α, p38β and p38β2. MG132 is a proteasome inhibitor that prevents κB degradation and BMS-345541 is a highly selective IKKβ inhibitor which prevents IκBα phosphorylation and thereby blocks NF-κB dependent transcription. As shown in Fig. 3C and D, TNF-α synthesis at both the mRNA and protein level was significantly reduced in MH-S cells which had been pre-treated with both of the NF-κB inhibitors, but not with the p38 inhibitor. Collectively, our data strongly suggests that PVL has the ability to induce pro-inflammatory cytokine synthesis in MH-S cells and although PVL induces MAP-kinase pathways in this cell type, it is the NF-κB pathway that is required for synthesis of pro-inflammatory mediators, such as TNF-α.

The ability of PVL to induce inflammatory gene expression is independent of pore formation.

One of the first effects of pore forming toxins is the permeabilization of the plasma membrane to ions, leading to changes in cytoplasmic ion composition, which have been previously shown to modulate inflammatory gene expression [32]. To determine whether pore formation is a pre-requisite for inflammatory cytokine synthesis following PVL treatment, we stimulated MH-S

cells with different doses of single subunits of PVL (LukS or LukF) or an equimolar combination of both subunits (PVL) and performed whole cell patch clamp. No pore formation was observed following treatment of MH-S cells with single subunits of toxin but multiple ion channels were opened following a short treatment with both subunits (Fig. 4A). These data are in line with previous observations, showing that both subunits of PVL in an equimolar ratio are required to perform a pore [10]. Significantly, although single subunits were incapable of forming a pore in MH-S cells, LukS was capable of inducing TNF-α gene expression (Fig. 4B). Further, LukS, but not LukF, was able to induce an inflammatory response by primary AMs (Fig. 4C). These data indicate that inflammatory gene expression relies on cellular pathways which are independent of pore formation.

TLR2 plays an important role in PVL mediated cytokine secretion and this response is enhanced in the presence of CD14.

Bacterial cell wall components from *S. aureus* have very potent proinflammatory activities in vitro and in vivo [30,33], and are being sensed by different host receptors including TLRs. Recently, it has been observed that bacterial pore forming toxins can also be recognized by TLRs [34-36]. Since PVL treatment induced a specific set of genes associated with TLR signaling (Supplemental Table S3), we wondered if TLRs are important for inflammatory cytokine synthesis in response to PVL. For this purpose, we used stably transfected HEK293 cells overexpressing TLR2, TLR4 or mock transfected cells with/without CD14. TLR4/CD14 cells exhibited absolutely no response to PVL or the TLR2-ligand LTA, while TLR2 cells responded to LTA as previously described (Fig. 5A) [30]. Importantly, we observed that in response to PVL treatment, HEK cells stably transfected with TLR2 secreted significantly higher amounts of IL-8, with synthesis being enhanced in the presence of CD14 (Fig. 5A). These over-expression data suggest that PVL has the ability to induce pro-inflammatory cytokine synthesis via TLR2 and that this response is enhanced by CD14, a previously described co-receptor for TLR2 [37].

Since LPS induces TLR4 signaling and is a potent activator of NF-κB [31], we wanted to rule out LPS contamination as a possible cause of NF-κB activation and inflammation. While HEK cells overexpressing TLR4/CD14 exhibited no response to PVL (Fig. 5A) IL-8 was secreted following LPS treatment and this was significantly reduced by polymyxin B (Supplemental Fig.

S1B). Stimulating TLR2/CD14 HEK cells with PVL in the presence of polymyxin B showed that IL-8 synthesis was not affected by polymyxin B, conclusively ruling out LPS contamination as a cause of inflammation mediated by PVL (Supplemental Fig. S1C). Furthermore, we discovered that stimulating TLR2/CD14 HEK cells with single subunits (LukF, LukS) or PVL induced IL-8 secretion in the presence of LukS or PVL, but not in response to LukF (Fig. 5B). Because we earlier observed that, although both subunits of PVL were generated in an identical way, only LukS, was able to activate macrophages via TLR2, whereas LukF did not induce substantial inflammation (Fig. 4C), we concluded that our recombinant proteins were not contaminated with TLR2-ligands. To furthermore conclusively demonstrate that the proinflammatory effects of LukS and PVL are depended on the proteins and not contaminating lipoproteins, we stimulated MH-S cells with LukS, PVL and the lipoprotein Pam_3CSK_4 in their native forms or after treatment with proteinase K or NaOH, which is known to hydrolyze the N-terminal acyl chains of bacterial lipoproteins [38]. As shown in Supplemental Fig. 1D, proteinase K abolished the TNF-inducing capacity of LukS and PVL, whereas alkalizing agents did not reduce TNF-α levels in supernatants.

We next sought out to determine which transcription factors are important in PVL induced inflammation, and transiently transfected TLR2/CD14 HEK cells with NF-κB, AP-1 and CRE reporter plasmids and could show that only NF-κB was activated by PVL (Fig. 5C) and that this activation was enhanced in the presence of CD14 (Fig. 5D). Again, LukS was the responsible PVL-subunit that activated NFκB in CD14/TLR2 HEK cells (Supplemental Fig. S2). Activation of NF-κB through TLR2 engages MyD88 and TIRAP, enrollment of IRAK4 and IRAK1, followed by formation of complexes of TRAF6 with TGF-β activated kinase 1 (TAK1) and TGF-β-activated protein kinase 1-binding protein 1 (TAB1) leading to activation of the IKK complex [39]. To identify cellular signaling molecules needed for NF-κB activation upon PVL challenge, we transfected TLR2/CD14 HEK cells with dominant negative plasmids for MyD88, TIRAP, IRAK1 and TRAF6. Cells treated with LTA in the presence of these plasmids exhibited significantly less NF-κB activation (Fig. 5E). Importantly, all dominant negative plasmids (other than the vector control) also inhibited PVL mediated NF-κB activation (Fig. 5E), suggesting that PVL signals to NF-κB via a TLR2-MyD88-IRAK1 and TRAF6 pathway, in a manner analogous to LTA.

PVL binds to the extracellular domain of TLR2.

Since we observed that LukS and PVL had the ability to induce IL-8 secretion in HEK 293 cells overexpressing TLR2, we next hypothesized that LukS or PVL could directly interact with TLR2. For this purpose, we developed a modified ELISA assay where we coated plates with increasing doses of PVL, LukF, LukS, the TLR2 ligand Pam_3CSK_4 [40] or the TLR4 ligand KDO-lipid A [41] and subsequently used either a chimeric molecule containing the extracellular domain of TLR2 or TREM-1, fused to human Fc (IgG) to capture ligands as described in the methods section. TLR2 interacted with Pam_3CSK_4 in a dose dependent manner, as evidenced by increased signal intensity above background, but not with KDO-lipid A (Fig. 6). Importantly, PVL and LukS also interacted with TLR2 in a dose dependent manner comparable to wells coated with Pam_3CSK_4. Not surprisingly, LukF, which was unable of inducing an inflammatory response in macrophages (Fig. 4B and 4C) or CD14/TLR2 HEK cells (Fig. 5B), did not bind to TLR2. Binding of PVL and LukS to TLR2 was specific, since PVL or LukS did not bind to TREM-1, a receptor which plays an important role in innate immunity, but whose ligand is unknown [42]. These biochemical data suggest that the ability of LukS and PVL to induce inflammation in HEK cells overexpressing TLR2 (Fig. 5), is due to its ability to directly bind to the extracellular domain of TLR2 and to induce downstream signal transduction events.

Lack of PVL-induced activation in CD14/ TLR2 deficient macrophages.

Dimerization of the TIR domain of TLRs is essential for TLR mediated inflammation [39]. To verify our over-expression data we stably transfected MH-S cells with a dominant negative mutant of TLR2, where the TIR domain is deleted (MHS TLR2-ΔTIR), producing MH-S cells which are still capable of recognizing TLR2 ligands but are not capable of signaling [43]. MHS TLR2-ΔTIR cells treated with PVL exhibited significantly lower levels of TNF-α than control cells in response to both LTA and PVL, showing that an intact TIR domain of TLR2 was essential for PVL mediated inflammation (Fig. 7A). Since we had earlier shown that PVL has the ability to activate NF-κB signaling and NF-κB was crucial for PVL mediated inflammation (Fig. 3 and 5), we hypothesized that NF-κB activation may be defective in $TLR2^{-/-}$ cells following PVL treatment. Indeed, while WT BMDM exhibited a prolonged phosphorylation of IKKβ in its activation loop at Ser177/181, beginning 15 min post PVL treatment and becoming stronger at 60 min, $TLR2^{-/-}$ BMDMs exhibited only a transient phosphorylation in response to PVL 15 min

post treatment (Fig. 7B). Activation of IKKβ precedes phosphorylation of IκB-α at conserved serine residues (Ser32/36), which primes IκB-α for degradation via the 26S proteasome [15]. We could show that the reduced IKKβ activation observed in response to PVL in TLR2$^{-/-}$ cells translated to defective IκB-α phosphorylation and degradation (Fig. 7B). This resulted in lower NF-κB translocation to the nucleus (Fig. 7C). Decreases in NF-κB activation in TLR2$^{-/-}$ BMDMs were specific for PVL since IKKβ, IκB-α phosphorylation, degradation and NF-κB translocation to the nucleus were equivalent in TLR2$^{-/-}$ BMDMs following LPS or KDO-lipid A treatment (Fig. 7B and 7C, Supplemental Fig. S3 and data not shown).

Interestingly, although there was a significant decrease in NFκB activation and cytokine synthesis in the absence of functional TLR2, the inflammatory response was not completely abolished. Arguing that additional receptors might be involved in these PVL-mediated responses, we reasoned that overexpression studies with HEK cells suggested a role for CD14 and therefore studied the importance of CD14 alone and in conjunction with TLR2. While WT AMs responded robustly to PVL, TLR2$^{-/-}$ and CD14$^{-/-}$ AMs secreted lower levels of MIP-2. Importantly, the inflammatory response to PVL was completely abolished in the absence of both CD14 and TLR2 (Fig. 7D). In line with our observation of LukS being the proinflammatory component of PVL, LukS induced MIP-2 secretion showed the same CD14/TLR2 dependency as PVL. These results illustrate that CD14 and TLR2 play important roles in PVL induced cytokine synthesis by AMs and strongly suggest that these receptors could be important in PVL induced lung inflammation *in vivo*.

CD14 and TLR2 are essential for PVL mediated lung inflammation *in vivo*.

As we discovered CD14 and TLR2 to be essential for the induction of an inflammatory response in AMs *in vitro*, we sought to gain insight into the nature of PVL induced lung inflammation *in vivo*. Intranasal administration of PVL or LukS resulted in an enhanced neutrophil influx to the alveolar space and increased pulmonary levels of IL-1β, TNF-α, MIP-2, and KC 6h post treatment (Fig. 8A and data not shown). These data not only illustrate the in vivo effects of PVL, but also confirm that LukS is the active subunit. We next hypothesized that PVL mediated lung inflammation would be reduced in TLR2$^{-/-}$ mice and indeed observed reduced PMN influx as

well as lower TNF-α, MIP-2, KC and IL-1β levels in TLR2$^{-/-}$ mice as compared to WT animals 6h after intranasal inoculation (Supplemental Fig. S4).

Considering the *in vitro* importance of TLR2 and CD14, we next investigated the contribution of these receptors to lung inflammation *in vivo*. Our findings confirm that TLR2 and CD14 partially contribute to PVL or LukS induced lung inflammation, and that no inflammatory response can be initiated in the absence of both receptors (Fig. 8B). Together, these data show that CD14 and TLR2 play indispensable and previously unappreciated roles in PVL induced lung inflammation *in vivo*.

Discussion

In this study, using microarray profiling and biochemical studies, we could show that PVL and LukS bind to the extracellular domain of TLR2 and induce inflammation in a TLR2 and CD14 dependent manner. PVL induced gene expression could be blocked with inhibitors of the NF-κB pathway suggesting NF-κB activation is central to PVL's ability to induce inflammation. Consistent with this, we could show that PVL signals to NF-κB via a TLR2/MyD88/TIRAP/TRAF-6 axis. Finally, experiments in TLR2, CD14, and TLR2/CD14 deficient mice and cells ratify our overexpression experiments and show that PVL induces inflammation and NF-κB activation via TLR2 and CD14 *in vivo*.

The early transcriptional response activated by PVL in AMs is surprisingly specific as only 29 genes were significantly expressed following a 1h treatment. This is in contrast to LPS treatment of macrophages which activates the expression of hundreds of genes [25,44] and induces activation of a plethora of transcription factors such as NF-κB, CREB, EGR-1 and members of the AP-1 family [45]. Inflammatory gene transcription is a combinatorial process and NF-κB family transcription factors can frequently associate with other transcription factors, such as AP-1, to influence gene expression following a particular stimulus such as LPS or TNF-α [46]. Since PVL only activates NF-κB but not other transcription factors such as AP-1, the specificity of the early gene signature activated by PVL could simply be explained by its ability to solely induce NF-κB activation. This is highlighted by the observation that while PVL induced transcription of TNF-α in AMs is almost completely abolished by a specific IKKβ inhibitor, its protein levels, although significantly reduced, are not completely abolished, possibly due to positive feedback by PVL induced cytokines. In line with this hypothesis, the number of genes influenced by PVL after 8h treatment goes up from 29 to 136 (data not shown).

We show that the ability of PVL to induce inflammation is uncoupled from its pore forming properties and that single subunits of PVL are not able to induce a pore even at higher concentrations. This situation is different to other toxins such as α-hemolysin of *E. coli*, which have been shown to induce inflammation in a dual dose dependent manner, where low concentrations of α-hemolysin induce calcium oscillations and inflammation, but high concentrations induce cell death [47]. For our microarray studies we used an identical dose of PVL that has the ability to cause cell death in MH-S cells and primary AMs and could show that

inflammation and NF-κB activation precede cell death. In this respect it is of interest that NF-κB is generally believed to be an anti-apoptotic transcription factor [48]. Many cancer cell lines produce high levels of inflammatory cytokines, which results in constitutive NF-κB activation and resistance to chemo- or radiotherapy [49,50]. It is tempting to speculate that the activation of NF-κB observed in response to PVL is a strategy of AMs to combat the death inducing capability of PVL. In line with this hypothesis we find that expression of anti-apoptotic genes regulated by NF-κB increased in AMs at later time points (data not shown). Cell death following PVL seems to be more complicated and dependent on cell type. Human and mouse neutrophils are more susceptible to PVL than AMs, which could be enlightened by differences in the induction of anti-apoptotic genes, as well as the activation of cell repair mechanisms [51].

Computational analysis of our microarray experiments suggested that TLR signaling was crucial for PVL's ability to induce transcription in AMs. The idea that TLRs could play an important role in bacterial toxin recognition is not uncommon as other pore forming toxins have been shown to mediate inflammation via TLRs, particularly TLR2 and 4. Peritoneal macrophages from TLR4 null mice exhibited blunted TNF-α secretion accompanied by decreased NF-κB activation following pneumolysin challenge compared to WT mice, suggesting that pneumolysin activates inflammation in a TLR4 dependent manner [52]. Stimulation of WT peritoneal macrophages with *Haemophilus influenzae* porin induced an increase in cytokine secretion compared to $TLR2^{-/-}$ and $MyD88^{-/-}$ macrophages [35]. Porin from *Neisseria meningitidis* (PorB) has been shown to induce activation of dendritic cells in a TLR2 and MyD88 dependent manner [53]. In addition it was shown that PorB binds TLR2 directly and that the presence of TLR1 enhanced this binding while TLR6 did not seem to play a role [36]. Here we show that PVL induced inflammatory cytokine synthesis via TLR2 and CD14. While the absence of either TLR2 or CD14 reduced the inflammatory response to PVL, CD14/TLR2 double-deficient cells and mice were incapable of responding to PVL at all. However, our study is in contrast to data showing that LukF from *S. aureus* is able to induce inflammation in a TLR4 dependent manner in bone marrow derived dendritic cells [54]. We could not confirm a role for TLR4 in the inflammatory response to LukF, at least in our HEK TLR4-CD14–MD2 over-expressing system (data not shown). Quite the opposite, we discovered that LukS is the active component of the toxin, as LukS stimulation of macrophages resulted in an inflammatory response while LukF induced no such effect. Furthermore, LukS bound to TLR2 and required CD14 and TLR2 for

signaling *in vitro* as well as *in vivo*. Overexpression of TLR2 but not CD14 was sufficient for LukS to induce an inflammatory response, indicating that TLR2 is required for signaling while CD14 might act as a co-receptor.

The recent emergence of CA-MRSA and rapid expansion of highly virulent strains carrying PVL such as USA300 [3] dramatically enhanced the number of studies performed on PVL. Patients with PVL positive *S. aureus* in their lungs develop necrotizing pneumonia and have exceedingly high mortality rates, indicating that PVL might be an important virulence factor [4]. However, the precise role of PVL as a virulence factor of CA-MRSA has only been recently investigated and resulted in controversial observations. Whereas some studies used WT and isogenic ΔPVL *S. aureus* strains and failed to show any harmful effects of PVL [55,56], others that used clinical isolates and isogenic strains carrying PVL, as well as purified PVL, clearly demonstrated the tissue damaging properties of PVL and were able to mimic the necrotizing pneumonia observed in humans [57]. A recent study performed vaccination experiments using LukS and/or LukF before infection with USA300 and disclosed improved survival in vaccinated mice, suggesting that PVL significantly contributes to *S. aureus* pathogenesis [58]. Arguing that mouse models might not be ideal to study the *in vivo* role of PVL, Olsen *et al.* infected nonhuman primates with USA300 and isogenic PVL deletion-mutant strains, and ultimately did not identify a major role for PVL in aggravating pneumonia *in vivo* [59] . Nevertheless, no previous report studied the precise innate immune mechanisms associated with PVL. Of great interest, while this work was in progress, [60] Yoong and Pier investigated the impact of PVL on bacterial replication *in vivo* and discovered that PVL-positive MRSA strains replicated less efficiently when compared to isogenic PVL-negative bacteria. Using blocking Abs the authors could illustrate that PVL induced an inflammatory response and activated neutrophils, which ultimately counteracted bacterial multiplication. The authors argue that at first PVL induces a protective immune response and that neutralizing Abs that are generated upon first encounter of this bacterium might enhance the bacterial spread during subsequent infections. Hence, this work was the first to predominantly shed light on the proinflammatory role of PVL. Significantly, we hereby show the molecular mechanisms underlying PVL's proinflammatory properties within the lung *in vivo* and disclose that both TLR2 and CD14 are required for this response.

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Footnotes

1. Correspondence address:
   Sylvia Knapp M.D., Ph.D, Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, and Department of Medicine I, Div. of Infectious Diseases and Tropical Medicine, Medical University Vienna, Austria, Waehringer Guertel 18-20; 1090 Vienna, Austria, Phone: +43-1-40400-5139; Fax: +43-1-40400-5167, E-mail: sylvia.knapp@meduniwien.ac.at
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3. Abbreviations used in this paper:

AM, alveolar macrophage; BALF, broncho-alveolar lavage fluid; BMDMs, bone marrow derived macrophages; CA-MRSA, community-acquired MRSA; DKO, double knock-out; HA-MRSA, healthcare-associated MRSA; HEK cells, human embryonic kidney cells; IKK, IkappaB kinase; IRAK1, IL-1 receptor-associated kinase 1; KC, keratinocyte derived chemokine; KDO, 3-Deoxy-D-manno-oct-2-ulosonic acid; LDH, lactate dehydrogenase; LTA, lipoteichoic acid; MRSA, methicillin resistant S. aureus; MSSA, methicillin sensitive S. aureus; PAMP, pattern associated molecular patterns; PMN, polymorphonuclear cells; PVL, Panton Valentine Leukocidin; TAB1, TGF-β activated protein kinase 1 binding protein 1; TAK1, TGF-β activated kinase 1; TIRAP, Toll-IL 1 receptor domain containing adaptor protein; TRAF6, TNF Receptor Associated Factor 6; TREM-1, triggering receptor expressed on myeloid cells 1;

Figure Legends

Figure 1. PVL has the ability to cause cell death. (A) Human or mouse PMN were treated with 280 nM PVL and lactate dehydrogenase (LDH) release was quantified after 1h. **(B)** MH-S cells were incubated with 280 nM PVL and LDH release was quantified over time. **(C)** MH-S cells were treated with 280 nM PVL or 1 μM staurosporine for 6h and/or 16h and DNA-laddering was visualized on a 1% agarose gel. **(D)** MH-S cells were incubated with indicated amounts of PVL and LDH release was measured after 16h. **(E)** 280 nM PVL, LukF or LukS subunits were pre-treated with proteinase K before adding to MH-S cells for 16h, followed by LDH measurements. **(F)** Human or mouse AM were treated with 280 nM PVL and LDH release was quantified after 6h and 16h. Data are presented as mean ± SD of triplicate samples and representative of at least 2 independent experiments. Significance was calculated versus untreated (CTR) sample.

Figure 2. Gene expression and validation of data from MH-S cells treated with PVL. (A) MH-S cells were treated with 280nM PVL for 1h and analyzed by microarray versus untreated cells. Quantile-Quantile (Q-Q) plot comparing the exponential values of gene expression analysis. Quantiles of each gene from untreated samples were plotted against the quantiles of treated samples. Data presented in red illustrate genes which are significantly up-regulated upon treatment and green dots represent down-regulated genes. The confidence interval was set at 0.01. **(B)** Heat map of up-regulated genes obtained from microarray analysis**. (C)** Gene expression levels of *MIP-2, NFκBiz, TNF-α, ZFP36, MAP3K8 or GADD45β* normalized to HPRT as determined by RT-PCR. Data are the presented as mean ± SD of triplicate samples and representative of at least 2 independent experiments. Significance was calculated versus control samples.

Figure 3. PVL induced NF-κB is important for pro-inflammatory cytokine synthesis. (A) Immunoblot analysis of MH-S cells treated with 280nM PVL for indicated time-points and blotted against phospho-IκBα (S32/36), total IκBα, phospho-p38 (T180/Y182), total p38, phospho-SAPK/JNK (T183/Y185), total SAPK/JNK and β-actin. **(B)** For EMSA experiments, fluorescently labeled oligonucleotides containing the NF-κB consensus binding site were incubated with nuclear extracts of MH-S cells treated with the 280nM PVL or LPS for indicated times. **(C and D)** MH-S cells were pretreated with 10μM of indicated inhibitors for 1h and

subsequently stimulated with 280nM PVL for 1h **(C)** or 6h **(D).** TNF-α induction was quantified by RT-PCR **(C)** or ELISA **(D).** Data are the presented as mean ± SD of 2 independent experiments Significance was calculated versus PVL treated samples without inhibitors; ns- not specific.

Figure 4. PVL creates a pore but single subunits alone can induce an inflammatory response. (A) Whole-cell patch clamp recordings of MH-S cells treated with LukS, LukF or PVL at indicated doses are presented at holding potentials of +80 mV (upper row) and –80 mV (lower row). The closed state of the channels is indicated by the dashed lines and is marked with "C". Up- and downward deflections indicate channel openings. **(B)** TNF-α induction measured by RT-PCR from MH-S cells treated with 280nM LukF, LukS or PVL for 1h. (**C**) Primary AMs were stimulated with 280nM LukF, LukS or PVL and cytokine release was assayed by ELISA after 6h. Data are presented as mean ± SD of triplicates and representative of at least two independent experiments. Significance was calculated versus untreated (CTR) samples.

Figure 5. Effect of PVL on HEK293 cells over-expressing different TLRs. (A) IL-8 secretion of HEK293 cells stably transfected with TLR2 or TLR4 and/or CD14 and treated with 280nM PVL for 6h. (**B**) IL-8 secretion of HEK293 cells stably transfected with TLR2 or and CD14 and treated with 280nM LukF, LukS or PVL for 6h. **(C)** Luciferase activity of HEK293-CD14/TLR2 cells transiently transfected with reporter plasmids for NF-κB, AP1, CRE or control plasmid, 24h upon addition of PVL**. (D)** NF-κB luciferase activity following stimulation of HEK293-TLR2 or HEK293-CD14/TLR2 cells with PVL for 24h. **(E)** Gene reporter assay of HEK293 CD14/TLR2 cells transiently transfected with dominant-negative plasmids for MyD88, TIRAP, IRAK1, TRAF6 or control plasmid, respectively, together with NF-κB reporter and stimulated with PVL for 16h. All reporter assays are expressed as fold-activation versus non-stimulated cells. Data are presented as the mean ± SD of triplicate samples and are representative of at least 2 independent experiments. Significance was calculated versus mock treated cells **(A, D)**, non-stimulated cells (**B**) or control vector **(C, E)**; AU, arbitrary units.

Figure 6. PVL binds to TLR2. LukF, LukS, PVL, Pam_3CSK_4 or KDO-lipid A, respectively, was immobilized and binding to TLR2-Fc and TREM-1-Fc was quantified using biotin-conjugated anti-Fc Ab followed by colorimetric detection (OD450). Data are presented as mean

± SD of triplicate samples of 2 independent experiments. Significance was calculated versus background.

Figure 7. TLR2 and CD14 are essential for PVL-dependent macrophage activation. (A) TNF-α levels of MH-S stably transfected with a dominant-negative TLR2 lacking the TIR domain (TLR2-ΔTIR) treated with 280nM PVL or 10µg/ml LTA for 6h (**B)** Immunoblot analysis of lysates from WT and TLR2$^{-/-}$ BMDMs stimulated with 280nM PVL for indicated time-points and probed with antibodies against pIKKα/IKKβ, pIκB-α, total IκB-α and β-actin loading control. **(C)** For EMSA, fluorescently labeled oligonucleotides containing the NF-κB consensus binding site were incubated with nuclear extracts of WT or TLR2$^{-/-}$ BMDMs treated with 280nM PVL or 100ng LPS for the indicated time. **(D)** MIP-2 levels released by primary AMs from WT, TLR2$^{-/-}$, CD14$^{-/-}$ and CD14/TLR2 DKO mice 6h after treatment with 280nM LukF, LukS or PVL. Data presented are mean ± SD of triplicate samples of at least two independent experiments. Significance was calculated versus mock MH-S cells **(A)** or versus WT cells **(D)**.

Figure 8. CD14/TLR2 DKO mice exhibit reduced lung inflammation following challenge with PVL or LukS. (A) WT mice (n=6) were inoculated with 1µg/g LukF, LukS or PVL and PMN influx and cytokine induction was measured after 6h. **(B)** WT, TLR2$^{-/-}$, CD14$^{-/-}$, or CD14 TLR2 DKO (n=5-6) mice were intranasally inoculated with 1µg/g LukF, LukS or PVL and neutrophil counts were enumerated in BALF at t=6h. Levels of proinflammatory IL-1β were determined in lung homogenates using ELISA Data are presented as mean ± SD and representative of two independent experiments. Significance was calculated versus WT mice.

Figure 1.

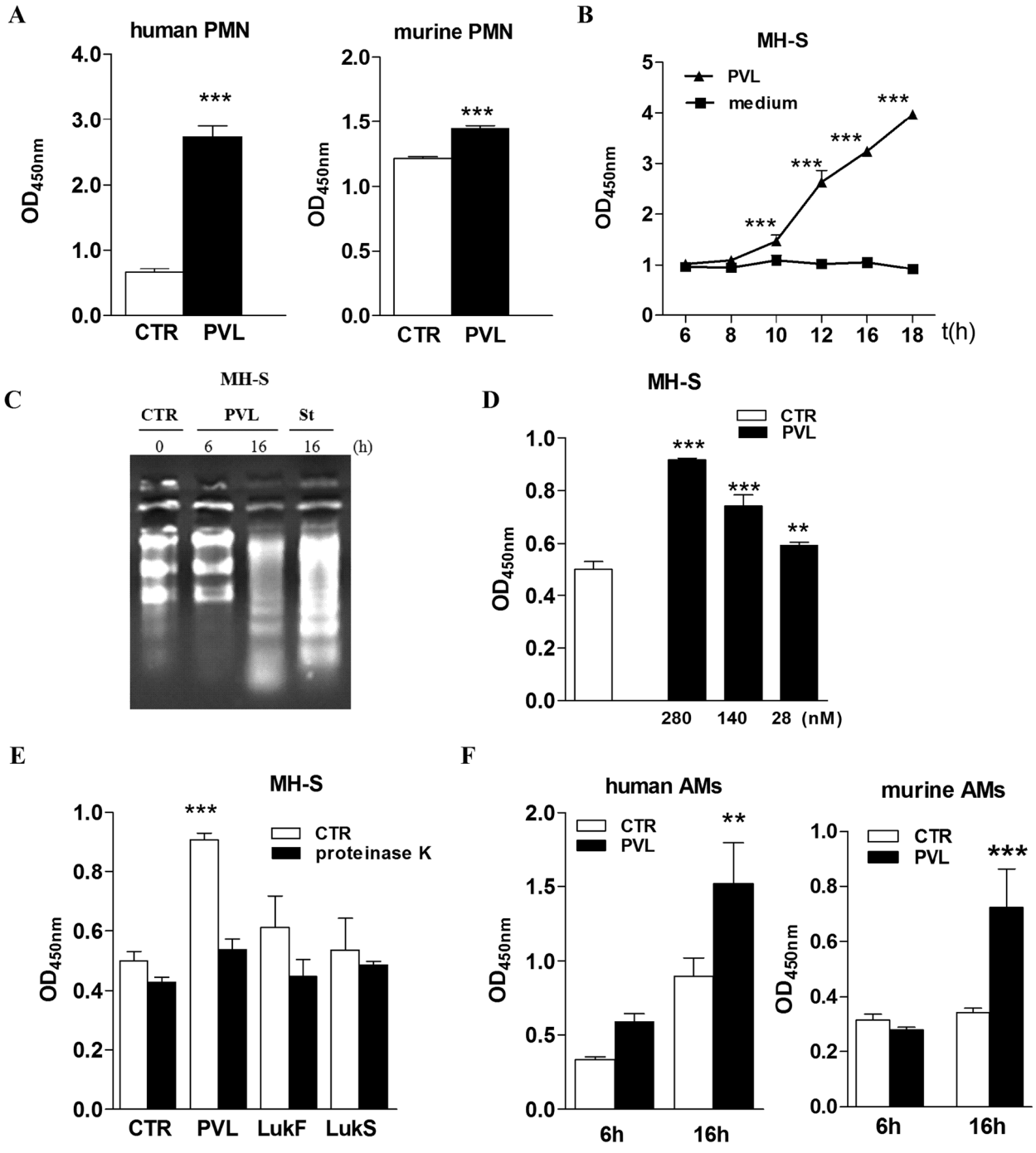

A
human PMN
murine PMN
OD450nm
CTR
PVL
***
B
MH-S
PVL
medium
t(h)
C
MH-S
CTR
PVL
St
0
6
16
16
(h)
D
MH-S
CTR
PVL
280
140
28 (nM)
**
E
MH-S
CTR
proteinase K
CTR
PVL
LukF
LukS
F
human AMs
murine AMs
6h
16h

Figure 2.

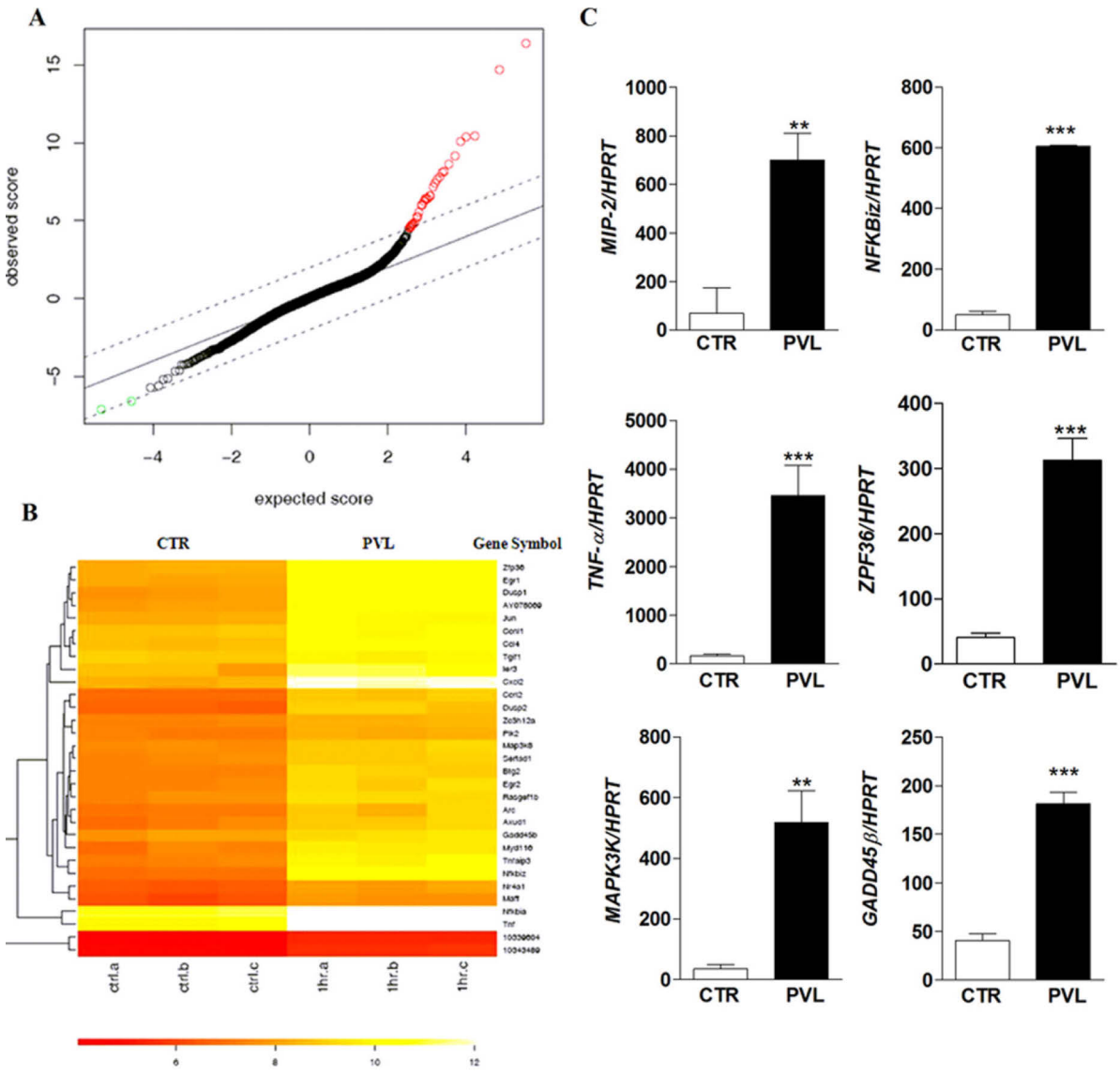

A
observed score
expected score
B
CTR
PVL
Gene Symbol
ctrl.a
ctrl.b
ctrl.c
1hr.a
1hr.b
1hr.c
C
MIP-2/HPRT
NFKBiz/HPRT
TNF-α/HPRT
ZPF36/HPRT
MAPK3K/HPRT
GADD45β/HPRT
CTR
PVL

Figure 3.

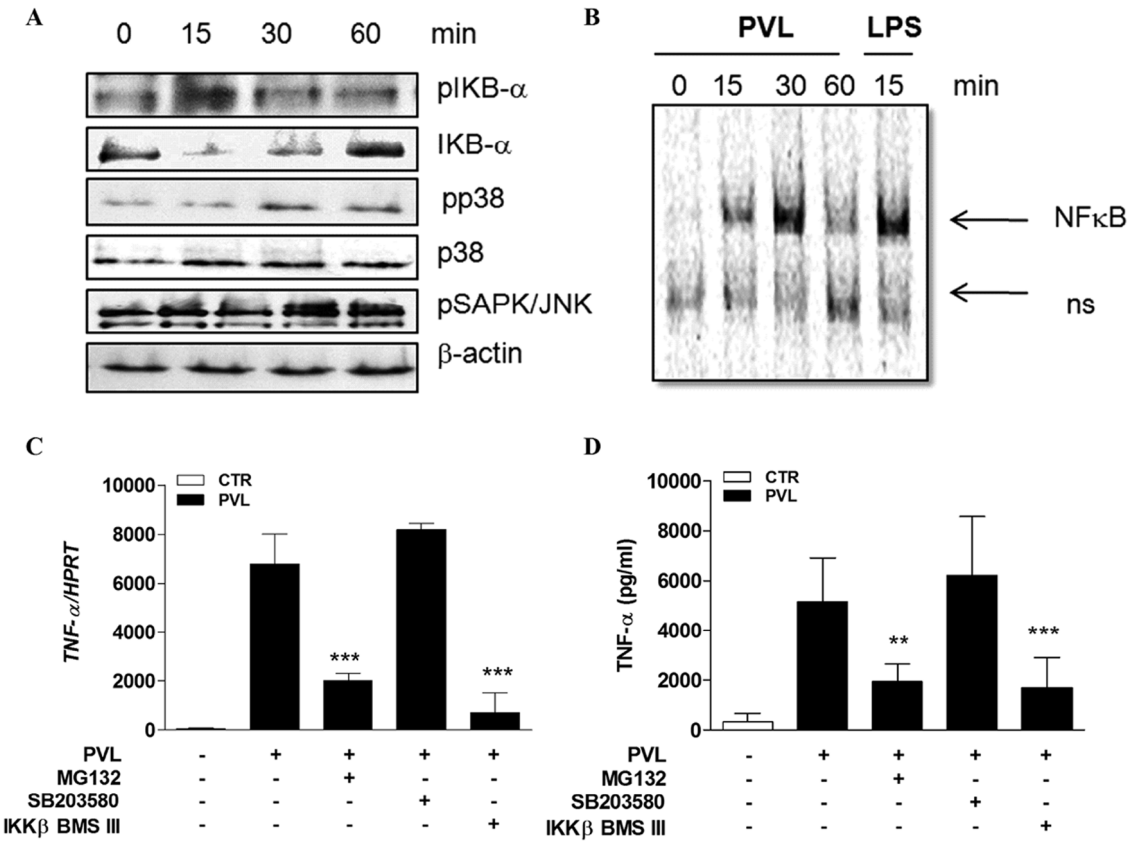
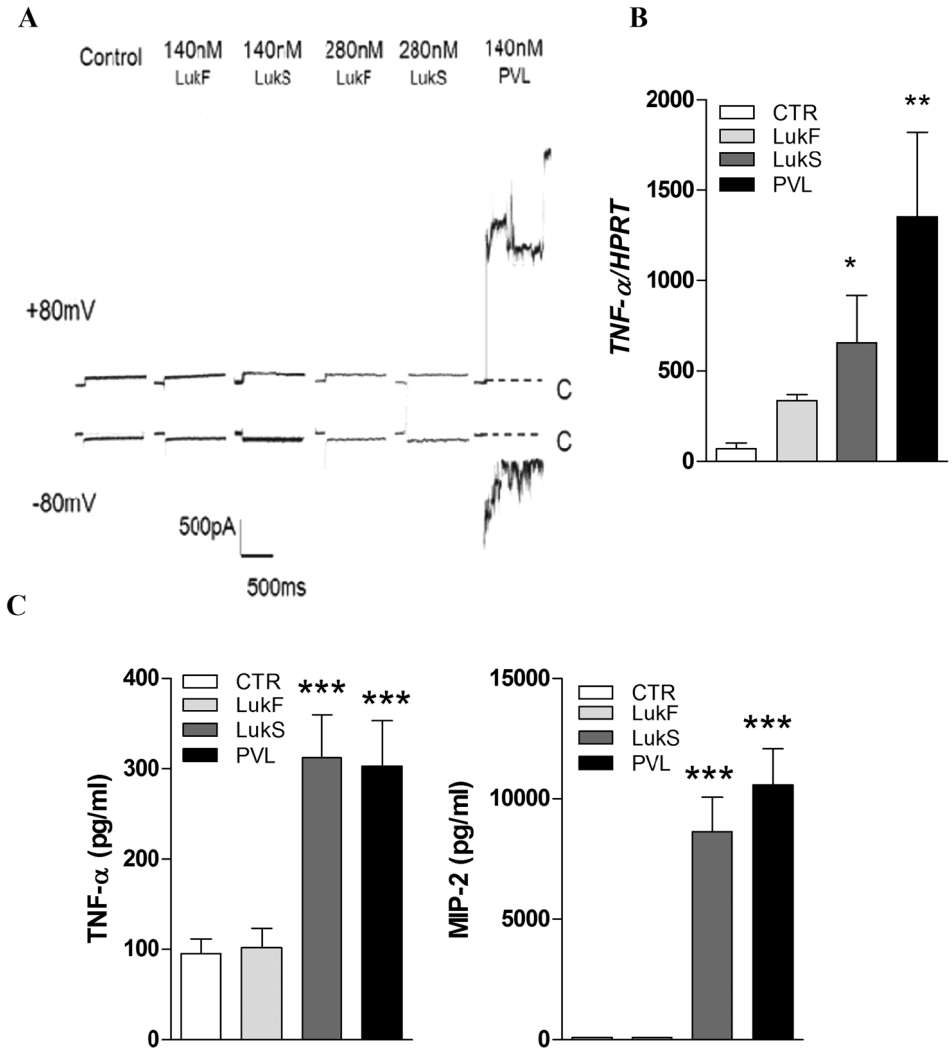

A
0 15 30 60 min
pIKB-α
IKB-α
pp38
p38
pSAPK/JNK
β-actin
B
PVL
LPS
0 15 30 60 15 min
NFκB
ns
C
CTR
PVL
TNF-α/HPRT
10000
8000
6000
4000
2000
0
***
***
PVL - + + + +
MG132 - - + - -
SB203580 - - - + -
IKKβ BMS III - - - - +
D
CTR
PVL
TNF-α (pg/ml)
10000
8000
6000
4000
2000
0
**
***
PVL - + + + +
MG132 - - + - -
SB203580 - - - + -
IKKβ BMS III - - - - +

Figure 4.


A
Control
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140nM LukS
280nM LukF
280nM LukS
140nM PVL
+80mV
-80mV
C
C
500pA
500ms
B
TNF-α/HPRT
2000
1500
1000
500
0
CTR
LukF
LukS
PVL
*
**
C
TNF-α (pg/ml)
400
300
200
100
0
CTR
LukF
LukS
PVL
***
***
MIP-2 (pg/ml)
15000
10000
5000
0
CTR
LukF
LukS
PVL
***
***

Figure 5.

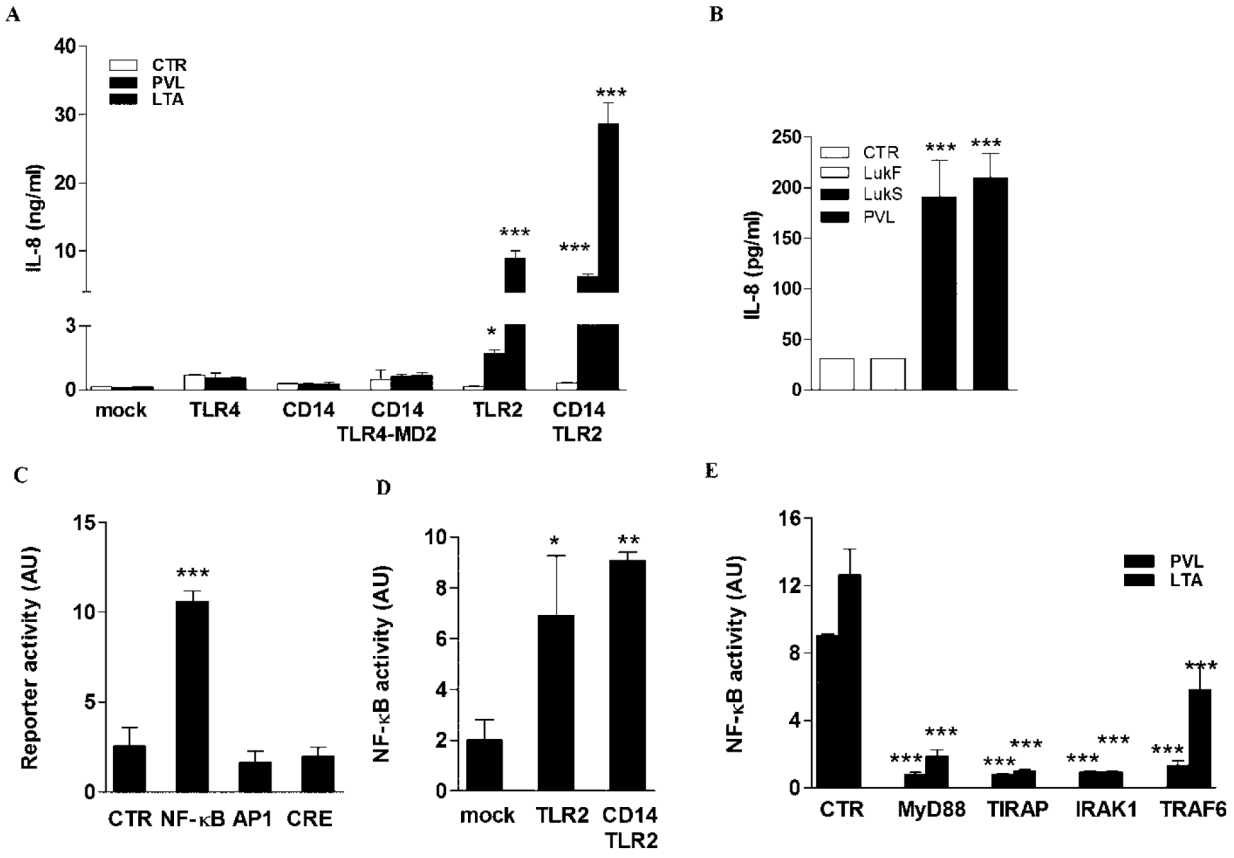

A
IL-8 (ng/ml)
CTR
PVL
LTA
mock
TLR4
CD14
CD14 TLR4-MD2
TLR2
CD14 TLR2
B
IL-8 (pg/ml)
CTR
LukF
LukS
PVL
C
Reporter activity (AU)
CTR NF-κB AP1 CRE
D
NF-κB activity (AU)
mock TLR2 CD14 TLR2
E
NF-κB activity (AU)
PVL
LTA
CTR MyD88 TIRAP IRAK1 TRAF6

Figure 6.

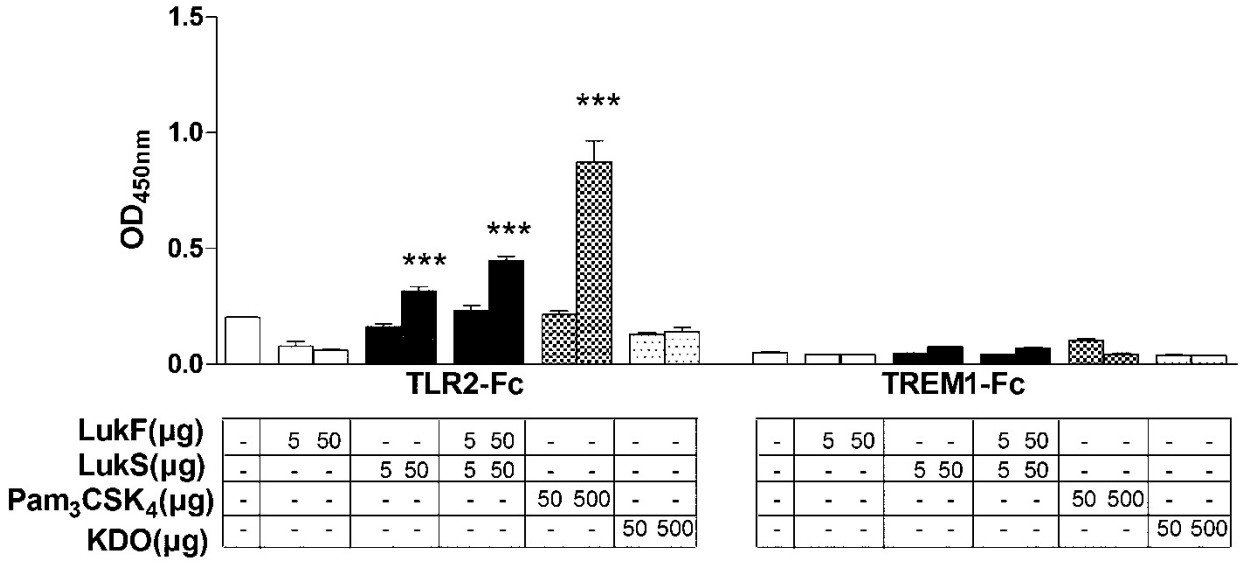

1.5
1.0
0.5
0.0
OD450nm
***
***
***
TLR2-Fc
TREM1-Fc
LukF(µg)
LukS(µg)
Pam3CSK4(µg)
KDO(µg)
- 5 50 - - 5 50 - - - -
- - - 5 50 5 50 - - - -
- - - - - - 50 500 - -
- - - - - - - - 50 500
- 5 50 - - 5 50 - - - -
- - - 5 50 5 50 - - - -
- - - - - - 50 500 - -
- - - - - - - - 50 500

Figure 7.

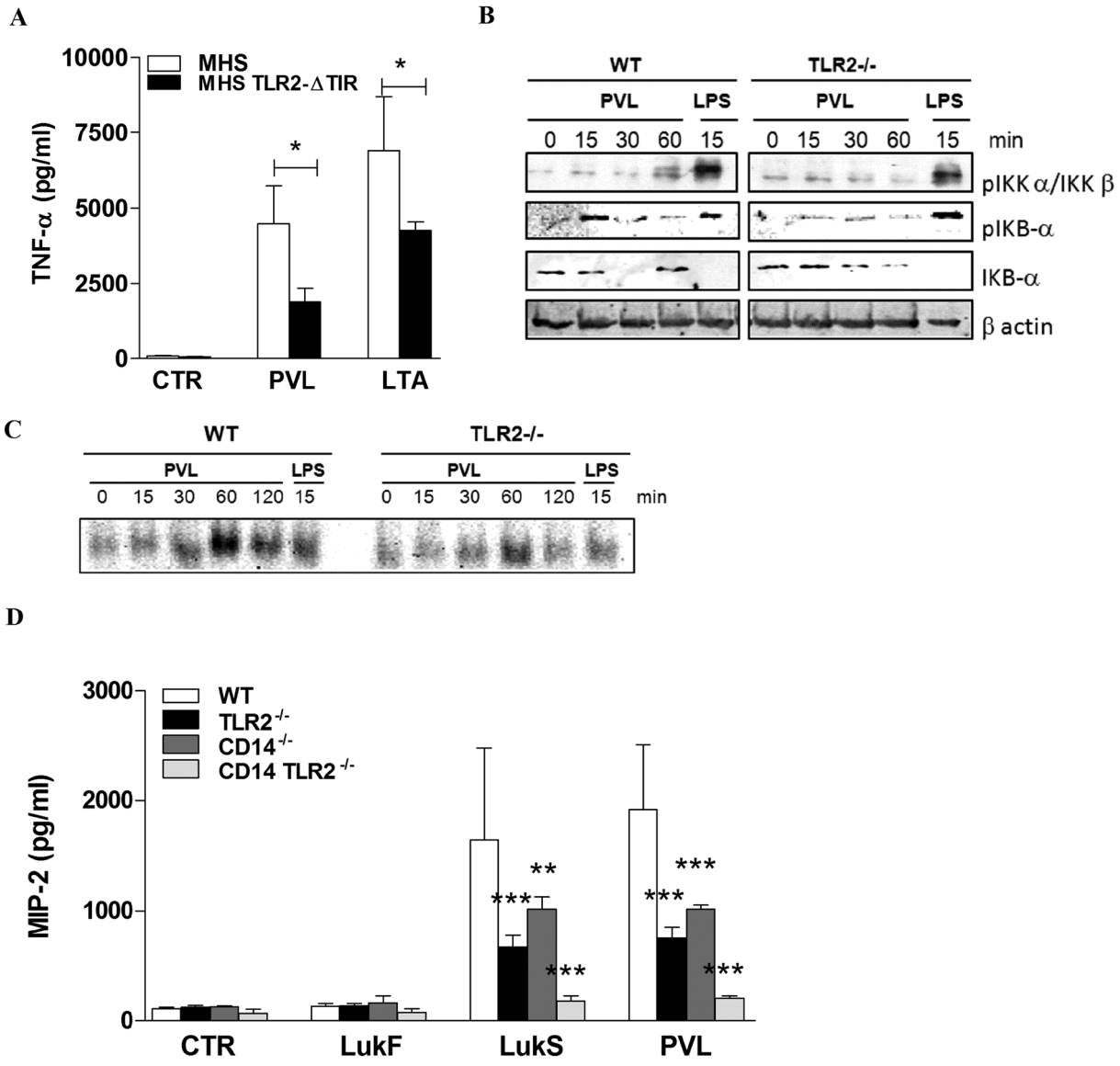
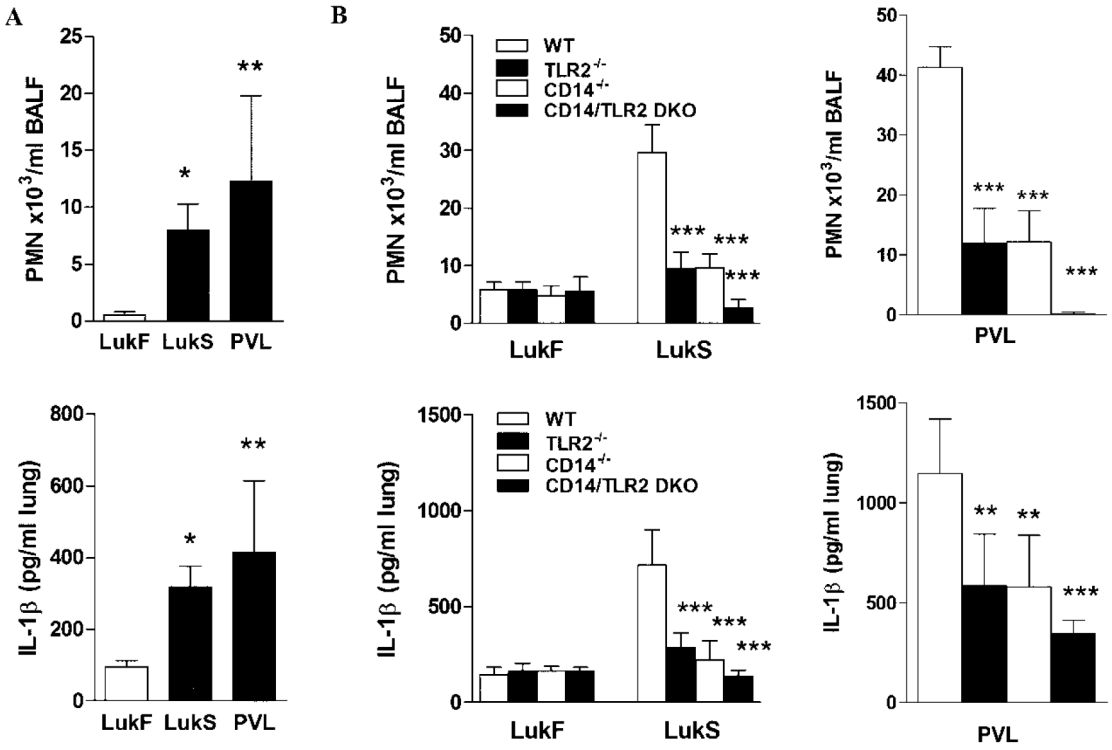

A
MHS
MHS TLR2-ΔTIR
TNF-α (pg/ml)
10000
7500
5000
2500
0
CTR
PVL
LTA
B
WT
TLR2-/-
PVL
LPS
0 15 30 60 15
min
pIKK α/IKK β
pIKB-α
IKB-α
β actin
C
WT
TLR2-/-
PVL
LPS
0 15 30 60 120 15
min
D
WT
TLR2-/-
CD14-/-
CD14 TLR2-/-
MIP-2 (pg/ml)
3000
2000
1000
0
CTR
LukF
LukS
PVL

Figure 8.


A
B
PMN x10³/ml BALF
IL-1β (pg/ml lung)
LukF
LukS
PVL
WT
TLR2-/-
CD14-/-
CD14/TLR2 DKO
*
**
***

Supplemental Information

Toll-like receptor 2 and CD14 mediate innate immunity and lung inflammation to Staphylococcal Panton Valentine Leukocidin in vivo.

Ana Zivkovic[*†], Omar Sharif[*†], Karin Stich[†], Bianca Doninger[*†], Mario Biaggio[*†], Jacques Colinge[*], Martin Bilban[‡], Ildiko Mesteri[§,] Parastoo Hazemi[¶], Rosa Lemmens-Gruber[¶] and Sylvia Knapp[*†]

Supplemental Figure Legends:

Figure S1. PVL is free of LPS. **(A)** Purified LukS and LukF were run on a 10% PAA gel and stained with silver solution. **(B)** $1x10^5$/well HEK 293 cells transfected with TLR4/CD14 and supplemented with soluble MD2 were stimulated with 100ng/ml LPS and 280nM PVL in the presence or absence of 10µg/ml of polymyxin B. IL-8 release was quantified in supernatants after 16h. **(C)** HEK 293 TLR2/CD14 cells were treated with 280 nM PVL with or without polymyxin B and IL-8 was measured in supernatants. ** indicates $p<0.01$ versus untreated controls (CTR) (for B and C). **(D)** $1x10^5$/well MH-S cells were treated with LukS, LukF, PVL or 10µg/ml Pam_3CSK_4, pretreated with 1µg/µl proteinase K or 0.2M NaOH. * indicates $p<0.05$, and *** indicates $p<0.001$ versus stimulation without proteinase K nor NaOH. Data presented are mean ± SD of triplicate samples and representative of at least 2 independent experiments.

Figure S2. LukS is the NF-κB activating subunit. NF-κB luciferase activity following stimulation of HEK293-CD14/TLR2 cells with LukF, LukS and PVL for 24h. Data are presented as fold activation compared to untreated sample; mean ± SD of triplicate samples are shown, data are representative of at least 2 independent experiments. * indicates $p<0.05$ and ** indicates $p<0.01$ versus LukF treated cells.

Figure S3. Quantification of NF-κB translocation. Band intensities from EMSA results depicted in Fig. 7C were quantified using the Quantiscan analysis software. WT and $TLR2^{-/-}$ BMDMs were treated with 280nM PVL or 100ng LPS for the indicated time, before nuclear

extracts were probed for binding to the NF-κB consensus binding site. Data are presented as mean ± SD and representative of three independent experiments. ***p<0.001, and *p<0.05 indicates differences between WT and TLR2$^{-/-}$ BMDMs at corresponding time points.

Figure S4. Decreased lung inflammation in TLR2$^{-/-}$ mice treated with PVL. WT and TLR2$^{-/-}$ (n=10/genotype) mice were intranasally inoculated with 1μg/g PVL. **(A)** Levels of proinflammatory cytokines were quantified in lung homogenates using ELISA and **(B)** neutrophil and macrophage counts were enumerated in BALF at t=6h. **(C)** Representative lung immunohistochemical stainings for neutrophils (Ly6) in lungs of WT and TLR2$^{-/-}$ mice 24h after administration of PVL (1μg/g). Magnification x20 (upper panel) and x40 (lower panel). **(D)** Inflammation score as described in Supplemental methods part. Data are presented as mean ± SD and representative of two independent experiments. . ***p<0.001, **p<0.01 and *p<0.05 indicates differences between WT and TLR2$^{-/-}$ mice.

Supplemental Tables:

Table S1. Primers used for RT-PCR

Primers	Sequence	Product size	Annealing Temp.
mHprt_FW	5´-GTT AAG CAG TAC AGC CCC AAA ATG -3`	96bp	72°C
mHprt_RV	5`- AAA TCC AAC AAA GTC TGG CCT GTA -3`		
mTnfα_FW	5`- GAA CTG GCA GAA GAG GCA CT -3`	200bp	68°C
mTnfα_RV	5`- GGT CTG GGC CAT AGA ACT GA -3`		
mMap3k8_FW	5'- CAG GAG GTT CCC TGG CTG TC -3'	169bp	68°C
mMap3k8_RV	5'- CGA CGA TTT GGT AGC GGC CAT TC -3'		
mZpf36_FW	5'- TTT GAG GCA GGG GTG TTT GG -3'	163bp	68°C
mZpf36_RV	5'- AGG TCC CCA CAG CAA TGA GC -3'		
mGadd45b_FW	5'- ACG CGG TTC AGA AGA TGC AG -3'	350bp	68°C
mGadd45b_RV	5'- TGT ATG ACA GTT CGT GAC CAG GAG -3'		
mNfkbid _FW	5'- TGG TCC AGC CAC TGA CTC CA -3'	298bp	68°C
mNfkbid_RV	5'- TGT GAC AGG GAA GGC TCA GG -3'		

Table S2. List of upregulated genes in MH-S cells upon PVL challenge (1h).

REFSEQ_ID	Gene Name	Gene Symbol	Fold change	DAVID annotated
NM_009140	Chemokine (C-X-C motif) ligand 2, MIP2-alpha, Gro-beta, Gro-2	*Cxcl2*	12.1	yes
NM_030612	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	*Nfkbiz*	9.3	yes
ENSMUST00000019997	Tumor necrosis factor, alpha-induced protein 3,	*Tnfaip3*	6.0	no

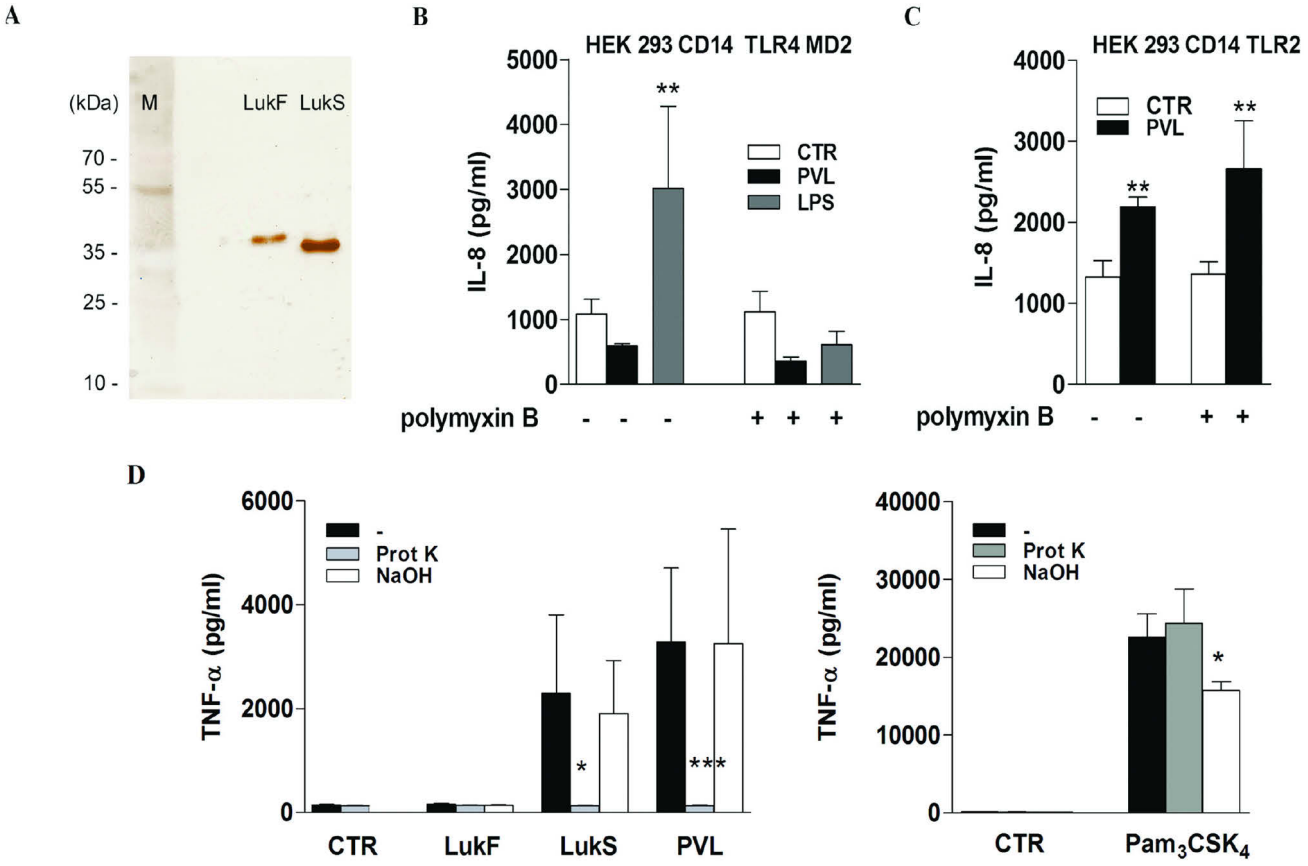
ENSMUST00000025025	Dual specificity phosphatase 1,	*Dusp1*	5.2	no
NM_013693	Tumor necrosis factors	*Tnf*	5.1	yes
NM_007913	Early Growth Response Protein 1	*Egr1*	4.9	yes
NM_010090	Dual specificity phosphatase 2	*Dusp2*	4.8	yes
NM_008654	myeloid differentiation primary response gene 116	*Myd116*	4.7	yes
M58691	Zinc finger protein 36	*Zfp36*	4.6	yes
NM_172142	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta IkappaBNS	*AY078069*	4.3	yes
ENSMUST00000111888	Chemokine (C-C motif) receptor-like 2	*Ccrl2*	3.9	no
NM_010118	Early growth response 2	*Egr2*	3.5	yes
NM_153287	AXIN1 up-regulated 1	*Axud1*	3.4	yes
NM_145839	GPIgamma4	*Rasgef1b*	3.2	no
NM_007570	BTG family, member 2	*Btg2*	3.1	yes
NM_010591	Jun oncogene	*Jun*	3.1	yes
ENSMUST00000023779	nuclear receptor subfamily 4, group A, member 1	*Nr4a1*	2.9	no
NM_013652	Chemokine (C-C motif) ligand 4,	*Ccl4*	2.9	yes
NM_010755	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*Maff*	2.8	yes
NM_007746	Mitogen-activated protein kinase kinase kinase 8	*Map3k8*	2.7	yes
ENSMUST00000008528	SERTA domain containing 1	*Sertad1*	2.6	no
NM_008655	Growth arrest and DNA-damage-inducible, beta,	*Gadd45b*	2.6	yes
ENSMUST00000021413	IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha)	*Nfkbia*	2.5	no
NM_019937	Cyclin L1	*Ccnl1*	2.4	yes
ENSMUST00000022212	Polo-like kinase 2	*Plk2*	2.2	no

Table S3. Gene ontology analysis of upregulated genes, as annotated by DAVID

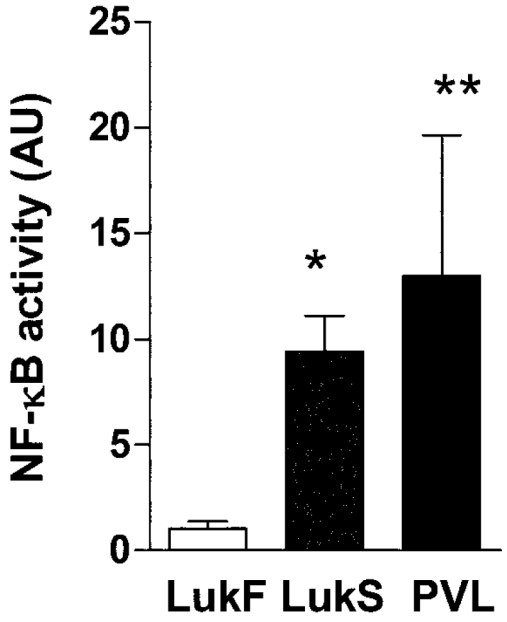
Biological process	p-value	FDR
Toll-like receptor signaling pathway	0.00029	0.4
MAPK signaling pathway	0.00019	0.2
Response to wounding	0.0003	0.6
Response to external stimulus	0.0016	3.0
Response to stress	0.0014	2.7
Inflammatory response	0.065	0.2

FDR: false discovery rate (%)

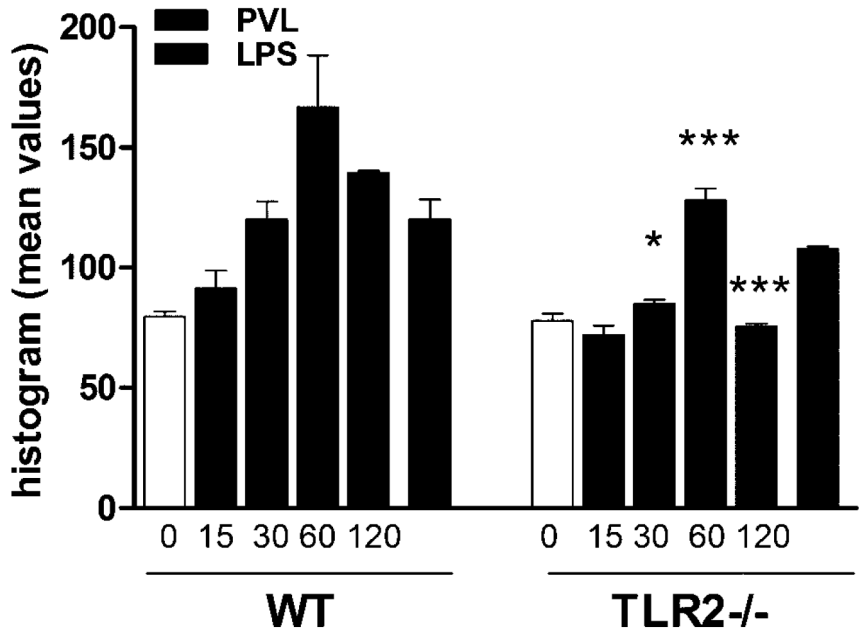
Supplemental Figure S1.



Supplemental Figure S2.



Supplemental Figure S3.



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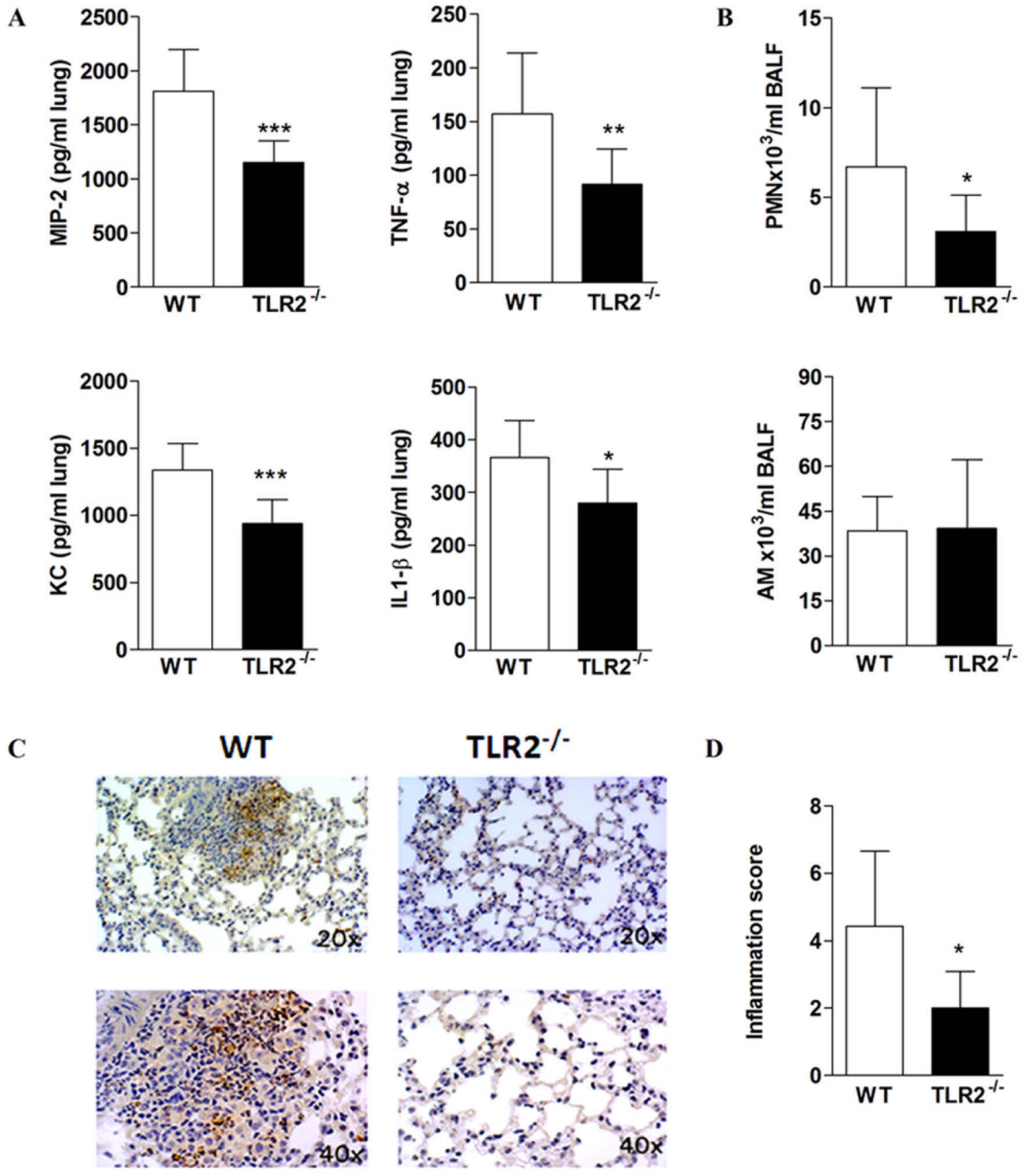


6.2 Staphylococcal Panton-Valentine Leukocidin and protein A synergistically inflame lungs via involvement of TNF-R1.

Staphylococcal Panton-Valentine Leukocidin and protein A synergistically inflame lungs via involvement of TNF-R1.

Zivkovic Ana[1, 2], Biaggio Mario[1, 2], Omar Sharif[1, 2], Karin Stich[2], Bianca Doninger[1, 2], and Sylvia Knapp[1, 2]

[1]Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, Vienna, 1090; Austria; [2]Department of Medicine I, Div. of Infectious Diseases and Tropical Medicine, Medical University Vienna, Vienna, 1090; Austria;

(Author's contribution: A.Z. designed research, performed experiments, analyzed data and wrote the manuscript, M.B. performed experiments and analyzed data, O.S. participated in the isolation of ECs, K.S. participated in BMT experiments, B.D. participated in BMT experiments and performed ELISAs, and S.K. designed and supervised the research, analyzed data and wrote the manuscript)

Running Title: PVL and protein A synergize to cause inflammation on epithelial cells

Corresponding author:

Sylvia Knapp M.D., Ph. D, Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, and Department of Medicine I, Div. of Infectious Diseases and Tropical Medicine, Medical University Vienna, Waehringer Guertel 18-20; 1090 Vienna, Austria, Phone: +43-1-40400-5139; Fax: +43-1-40400-5167, e-mail: sylvia.knapp@meduniwien.ac.at

Key words: PVL, protein A, TNF-R1, lung, epithelial cells.

Abbreviations:

Abstract

Panton-Valentine Leukocidin (PVL) is considered a potential virulence factor in community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections. We have previously shown that TLR2 and CD14 play essential roles in the recognition of the PVL subunit LukS, and that this interaction activates alveolar macrophages to secrete cytokines and chemokines. However, in our *in vivo* experiments we did not observe necrosis of tissue, which has been described in humans suffering from pneumonia. Therefore, we hypothesized that PVL might synergize with other staphylococcal virulence factors in aggravating inflammation. Since it has been described that protein A was up-regulated in PVL-carrying *S. aureus* strains, we tested the synergistic effects of PVL and protein A and evaluated the role of lung epithelial cells herein.

By first studying the role of epithelial cells in PVL-associated inflammation, we discovered that these cells respond via a TLR2-dependent mechanism to PVL and LukS, while LukF did not induce any inflammation. Addition of protein A synergistically enhanced the inflammatory response by respiratory epithelial cells and in a murine lung inflammation model *in vivo*. Because TNF-R1 has been described to mediate the inflammatory response to protein A earlier, we tested the requirement for TNF-R1 in causing this synergistic effect. Indeed, the synergistic effect was no longer visible when isolated primary epithelial cells or murine lung inflammation models were investigated in the absence of TNF-R1. Unexpectedly we also discovered that the inflammatory response to PVL itself was found reduced in the absence of TNF-R1. Further studies revealed that PVL-induced TNF secretion by epithelial cells (and alveolar macrophages) enhances the inflammatory response in a autocrine and paracrine fashion. Hence, TNF is a major mediator of PVL-induced lung inflammation and the synergism with the virulence factor protein A further augments the inflammatory response within the lungs.

Introduction

Panton-Valentine Leukocidin (PVL) is a staphylococcal pore-forming toxin [141]. An increased attention to this toxin arose during the last decade, due to the sudden emergence of life-threatening infections caused by community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) strains carrying PVL [162]. So far, PVL is the only known *S. aureus* virulence factor which is epidemiologically associated with the development of severe necrotizing pneumonia and necrotizing skin infections in humans [162, 164]. PVL preferably acts on polymorpho-nuclear cells (PMNs), which are highly susceptible to its pore forming properties and die quickly upon incubation with PVL [165, 166]. *S. aureus* gene expression analysis showed that bacteria, which express PVL show an increased expression of the cell-wall anchored protein, protein A [167]. Protein A and PVL represent two out of roughly 40 *S. aureus* virulence factors [168]. Virulence factors significantly contribute to the increased survival of *S. aureus* by counteracting the host's anti-bacterial response. As such, staphylococcal protein A has been shown earlier to interfere with host defense by binding to the Fc portion of immunoglobulins, thereby preventing opsonization and phagocytosis of bacteria [169]. In addition, protein A seems to play a crucial role during staphylococcal lung infections, as the group of Alice Prince could show some years ago that protein A is able to induce lung inflammation via TNF-R1 expressed on respiratory epithelial cells [123].

We recently studied the effects of PVL on alveolar macrophages and discovered that the LukS subunit of PVL exerts potent inflammatory properties *in vitro* and *in vivo* [170]. Of importance, this pro-inflammatory effect was independent of PVL's pore-forming properties. LukS is recognized by TLR2 and requires the presence of the co-receptor CD14 to potently induce an inflammatory response. Both of these receptors are widely expressed on myeloid cells in the lungs as well as on epithelial cells. Beside alveolar macrophages, epithelial cells play an important role as immune cells as they secrete a vast amount of chemokines, thus attracting neutrophils to the site of infection [6]. In a mouse model of necrotizing pneumonia caused by PVL positive MRSA an increased binding of bacteria to the damaged epithelium has been noticed [171, 172]. Furthermore, one of the highly abundant proteins on epithelial cells, TNF-R1, which normally binds to the proinflammatory cytokine TNF-α, was shown to play an important role in the recognition of protein A [123]. We hypothesized that epithelial cells participate in

PVL-associated lung inflammation as they express CD14 and TLR2. Since the expression of some staphylococcal virulence factors, like i.e. PVL and protein A is mutually regulated [167], we additionally hypothesized that PVL and protein A may act synergistically. Cooperation between these two virulence factors could augment local inflammation that might ultimately lead to an overwhelming inflammatory response in the lungs.

Results

1. Epithelial cells are insensitive to PVL's pore forming properties

PVL is a beta-barrel pore forming toxin, which binds to the cellular membrane and creates octamere-structured pores that can eventually lead to cell death [143]. Several cell lines have been investigated for their sensitivity to PVL's pore-forming capacity and so far human and rabbit granulocytes have been shown to be most sensitive [165]. We and others investigated the response of murine granulocytes to PVL earlier and showed an impaired viability upon PVL treatment [165, 170]. Furthermore, alveolar macrophages were also susceptible to pore-formation by PVL, although these cells died only after prolonged incubation, suggesting that alveolar macrophages are either less sensitive or able to repair pores more efficiently than granulocytes [165, 170]. Because extensive lung necrosis is a known feature of PVL associated pneumonia, we wished to investigate if respiratory epithelial cells were susceptible to pore-formation by PVL. We therefore treated MLE15 and primary epithelial cells with a dose of PVL that has been shown to induce pores earlier (0.1µg/µl PVL) for various time-points and measured cell viability. Staurosporine, a classical inducer of apoptosis [173], was used as positive control. Assaying LDH release, AnnexinV/Propidium-iodide staining (FACS) and DNA laddering (data not shown) we did not discover any death-inducing properties of PVL, even after prolonged incubations of up to 48h (**Figure 1**). These data suggest that murine epithelial cells are insensitive to PVL's pore forming properties.

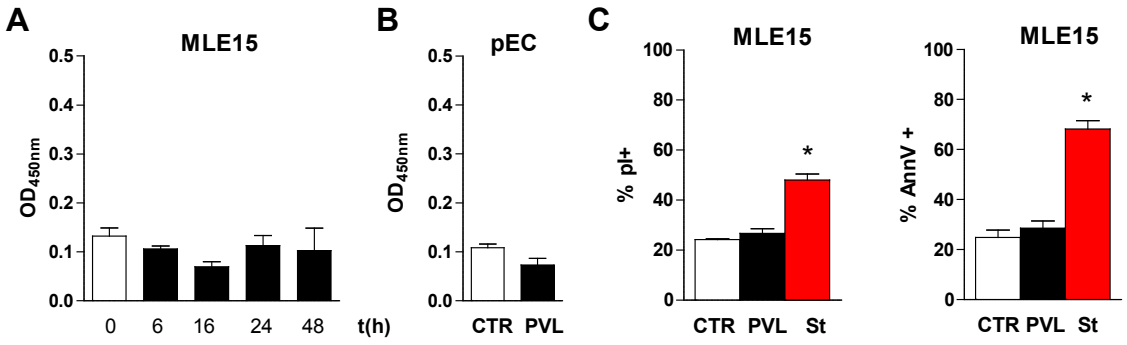


Figure 1. Murine epithelial cells do not undergo cell death upon treatment with PVL. (A) MLE15 cells and (B) primary epithelial cells were treated with 0.1µg/µl PVL for indicated timepoints (A) or 6h (B). Viability of cells was assessed by measuring LDH release. (C) MLE15 cells were treated with 0.1µg/µl of PVL or 10µM staurosporine (St) for 16h after which cells were stained for AnnexinV and Propidium-iodide and analyzed by FACS. Data

presented are mean ± SD of n=3 replicates. * indicates $p<0.05$ versus control. Data are representative of at least two independent experiments.

2. Epithelial cells respond to PVL by secretion of pro-inflammatory chemokines

Previously we have shown that alveolar macrophages sense PVL via the extracellular domain of TLR2 and that CD14 assists in triggering an inflammatory response [170]. To investigate the contribution of epithelial cells in PVL-induced pulmonary inflammation, we now studied the inflammatory response elicited by lung epithelial cells upon challenge with PVL. As has been published earlier, we verified that mouse respiratory epithelial cells express TLR2 (**Supplementary Figure 1**) and CD14 (**Supplementary Figure 2**) [174-176]. We then treated immortalized murine type II epithelial cells (MLE15) and primary epithelial cells from WT (C57Bl/6J) mice with 0.1µg/µl LukF, LukS or PVL and observed similar patterns of KC secretion by both cell types (**Figure 2A**). Additionally, we observed earlier that WT mice treated intranasally with 1µg/g of LukF, LukS or PVL for 6h induced KC releases *in vivo* [170], where LukS acted as the active component and induced KC while LukF treatment did not trigger any inflammation. To investigate if TLR2 mediates the inflammatory response to LukS and PVL by primary epithelial cells, we then stimulated WT and $TLR2^{-/-}$ primary epithelial cells with these compounds and quantified KC in supernatants. Doing so, we observed that, just like in alveolar macrophages [170], epithelial cell expressed TLR2 mediated the inflammatory response to LukS or PVL, respectively (**Figure 2B**).

3. PVL and protein A synergistically enhance inflammation.

The contribution of PVL to the development of necrotizing pneumonia, despite a strong epidemiologic link [162] remains uncertain and controversial. Since PVL alone obviously does not induce epithelial cell death, yet demonstrates inflammatory properties, we hypothesized that necrosis might be a result of an overwhelming inflammatory response. Because PVL alone induces lung inflammation, but not sufficiently to induce severe tissue damage, we hypothesized that PVL might interact with other staphylococcal virulence factors so that the potential synergism might cause overwhelming inflammation and tissue necrosis. A recent study discovered that the presence of PVL was associated with an increased expression of *spa*, which

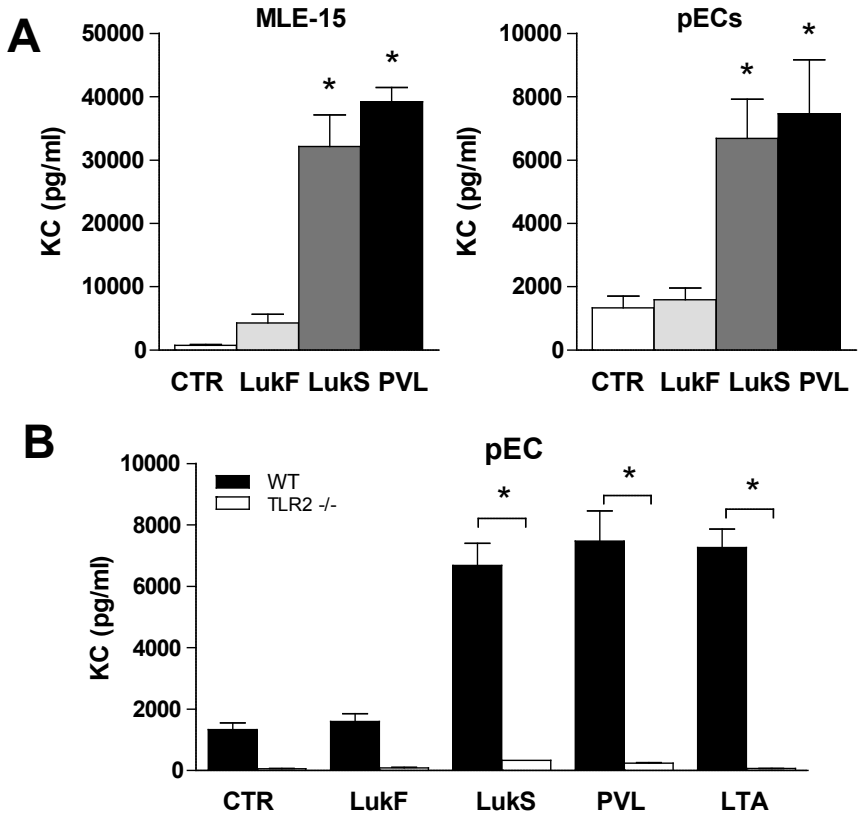


Figure 2. Epithelial cells secrete KC upon LukS/PVL stimulation. (A) MLE15 and primary respiratory epithelial cells (pEC) were treated with 0.1µg/µl LukF, LukS or PVL for 16h before KC was measured in supernatants. (B) Primary epithelial cells from WT and TLR2$^{-/-}$ mice were isolated and stimulated with indicated compounds for 6h before KC release in supernatants was measured. Data presented are mean ± SD of n=3 (A) or n=4 replicates (B). * indicates $p<0.05$ versus control (A) or WT (B), respectively.

codes for the staphylococcal virulence factor protein A [167, 177]. Protein A strongly impacts staphylococcal virulence as it interacts with host IgG and thereby interferes with phagocytosis [111] and also was shown to induce lung inflammation via TNF-R1 expressed on respiratory epithelial cells [123]. We hypothesized that since PVL and protein A expression were shown to be linked that these two virulence factors might synergistically inflame the lungs. To investigate this idea we treated MLE15 and primary epithelial cells with PVL and protein A and indeed observed higher levels of secreted KC than when cells were treated with PVL or protein A separately (**Figure 3A**). We also studied the possibility that protein A treatment renders epithelial cells sensitive to PVL's pore forming properties, and additionally assayed these cells for LDH release. No cell death was observed upon co-stimulation (**Supplementary Figure 3**).

To further verify the synergistic enhancement of inflammation, we performed *in vivo* experiment, where PVL, protein A or PVL and protein A together were administrated intranasally to WT mice (**Figure 3B**). The number of PMNs in BALF as well as chemokines in lung homogenates was clearly enhanced in mice who received both PVL and protein A, hence confirming synergistic effects *in vivo* (**Figure 3B**).

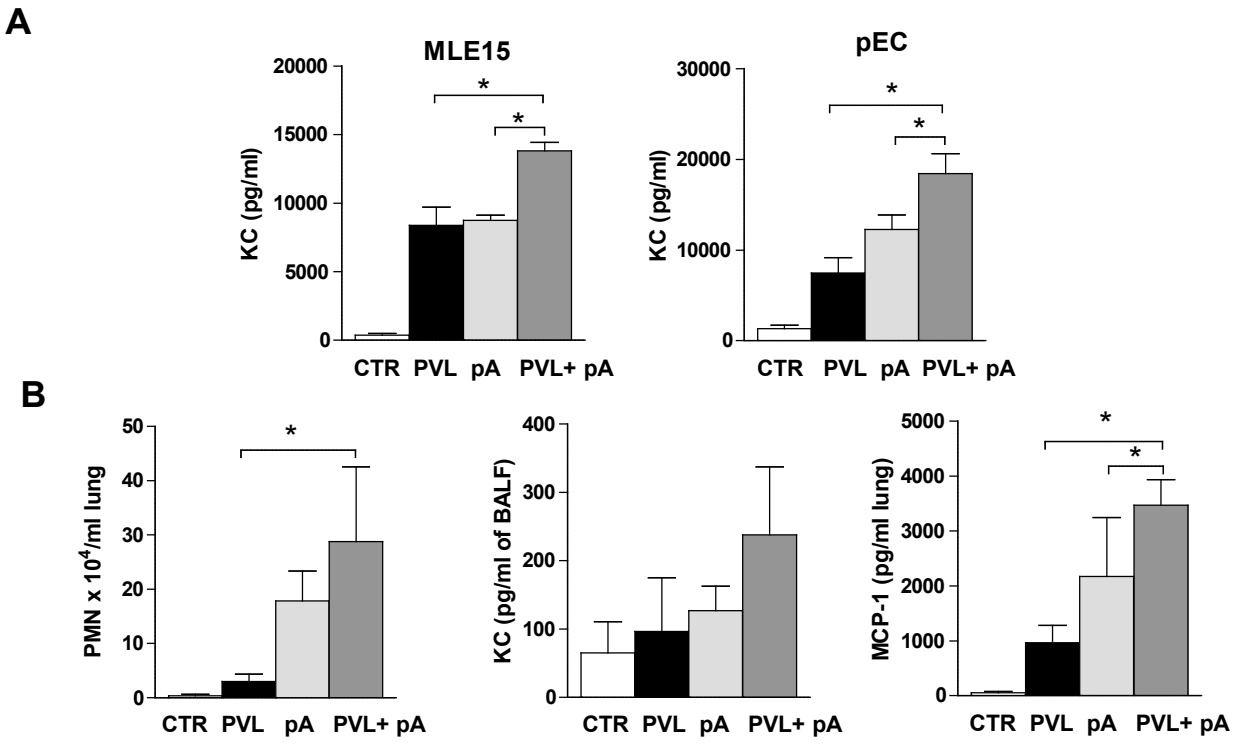


Figure 3. PVL and protein A synergistically augment inflammation in lungs. (A) MLE15 and primary epithelial cells were treated with 0.1µg/µl PVL and/or 200ng/µl of protein A for 6h. KC was quantified in supernatants. (B) WT mice were treated with 1µg/g of PVL, 2.5µg/g of protein A or both. PMNs were enumerated in BALF, KC levels were measured in BALF, and MCP-1 concentrations were quantified in lung homogenates. Data are presented as mean ± SD of n=3 replicates (A) and n=6 mice per group (B). * indicates $p<0.05$.

4. TNF-R1 is required for the synergistic effects of PVL and protein A

Given that PVL showed synergistic augmentation of the inflammatory response we sought to investigate this response in more details. Protein A is a surface molecule, covalently anchored to the bacterial cell wall that can interfere with phagocytosis by binding to the Fc-part of IgG [111]. Moreover it was reported recently that the IgG binding domain of protein A activates epithelial

cells via TNF-R1, resulting in NF-κB activation and IL-8 secretion by respiratory epithelial cells [122, 123]. We found TNF-R1 abundantly expressed on epithelial cells (**Supplementary Figure 2**), and therefore wanted to study if the synergistic enhancement of inflammation by PVL and protein A requires the presence of TNF-R1. For this purpose we treated primary epithelial cells from WT and TNF-R1$^{-/-}$ mice with PVL and/or protein A for 16h and quantified KC in supernatants. Doing so, we indeed observed that the synergistic effects were no longer present when TNF-R1 was missing (**Figure 4**). However, to our surprise we also discovered that the PVL-induced inflammation required the presence of TNF-R1, as epithelial cells from TNF-R1-deficient mice produced significantly less KC when compared to WT cells (**Figure 4**). To test if TNF-R1 was only required for the inflammatory response by epithelial cells, we also tested alveolar and peritoneal macrophages and discovered that these phagocytes released significantly less TNF in the absence of TNF-R1 (**Supplementary Figure 4**).

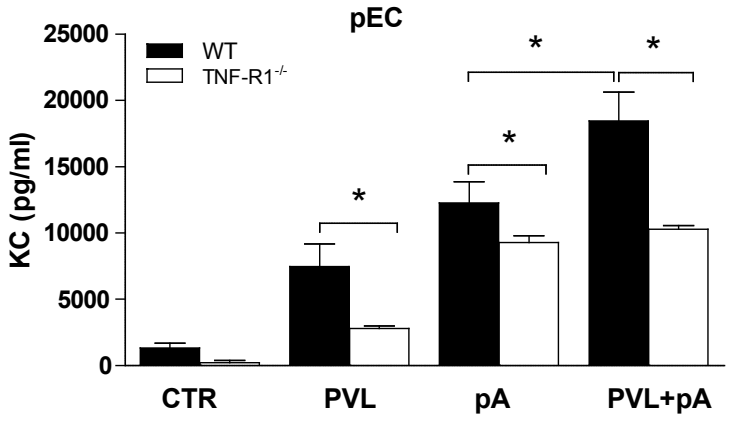


Figure 4. TNF-R1 is required for synergistic effects by PVL and protein A. Primary epithelial cells isolated from WT and TNF-R1 knock-out mice were treated with 0.1µg/µl PVL and/or 200ng/µl of protein A for 16h. KC was quantified in supernatants by ELISA. Data are presented as mean ± SD of n=4 replicates. *indicates $p<0.05$.

To additionally evaluate the synergistic potential of PVL and protein A and the role of TNF-R1 herein *in vivo*, we administrated both virulence factors to WT and TNF-R1$^{-/-}$ mice. We observed that TNF-R1 was required for the synergistic effects of PVL and protein A, as no enhanced inflammation was observed in mice lacking TNF-R1 (**Figure 5**). Intriguingly, the influx of neutrophils was severely diminished in TNF-R1$^{-/-}$ mice treated with either PVL, protein A or the combination of PVL and protein A.

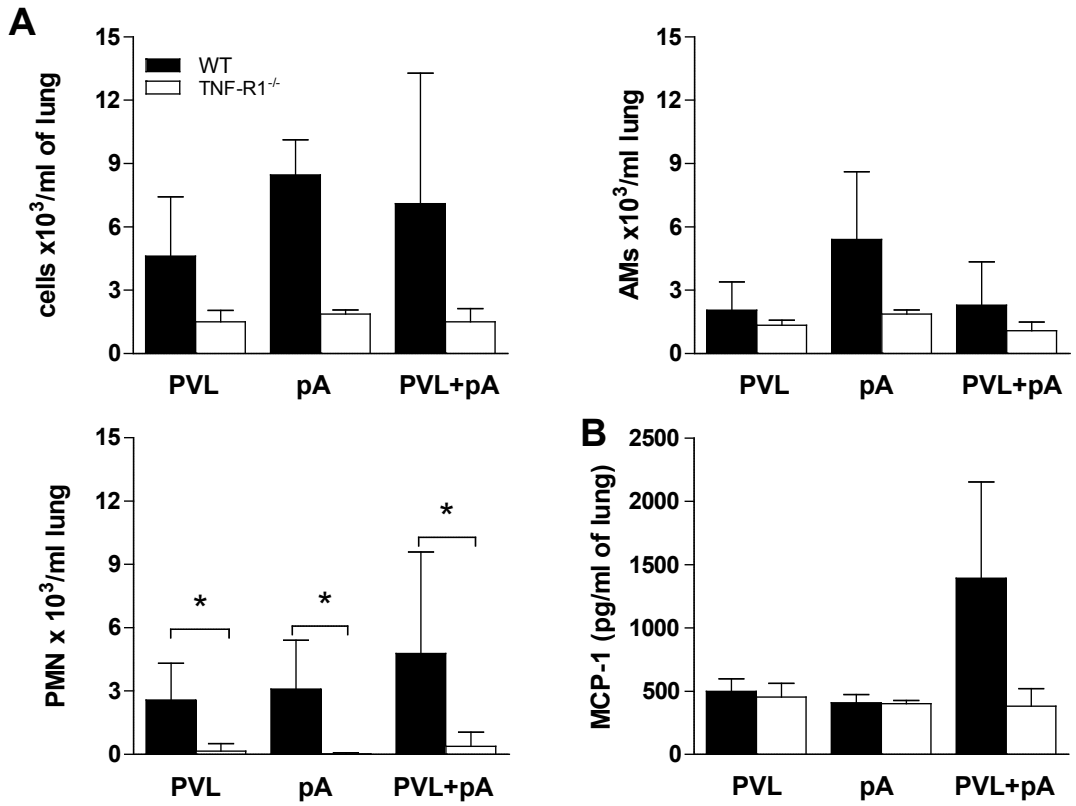


Figure 5. No synergistic effects in the absence of TNF-R1 *in vivo*. A. WT and TNF-R1$^{-/-}$ mice were treated with indicated compounds intranasally. After 6h total and differential cell counts were performed in BALF samples (A) and cytokines were quantified in lung homogenates (B). Data are presented as mean ± SD of n=5 mice per group. * indicates <0.05 versus WT mice.

5. Presence of TNF-R1 contributes to PVL induced inflammation

Because PVL-induced inflammation was repeatedly found reduced in the absence of TNF-R1 **(Fig. 4, 5 and Supplementary Figure 4)** we wondered why PVL-induced inflammation requires the presence of TNF-R1. Since in all previous experiments we quantified cytokines and chemokines in supernatants of cells or in lung homogenates from *in vivo* experiments 6h after stimulation, we decided to investigate mRNA induction at earlier timepoints, which should help us answer if TNF-R1 is also required for gene induction. For this purpose we stimulated primary alveolar macrophages from WT and TNF-R1$^{-/-}$ mice with PVL and used TNF-α as a positive control. Doing so, we discovered that PVL does not require the presence of TNF-R1 to induce TNF-mRNA, as values were identical between WT and TNF-R1$^{-/-}$

cells. However, TNF-induced inflammation was significantly reduced in the absence of TNF-R1. Hence, PVL induces inflammatory genes independent of the presence of TNF-R1.

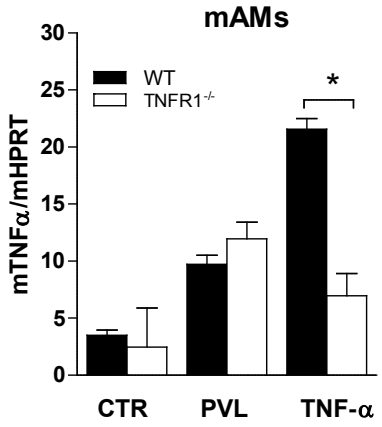


Figure 6. TNF-R1 is not required for inflammatory gene induction by PVL. Primary alveolar macrophages from WT and TNF-R1$^{-/-}$ mice were isolated and stimulated with PVL or TNF at indicated doses. 1h later RNA was isolated and TNF-α mRNA was quantified by RT-PCR. Data are presented as mean ± SD of n=4 replicates. * indicates $p<0.05$ versus WT.

6. TNF contributes to PVL induced inflammation

To finally understand the contribution of TNF-R1 in PVL-induced lung inflammation, we considered the possibility that TNF might enhance the PVL-induced inflammatory response in an autocrine/paracrine fashion. We already knew from earlier experiments, that PVL treatment of alveolar macrophages induced a very selective set of only 29 inflammatory mediators, and that TNF was among the highest induced genes in this microarray experiment [170]. It thus seemed reasonable to hypothesize that TNF is a main amplifier of PVL-induced lung inflammation. To investigate this possibility and to understand the respective contribution of different cell types within the lungs, we generated chimeric mice by transplanting bone marrow from WT or TNF-R1$^{-/-}$ mice into radiated WT or TNF-R1$^{-/-}$ animals (thus generating 4 groups of mice WTxWT, WTxTNF-R1$^{-/-}$, TNF-R1$^{-/-}$xWT, TNF-R1$^{-/-}$xTNF-R1$^{-/-}$). We verified engraftment after 6 weeks by testing isolated alveolar macrophages for the presence of TNF-R1 **(Supplementary Figure 5)**, after which we administered PVL intranasally and harvested BAL and lungs after 6h. The results are depicted in **Figure 7** and indicate that the absence of TNF-R1 on either lung structural

cells or bone-marrow-derived cells modestly reduced the inflammatory response to PVL. However, TNF-R1$^{-/-}$ mice that received TNF-R1$^{-/-}$ bone marrow showed the most impressive reduction in PMN influx into the lungs. These data indicate that TNF-α is a major mediator of PVL-induced lung inflammation and that this effect is mediated via TNF-R1 expressed on both epithelial cells and macrophages.

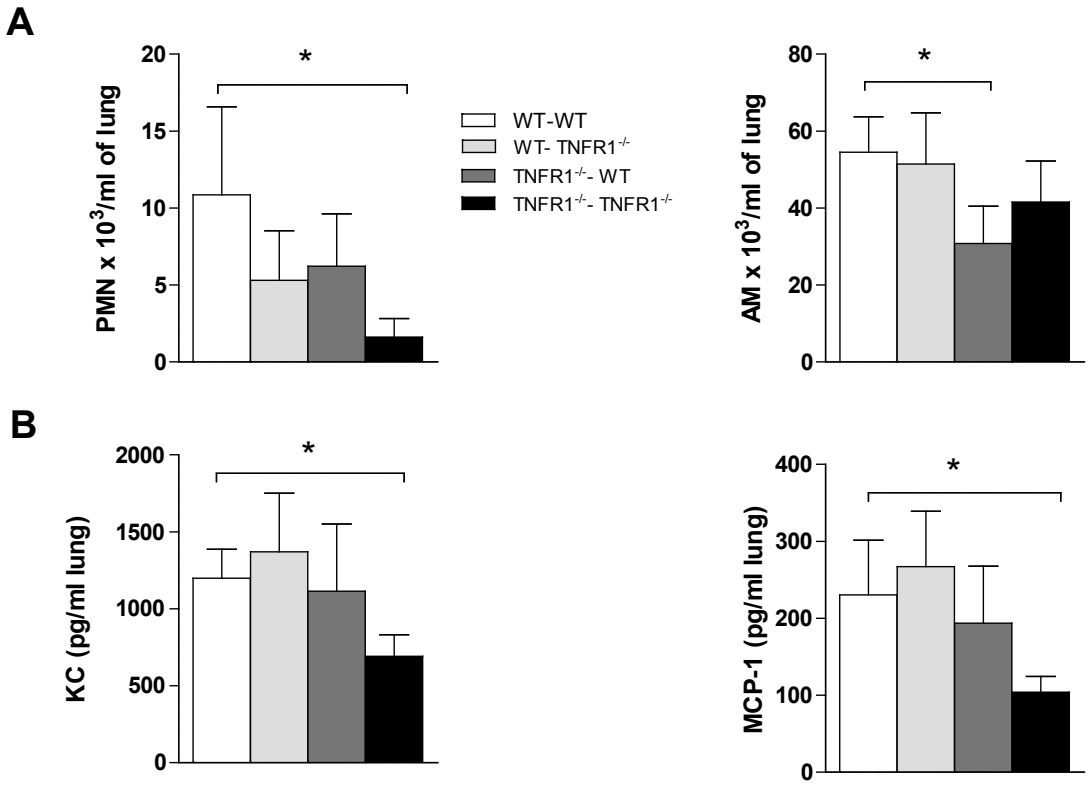


Figure 7. Role of TNF-R1 in PVL-mediated lung inflammation. Chimeric mice were treated with 1µg/g of PVL intranasally for 6h. Cell counts (A) and chemokines levels (B) in lungs were determined from BALF or lung homogenates. Data are presented as mean ± SD of n=9 replicates. * indicates $p<0.05$ versus WT-WT

Discussion

Bacterial infections cause a complex set of inflammatory responses that are often induced by an array of different bacterial molecules. It is part of the quorum sensing capacity of microbes to adjust to local environments and induce e.g. different virulence factors to improve their invasiveness and survival in a hostile environment. While the host's response is primarily targeting bacteria to eliminate them, the inflammatory response associated with this process unequivocally causes tissue damage that can eventually harm the host. We hereby investigated the precise role of lung epithelial cells in their capacity to sense the presence of PVL and to induce an inflammatory response. We show that epithelial cell-expressed TLR2 is required for these cells to induce inflammation, and that epithelial cells are insensitive to the pore-forming properties of PVL. Because we could not identify an overwhelming inflammatory response to PVL *in vivo*, we considered the possibility that PVL requires the presence of other staphylococcal proteins in causing this. Based on earlier reports it was evident, that PVL-carrying *S. aureus* strains show specifically enhanced expression of surface protein A, an important virulence factor of *S. aureus*. We here show that PVL and protein A synergistically enhance the inflammatory response by epithelial cells and during lung inflammation *in vivo*.

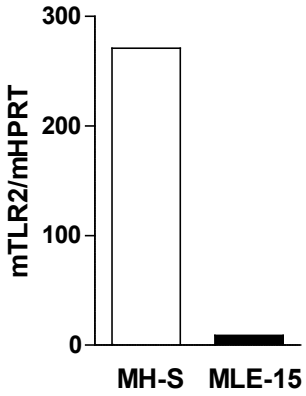
Synergistic effects of different bacterial molecules are a well-established phenomenon in infectious diseases. In fact, many staphylococcal virulence factors act synergistically, thus promoting survival of the pathogen [178]. As such, it was shown earlier that the *S. aureus* virulence factors lipoteichoic acid (LTA) and peptidoglycan (PGN) act synergistically through TLR2 in order to promote PMN influx in lungs of mice [179]. Additionally, α-toxin was shown to act synergistically with bacterial components from *S. aureus in vivo*, causing decreased phagocytosis by peritoneal macrophages and enhanced IL-6 releases [180, 181]. Our results add to these findings as we now show a synergistic effect of the staphylococcal virulence factors PVL and protein A, where both factors administered simultaneously to lung epithelial cells or directly into lungs of mice, caused significantly higher chemokines levels. When we investigated this synergism in mice deficient for TNF-R1, a known receptor for protein A [123], the additive effect of PVL and protein A was abolished.

Since PVL is a known TLR2 ligand [170], it seems interesting to note that a previous publication investigated the role of protein A in modulating the B-cell response in the presence

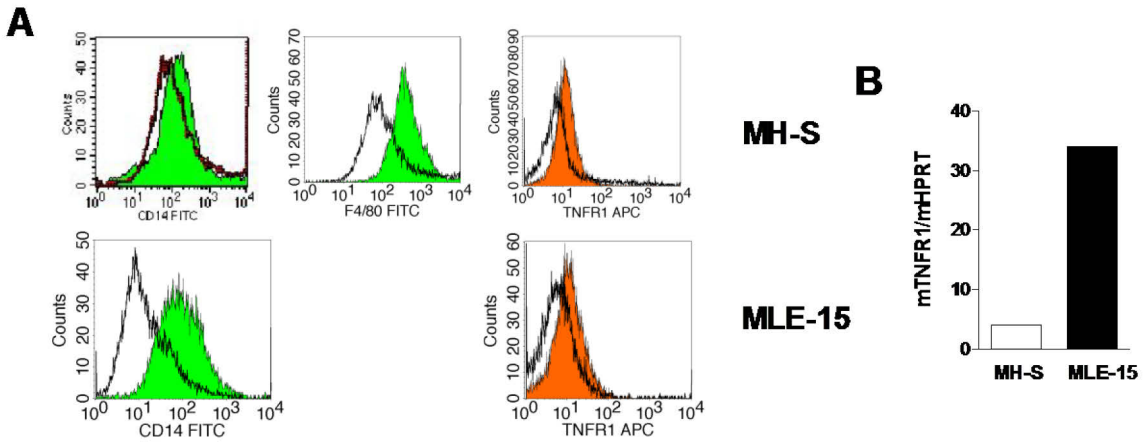
of TLR2 ligands. Bekeredjian-Ding et al. discovered that treatment of B cells with protein A sensitizes these cells for the recognition of lipopeptides recognized by TLR2, thus promoting B cell proliferation [182]. It would be interesting to know if protein A also impacts the B-cell response and possibly antibody induction to the TLR2 ligand PVL. The role of antibodies in PVL-associated diseases has been investigated recently. From data presented so far, it surprisingly seems that the presence of anti-PVL antibodies is not associated with protection but rather aggravation of disease [183-185].

The here described *in vivo* experiments with TNF-R1 knock-out animals revealed in addition that the absence of TNF-R1 diminished the influx of PMNs upon stimulation with PVL alone, indicating an important role for TNF-R1 in the propagation of PVL-mediated inflammation. Since the treatment of alveolar macrophages with PVL results in high levels of TNF-α, we postulate that secreted TNF-α might augment inflammation in an autocrine/paracrine fashion. TNF-α is bound by two receptors, TNF-R1 and TNF-R2, where the former is found to be prominently expressed on bronchial epithelial cells and primarily responsible for initiating inflammatory responses, whereas TNF-R2 serves rather as a molecular switch in the regulation of inflammatory or apoptotic pathways [186, 187]. As to our knowledge, the effects of PVL on epithelial cells until now were not investigated in detail and therefore our results presented here illustrate the path PVL might utilize in combination with other staphylococcal virulence factors and lead to the overwhelming inflammatory response in the lungs.

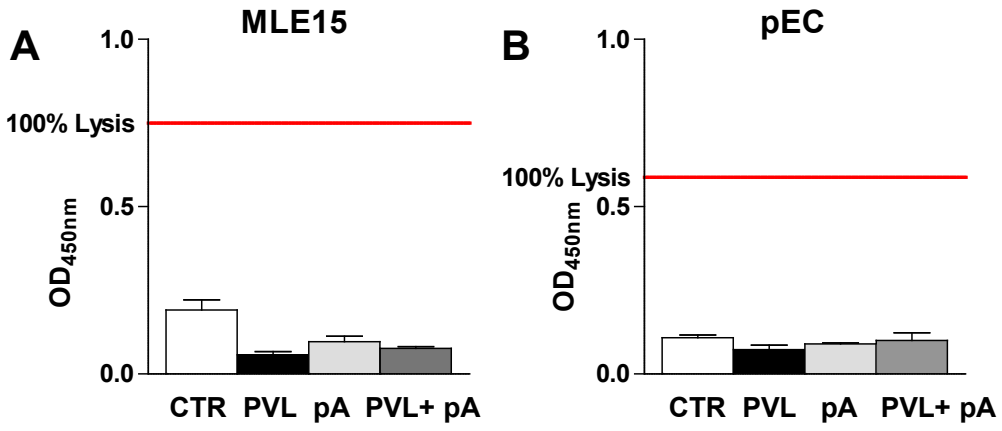
Supplementary figures



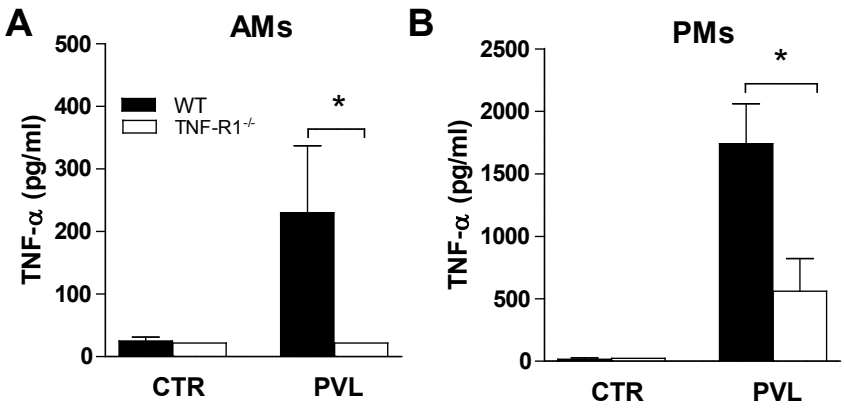
Supplementary Figure 1. Immortalized murine alveolar macrophages (MH-S) and epithelial cells (MLE-15) express TLR2. RT-PCT determined mRNA values of TLR2 in MHS and MLE15 cells.



Supplementary Figure 2. Immortalized murine alveolar macrophages and epithelial cells express TNF-R1. FACS analysis of MHS and MLE15 cells was performed using antibodies against CD14, F4/80 and TNF-R1.



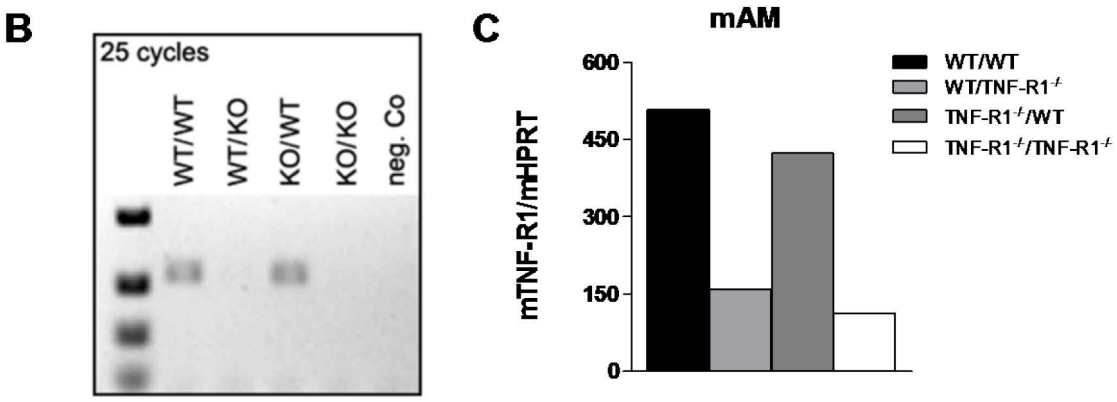
Supplementary Figure 3. Epithelial cells do not die upon treatment with PVL and/or protein A. MLE15 and primary epithelial cells were treated with PVL, protein A or PVL and protein A for 24h (A) and16h (B), respectively. Supernatants of treated cells were analyzed for LDH release. Data are presented as mean ± SD of n=4 replicates.



Supplementary Figure 4. Lack of TNF-R1 reduces TNF-release by macrophages. (A) Primary alveolar macrophages (AMs) and (B) peritoneal macrophages (PMs) from WT and TNF-R1$^{-/-}$ mice were treated with 0.1µg/µl PVL for 6h. TNF concentrations were measured in supernatants by ELISA. Data are presented as mean ± SD of n= 4 replicates. * indicates $p<0.05$ vs. WT.

A

	Group 1	Group 2	Group 3	Group 4
Recipient Genotype	WT	WT	TNF-R1$^{-/-}$	TNF-R1$^{-/-}$
Donor Genotype	WT	TNF-R1$^{-/-}$	WT	TNF-R1$^{-/-}$



Supplementary Figure 5. Verification of engraftment following bone marrow transplantation. (A) Overview of experimental groups. (B) mRNA levels of TNF-R1 by PCR and (C) RT-PCR in AM 6 weeks after bone marrow transplantation.

Materials and Methods

Bacterial Reagents

LukS and LukF were produced recombinantly in *E. coli* as described recently (10). Briefly, Luk-S and Luk-F constructs were transformed into competent *E. coli* DH5-α TM cells and sequence-verified clones were expressed in BL21 (DE3) cells exactly as described (10). Proteins were purified using Ni-NTA resin columns, LPS was removed by DetoxiGel columns (Thermo Scientific) until a final LPS concentration of <0.02 EU/ml was ensured (Charles River Analytics). All proteins were checked on silver stains before use. LukS and LukF were used at equimolar concentrations of 1µg/µl (280nM). *S. aureus* protein A was obtained from Sigma and used at a concentration of 200µg/ml.

Cells

MLE-15 cells, type II lung epithelial cell were kindly provided by Jeffrey A. Whitsett, University of Cincinnati, OH [188] and grown in RPMI 1640 (Gibco), supplemented with 2% FCS, 1% penicillin/streptomycin, 1% insulin-transferrin-sodium selenite, 5µg/ml transferrin, 10nM hydrocortisone, 10nM β-estradiol (all from Sigma). MH-S cells (ATCC) were grown in RPMI 1640, supplemented with 10% FCS, 1% penicillin/streptomycin and 0.01 % β-mercaptoethanol. Bone marrow derived macrophages (BMDMs) were isolated and differentiated in L929 conditioned medium for seven days as previously described [170]. Primary epithelial cells were isolated from blood-free lungs by intratracheal instillation of dispase and low-melting agarose (1% w/v) (Sigma). Lungs were subsequently removed and placed in dispase for 45 min, after which lungs were dissected, using forceps in DMEM containing 0.01% DNase I, filtered and resuspended in DMEM containing 10% FCS. Isolated cells were then plated in petri-dishes coated with antibodies against CD45/CD16/CD32 (eBioscience) for 2h at 37°C. Non-adherent cells were collected and enumerated using a hemocytometer. After this cells 5x105/well were plated on collagen-coated 96-well plates in HITES medium over night at 37°C.

Cell death assays

LDH release in supernatants was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instruction. To analyze the proportion of apoptotic and dead cells, $5x10^5$ cells were treated with 280nM PVL or 10µM staurosporine and

stained with AnnexinV and Propidium-iodide (BD Biosciences). Cells were subsequently anylzed on a FACScalibur (BD Biosciences).

FACS analysis

For FACS analysis $5x10^5$ MH-S and MLE-15 cells were stained with rat-anti-mouse CD14 mAb (1:50 and 1:10; BD Biosciences), rat-anti-mouse F4/80 mAb (1:50; Serotec) and goat-anti-mouse TNF-R1 mAb (1:50; RnD Systems), followed by incubations with respective secondary anti-rat Fab FITC (1:50; Jackson) and anti-goat Ig APC (1:50; RnD Systems). Samples were analyzed on a FACSCalibur using CellQuest software.

RT-PCR

mRNA levels were quantified by real time (LightCycler, Roche Diagnostics) exactly as described recently [170]. Data were normalized to HPRT gene expression levels. Mouse gene specific primer sequences used were: HPRT_FW (5´-GTT AAG CAG TAC AGC CCC AAA ATG -3`) and HPRT_RV (5`-AAA TCC AAC AAA GTC TGG CCT GTA-3`); TNF-α_FW (5`-GAA CTG GCA GAA GAG GCA CT-3`) and TNF-α_RV (5`-GGT CTG GGC CAT AGA ACT GA-3`); TNFRSF1a_FW (5`-TGT CAA TTG CTG CCC TGT CC-3`) and TNFRSF1a_RV (5`-CCC ATC AGCAGA GCC AGG AG-3`); TLR2_FW (5`-CGA AAC CTC AGA CAA AGC GTC AAC ACG CTC GGA GGT CAC CAT-3`); with annealing temperatures of 72°C for HPRT, 68°C for TNF- α, 70°C for TNFRSF1a and 70°C for TLR2.

Cytokine and chemokine measurement

Cytokines and chemokines (TNF-α, KC and MCP-1) in cell supernatants and mouse tissues were quantified using commercially available ELISAs (RnD Systems) according to the manufacturer's instructions.

Animal experiments

All animal experiments were approved by the Animal Review committee of the Medical University Vienna and the Austrian Ministry of Sciences. For *in vivo* experiments 9 week old female C57BL/6 and TNF-R1$^{-/-}$ mice were treated with LukF, LukS or PVL (all 1µg/g), *S. aureus* protein A (2.5µg/g) or saline intranasally. For bone marrow transplant experiments 9-week old female C57BL/6 and TNF-R1$^{-/-}$ mice were irradiated with a dose of 9Gray, after which bone marrow cell suspensions ($2x10^6$cells/200µl) were injected via the retro-orbital route. Mice

were left for 6 weeks to let cells repopulate, after which alveolar macrophages were tested for donor DNA. Following verification of repopulation, mice were intranasally challenged with 1µg/g PVL. The inflammatory response was evaluated after 6h by quantifying cell influx, cytokine and chemokine release as described earlier [154, 170, 189].

Statistics

All data are expressed as mean ± SD. Differences between groups were analyzed using unpaired t test or one-way ANOVA followed by Bonferroni posthoc analyses, where appropriate, using GraphPad Prism software. A p-value <0.05 was considered statistically significant.

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7. CONCLUDING REMARKS

During my doctoral thesis I investigated the role of *staphylococcal* PVL, which is considered a virulence factor in *S. aureus* induced pneumonia and believed to contribute to the development of life threatening necrotizing pneumonia. Although the role of TLR2 in *S. aureus* infections is well established as TLR2 is indispensible in the recognition of bacterial LTA [111, 154, 190], our discovery of PVL being a ligand for TLR2 is novel and opens a new perspective on the properties of pore forming toxins and host response. Together with this, lung epithelial cells respond to PVL treatment with the production of chemokines, resulting in the recruitment of PMNs to the site of inflammation. In our experiments we could not confirm that PVL alone can induce extensive lung inflammation. Therefore, we hypothesized that staphylococcal protein A might have a role in PVL mediated inflammation, since PVL regulates its expression. Interestingly, we discovered that these two well known virulence factors act cooperatively and that TNF-R1 has an important role in the propagation of PVL inflammatory properties. Interestingly, we could conclude that different lung cells, epithelial, macrophages and neutrophils recognize PVL and act in a cell-specific manner.

In light of these discoveries there are several points which need to be discussed to fully understand our results in the context of previous studies performed to comprehend PVL's role in the inflammatory response during pneumonia. First of all, there is a big controversy on studying the virulence of PVL positive *S. aureus* in different animal models since many investigators do not agree on the impact of PVL on the survival of infected animals [191, 192]. The main reason for this lies in the fact that PVL's toxic properties were mainly based on measurements of neutrophil viability [147, 165, 193]. The current conclusion is that different species have different susceptibility to PVL's pore forming properties [165], where the most sensitive cells to PVL are human neutrophils [165, 166]. Other mammalian cells, like rabbit cells also show high sensitivity in terms of cell death, while mouse PMNs do not seem to be sensitive [165, 194]. This difference in PMN susceptibility to PVL's pore forming properties is interesting and may origin from special lipid compositions of the cellular membrane in different species, as has been shown for γ-hemolysin dependent effects earlier [134]. The most interesting studies are the ones investigating PVL's impact on survival in models mimicking human disease. Although PVL is the only virulence factor from *S. aureus* that has been epidemiologically associated with the development of necrotizing pneumonia [162], very few papers managed to show that PVL

actually contributes to the severity of the disease in animals, leaving the role of PVL inconclusive. The majority of these studies were performed with CA-MRSA strains and showed that the presence or absence of PVL did not alter survival. For instance, Bubeck-Wardenburg et al. stated that during USA300 / USA400 lung infections (C57Bl/6J mice), PVL was not a virulence factor, since the presence of PVL did not impact bacterial burden [195]. Balb/c mice seem to be more sensitive to PVL's toxic effects [167]. Other papers, report skin and lung infection model, in which PVL did not display any major virulence [196, 197]. However, at the same time there are several reports clearly demonstrating that PVL is an important virulence factor. As such PVL contributes to the development of inflammatory lesions in skin infection models [198, 199], PVL's presence in *S. aureus* enhances the severity of disease during osteomyelitis [200] and in a muscle injury model, where PVL indirectly contributes to the outcome by destroying PMNs [201]. Moreover, Labandeira-Ray et al., demonstrate that LukS promotes staphylococcal lung infection, and that PVL dose-dependently triggers lung necrosis [167].

Of importance, immunization against PVL was shown earlier to have fairly protective role in the mouse model of lung necrotizing pneumonia and skin infections, where the animals were challenged with a rather high inoculum of bacteria ($\sim 10^7$-10^8) [198, 202]. The animals subcutaneously injected with LukF and LukS show protection against dermal infections, while intranasal administration of LukS behaves defensive in the model of pneumonia, suggesting a host immune response to LukS [198]. On the contrary, recent investigations from surprisingly show that anti-PVL antibodies, in the presence of low bacterial inoculum ($\sim 10^4$) are not associated with protection but rather aggravation of disease [183]. Yoong group found that the antibody to PVL in the model of skin abscess decreases the ability of PMNs to control the replication of *S. aureus*, since bacterial proliferation and survival in the presence of anti-PVL antibody was augmented [184]. Additionally, PVL seems to trigger a systemic activation of the host immune response leading to the production of the anti-PVL antibodies, abating the Ca^{2+} channel activation of PMNs and secretion of pro-inflammatory mediators, which we also observed earlier (data not shown), implying that the antibodies to PVL could interfere with the triggering of the appropriate host innate immune response. These findings together correlate with the earlier observed recurrence of MRSA infections in humans, which most likely have high

levels of anti-PVL antibody, suggesting the ineffectiveness of the vaccines composed of PVL [185].

Together, despite of some controversy as to how much PVL is present during *S. aureus* infection there are indications that PVL itself exerts some effects in various inflammatory models. The precise molecular mechanisms underlying these effects are less well understood. The findings presented in my thesis clearly indicate that mice directly recognize PVL via TLR2. This recognition results in the classical TLR2 mediated NF-κB activation and induction of inflammatory mediators secreted by AMs. We furthermore studied the sensitivity of murine PMNs to PVL and found that these cells rapidly succumb due to pore-formation. Having in mind that the creation of a membrane-pore is a dynamic process, with continuous repair attempts counteracting the pore [203] as well as the assembly of larger clusters of pores that eventually lead to cell death [135, 144] the individual susceptibility to toxins certainly depends on the cell subset and its potential for repair and recovery.

In parallel to the investigations above, Montgomery et al. analyzed the gene expression pattern of USA300 and he discovered that PVL is not expressed in the exponential growth phase of bacteria under laboratory conditions [140]. This brought up some important questions: How and when is PVL expressed and could the lack of PVL's expression be the reason for the inability to prove a role for PVL in several infection models? Expression of virulence factors is tightly regulated and fine changes in quorum sensing or the host-interaction could alter expression levels of several bacterial factors [204, 205]. This fine tuning allows bacteria to survive in aggressive environments while at the same time gaining access to tissues with nutrients. A recent publication from Wirtz et al. showed that the expression of virulence factors, such as PVL, can be induced upon stress or usage of antibiotics, resulting in higher amounts of PVL [206]. Furthermore, the presence of other bacteria and their internal communication, known as quorum sensing, significantly contributes to altered gene expression [204]. Many genes encoding virulence factors are regulated by operons, which are controlled by quorum sensing mechanisms. These mechanisms allow bacteria to choose the ideal moment to secrete virulence factors. Therefore, growing bacteria in inappropriate medium or inappropriate handling of bacteria will probably result in data that give an incomplete picture.

Another interesting connection was made between patients who had influenza and subsequently developed staphylococcal necrotizing pneumonia [162, 207]. DeBentzmann et al. showed that PVL-positive *S. aureus* strains have an increased capacity to attach to collagen and laminin [172], which is more likely to occur upon viral infection and resulting desquamation of airway epithelium [208]. Likewise, the signaling peptide of LukS was shown to be recognized by heparan sulfate, a highly abundant protein in the respiratory epithelium [171]. How LukS binding to the lung epithelium would contribute to the increased susceptibility to *S. aureus* lung infection is still not well investigated.

Moreover, studies on MRSA and MSSA strains showed that different *S. aureus* strains produce variable amounts of PVL [209, 210], which might affect the severity of disease. Additionally, analysis of *S. aureus* isolates disclosed 12 polymorphysisms in the PVL gene, of which 10 are synonymous and 2 nonsynonymous mutations [209, 211]. One of the two nonsynonym mutations is called H177R, where the R isoform, carried by USA300, is frequently found in U.S. strains. [212]. Molecular modeling revealed that the H177R mutation is localized on LukS, close to the place of interaction with F, suggesting that this mutation might affect the pore formation of PVL [209].

Experiments performed during my PhD studies importantly contribute to the understanding of PVL's binding and signaling, because we identified the cellular receptors signaling the pro-inflammatory response to PVL. These inflammatory effects were independent of PVL's pore-forming properties and preceded any toxic effects of PVL on macrophages. By studying epithelial cells we found that PVL is not able to induce cell death in this cell type, which is in line with earlier reports showing that PVL is a so-called leukocidin, i.e. a toxin that kills primarily neutrophils. Furthermore, our data illustrate the importance of TNF-signaling during lung inflammation induced by PVL, and we propose that TNF is the key 'virulence' factor that is strongly induced by PVL and in turn mediates the inflammatory reaction within the lungs.

To sum up our findings, we created a scheme which describes the effects of PVL on different cell types within the lungs (**Figure 8**). The pulmonary presence of PVL activates resident alveolar macrophages and epithelial cells to secrete pro-inflammatory mediators in a TLR2/CD14-dependent manner. High levels of cytokines and chemokines induce the influx of

PMNs, which - due to their high sensitivity to PVL's pore forming properties - undergo cell death. At the same time high levels of proinflammatory cytokines could play an important role in activating *S. aureus* to release the cell wall-anchored protein A [213]. Both virulence factors together then act synergistically to amplify the inflammatory response, where high levels of pro-inflammatory cytokines such as TNF- α could further enhance inflammation in an autocrine/paracrine fashion. The resulting inflammation might contribute to tissue damage, which has been described in patients as necrotizing pneumonia. Further investigations on the precise mechanism of PVL induced inflammation and the patient-specific responses (i.e. TLR2, TNF-R1 polymorphisms, level of anti-PVL antibodies), could help us understand PVL associated diseases and possibly novel therapeutic interventions.

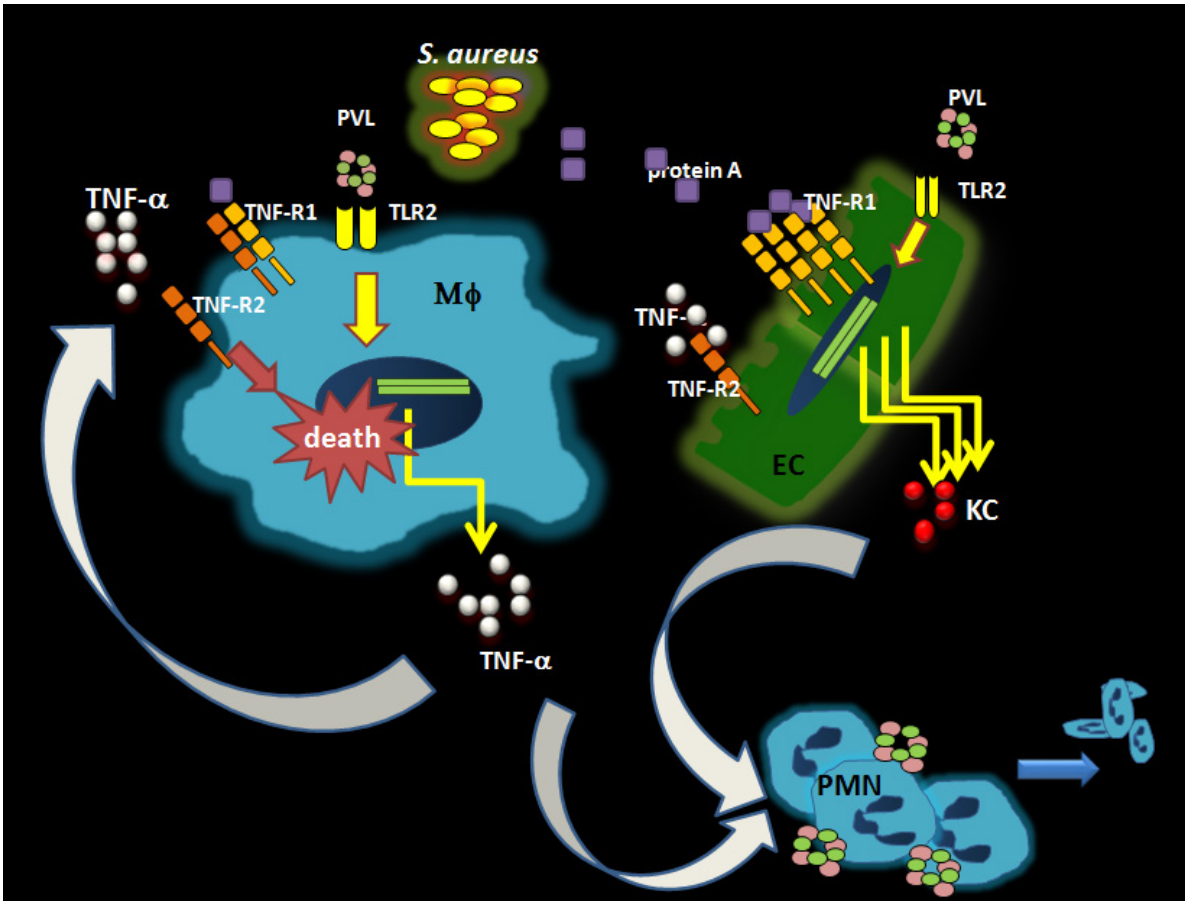

Figure 8. PVL effects on different cell types within the lung.

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10. CURRICULUM VITAE

Zivkovic Ana, Magistra rerum naturalium

Born on 06. November, 1980; married (Maiden name: Stevanovic); Serbian Citizenship

Career and Education

2010- currently	Clinical Operations, **Quintiles Eastern Europe and Middle East Holdings GmbH**, Vienna, Austria
2006-currently	PhD Studies, Medical University Vienna, Austria; **Center for Molecular Medicine (Ce-M-M) of the Austrian Academy of Sciences**, Laboratory for Innate Immunity and Infection; Thesis: *Molecular Mechanisms of Panton-Valentine Leukocidin in the development of necrotizing pneumonia*
2005-2006	**Institute for Molecular Genetics und Genetical Engineering (IMGGE)**, Laboratory for Molecular Hematology, Belgrade, Serbia; Project: P*olymorphisms in Chron's disease patients*
1999-2005	**Molecular Biology and Physiology Studies**, Major in Genetical Engineering and Biotechnology, Faculty for Biology, University of Belgrade, Serbia, Diploma thesis: *Optimization of DGGE-Method for genetic analysis of phenylketonuria patients*
2004-2005	**Institute for Nuclear Sciences Vinca**, Laboratory for Radiology and Molecular Genetics, Belgrade, Serbia; Project: *Optimization of Molecular Genetics Methods*
2003-2004	**Institute for physiology und biochemistry**, Faculty for Biology, Belgrade, Serbia; Project: *The effects of adrenalectomy, adrenocorticotropic hormone treatment and dexamethasone treatment on the activity of monoamine oxidase in the rat hypothalamus*
1995-1999	**St. Sava (VIII) Gymnasium**, Belgrade, Serbia; Matura exam: *Disturbances of purine bases metabolism*

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- **Zivkovic A**, Biaggio M, Sharif O, Stich K, Doninger B, Knapp S. *Staphylococcal Panton-Valentine Leukocidin and protein A synergistically inflame lungs via involvement of TNF-R1.* (in preparation)

- **Zivkovic A**, Sharif O, Stich K, Doninger B, Biaggio M, Colinge J, Bilban M, Mesteri I, Hazemi P, Lemmens-Gruber R, Knapp S. *TLR 2 and CD14 Mediate Innate Immunity and Lung Inflammation to Staphylococcal Panton-Valentine Leukocidin In Vivo* J. Immunol. 2011 Feb 1; 186[214]:1608-17.

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Other

Co-Supervision: Diploma of Mag. rer. nat Mario Biaggio

Languages: **Serbian** (native); **Croatian** (fluent), **Bosnian** (fluent)
English (fluent); **German** (advanced); **Russian** (good);

Conferences and Awards

2010 Contributions from scientific research to the risk assessment of GMO; Brussels, Belgium
2010 Women in Research and Technology (FEMtech); Vienna, Austria
2010 Forum of the Austrian Research Promotion Agency; Vienna, Austria
2010 Supervision in Doctoral Education, Center for Doctoral Studies; University of Vienna, Vienna, Austria
2010 Austrian Society for Infection Diseases; Saalfelden, Austria (Oral Presentation)
2010 European Congress of Clinical Microbiology and Infectious Diseases; Vienna, Austria (Oral Presentation)
2009 Complement, perforins and cholesterol dependent lysins; Prato, Italy (Oral Presentation)
2008 Best Abstract Award, Young Scientist Association Conference, Medical University of Vienna; Vienna, Austria
2008 Toll 2008 Meeting; Lisbon, Portugal (Poster Presentation)
2008 Joint Annual Meeting of Immunology of Austrian and German societies; Vienna, Austria (Poster Presentation)
2007 Austrian Proteomics Association, Technical University; Vienna, Austria
2007 Federation of the Societies of Biochemistry and Molecular Biology (FEBS) Meeting; Vienna, Austria
2006 Top 100 Students in Serbia with Foreign Doctoral studies, Ministry for Sciences, Serbia
2006 Best Diploma Thesis Award in the field of Molecular Biology, 'Dr. Goran Ljubljankic' Foundation; Belgrade, Serbia
1995 Diploma for academic Excellence ´Vuk Stefanovic Karadzic´; Belgrade, Serbia