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The role of lung epithelial cells and TNF-α in Panton-Valentine Leukocidin induced lung inflammation

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Mario Biaggio

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Betreuer:	Univ. Prof. Dr. Sylvia Knapp, PhD
	Mag.[a] Ana Zivkovic
	Univ. Prof. Dr. Pavel Kovarik

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Abstract

Panton Valentine Leukocidin (PVL) is a pore-forming toxin secreted by *Staphylococcus aureus*. PVL has gained enormous attention over the last few years, because clinical data revealed that the presence of this toxin was found associated with life-threatening cases of necrotizing pneumonia in young adults. Moreover, methicillin resistant *S. aureus* (MRSA) strains emerged in the community over the last years, most of which carrying the gene for PVL. Because these new bacterial strains have been shown to be far more virulent than hospital associated MRSA, we hypothesized that PVL might be an important virulence factor.

The lab of Sylvia Knapp studied the role of PVL in lung inflammation and recently identified that CD14 and TLR2 are required for PVL to induce an inflammatory response both *in vitro* and *in vivo*. This inflammatory response was shown to be independent of PVL's pore-forming properties and was induced by one subunit of PVL (called LukS-PV).

Because PVL alone was not capable of inducing lung necrosis, we hypothesized that PVL might enhance the activity of other bacterial virulence factors, and that only the combined effect can trigger pulmonary necrosis. The topic of my diploma thesis was to investigate the potentially synergistic effects of PVL and other known staphylococcal virulence factors during lung inflammation. As such I studied the potential synergism between PVL and protein A, a well-known virulence factor of *S. aureus*. Protein A has been earlier shown to importantly contribute to *S. aureus* pneumonia as it is capable of inflaming the lung via involvement of TNF-R1.

Based on experiments I performed we observed that PVL and protein A indeed synergize in causing lung inflammation both *in vitro* and *in vivo*. This synergistic effect depended on the presence of TLR2 (PVL receptor) and TNF-R1 (protein A receptor) *in vitro*. When studying this synergism during lung inflammation in mice, we unexpectedly discovered that PVL itself was almost incapable of inducing lung inflammation in the absence of TNF-R1 *in vivo*. Absence of TNF-R1 on macrophages did not prevent PVL from inducing an inflammatory response, hence ruling out the possibility that PVL triggers inflammation via TNF-R1 but rather suggesting that the *in vivo* phenotype was based on indirect effects of soluble TNF-α. In accordance,

microarray and PCR results confirmed that TNF-α is one of the strongest induced cytokines in response to PVL stimulation of macrophages. Because both macrophages and respiratory epithelial cells express TNF-R1 *in vivo*, we performed bone marrow transplant experiments to identify the respective contribution to PVL induced lung inflammation. While PVL-induced lung inflammation was present in WT/WT mice and abolished in KO/KO animals, both chimeric WT/KO and KO/WT groups showed an intermediate phenotype. These data suggest that TNF importantly contributes to PVL-induced lung inflammation and that TNF-R1 on macrophages and epithelial cells is required for this response.

Abstract auf Deutsch

Panton Valentine Leukocidin (PVL) ist ein Poren-bildendes Toxin, das von *Staphylococcus aureus* produziert wird. PVL ist ein seit langem bekanntes Toxin, mit Großteils unbekanntem Wirkmechanismus und hat in den letzten Jahren besondere Aufmerksamkeit erregt. Klinische Studien belegen, dass dieses Toxin mit lebensbedrohlichen Fällen von nekrotisierender Pneumonie in jungen Erwachsenen assoziiert ist. Des Weiteren ist eine auffällige Assoziation mit Methicillin resistenten *S. aureus* Stämmen (MRSA) gegeben, die auch außerhalb von Krankenhäusern verbreitet werden. Da diese neuen Varianten von *S. aureus* eine wesentlich höhere Virulenz besitzen als nosokomiale Stämme, vermuten wir, dass PVL hierbei eine tragende Rolle spielt.

Das Labor von Sylvia Knapp erforscht die Rolle von PVL in Entzündungsvorgängen der Lunge und entdeckte kürzlich, dass die Rezeptoren CD14 und TLR2 notwendig sind in der Initiation der Entzündungsreaktion *in vitro* als auch *in vivo*. Die Auslösung der Entzündung ist unabhängig der Fähigkeit von PVL, Poren in Zellmembranen zu bilden und wird von einer PVL Untereinheit ausgelöst, die lukS-PV genannt wird.

Da PVL alleinig nicht in der Lage ist eine Nekrose der Lunge hervor zu rufen, vermuteten wir, dass PVL die Aktivität anderer bakterieller Virulenzfaktoren verstärken könnte, und diese zusammengefasst in der Lage sind eine Nekrose hervorzurufen. Das Thema meiner Diplomarbeit beschäftigte sich mit den möglichen synergistischen Effekten von PVL und anderen, bereits bekannten Virulenzfaktoren von *S. aureus* bei Entzündungen der Lunge. Dies umfasste auch Protein A, einen weiteren Virulenzfaktor von *S. aureus*, bzw. die potentielle Verstärkung der Entzündung durch diesen und PVL. Es ist bekannt, dass Protein A erheblich zur Entstehung von *S. aureus* vermittelter Lungenentzündung beiträgt, und alleine in der Lage ist eine Entzündungsreaktion in der Lunge durch Interaktion mit TNF-R1 auszulösen.

Basierend auf *in vitro* und *in vivo* Experimenten, die ich durchführte, konnten wir tatsächlich einen synergistischem Effekt von Protein A und PVL bei der Auslösung einer Entzündungsreaktion in der Lunge beobachten. Dieser Effekt war in einem *in*

vitro Experiment abhängig von der Präsenz von TLR2 (PVL Rezeptor) und TNF-R1 (Protein A Rezeptor). Während der Untersuchung des synergistischen Effektes von PVL und Protein A entdeckten wir in einem *in vivo* Experiment überraschenderweise, dass die Entzündungsauslösung durch PVL bei Abwesenheit von TNF-R1 fast vollständig inhibiert wurde. Das Fehlen von TNF-R1 auf alveolären Makrophagen verhinderte nicht die PVL vermittelte Entzündungsreaktion was bedeutet, dass PVL keine Entzündungsreaktion direkt über TNF-R1 vermitteln kann. Wir vermuteten in unserem *in vivo* Modell allerdings einen wesentlich komplizierteren Mechanismus durch frei lösliches TNF-α, dessen Ausschüttung möglicherweise eine Folge der Präsenz von PVL sein könnte. In der Tat bestätigten weitere PCR und Microarray Experimente, dass TNF-α eines der durch PVL am stärksten induzierten Zytokine in alveolären Makrophagen ist. Da sowohl alveoläre Makrophagen, als auch Lungenepithelzellen *in vivo* TNF-R1 exprimieren, war es notwendig eine Knochenmarkstransplantation durch zu führen um die jeweilige Bedeutung in PVL induzierter Entzündungsreaktionen der Lunge aufzuklären. Während in WT/WT Mäusen PVL in der Lage war eine Entzündungsreaktion aus zu lösen war dies in KO/KO Mäusen nicht möglich. Beide chimären Genotypen (WT/KO & KO/WT) zeigten einen intermediären Phänotyp. Diese Daten deuten darauf hin, dass TNF-α wesentlich zur PVL-vermittelten Entzündungsreaktion beiträgt, und dass TNF-R1 auf alveolären Makrophagen als auch auf Epithelzellen für diesen Effekt notwendig ist.

Introduction

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium and is part of the normal flora of the skin and the upper airway tract [1]. It has a circular chromosome of approx. 2800bp and can harbor prophages, plasmids and transposons, which facilitate genetic exchange with other Gram-positive bacteria. The cell wall of *S. aureus* consists of peptidoglycan (up to 50%), lipoproteins and teichoic acids, which are all recognized by different receptors of the innate immune system. Based on the microcapsule present in most staphylococci, 11 serotypes have been described, of which serotype 5 and 8 count responsible for most infections in humans [2]. The microcapsule can be considered a virulence factor as it has the ability to inhibit phagocytosis. *S. aureus* also developed other strategies to prevent innate immune responses. As such, staphylococcal surface proteins like protein A prevent phagocytosis. Protein A binds to the Fc part of human IgG, consecutively preventing opsonization and phagocytosis [3].

S. aureus is responsible for a wide range of clinically important diseases, ranging from minor skin infections such as furunculosis, to life threatening diseases such as osteomyelitis, pneumonia, endocarditis, toxic shock syndrome and septicemia [4-9]. While osteomyelitis and endocarditis can occur in otherwise healthy people, pneumonia is mostly restricted to elderly patients with underlying disorders like cardiovascular and malignant diseases, chronic pulmonary diseases or diabetes mellitus [10]. All together, *S. aureus* is the single most important pathogen in causing infectious diseases in humans [5].

S. aureus harbors various toxins that contribute to its virulence and enhance progression as well as severity of diseases. The toxins of *S. aureus* can be categorized into three groups, namely 1) pore forming toxins, 2) exfoliative toxins, and 3) entero- & toxic shock syndrome toxins (TSST). Pore-forming toxins consist of hemolysins, the latter being further divided in α, β and γ hemolysins with different cell type specifity for their lysis capability [11]. Panton Valentine leukocidin (PVL) is part of the β hemolysin group, because of its beta- barrel- pore forming property and will be explained in more detail below [12]. Exfoliative toxins contain a protease activity

and can therefore cause blistering of the skin via cleavage of cadherins [13]. About 20 subtypes of *S. aureus* enterotoxins have been discovered over the last years and some of them cause severe gastrointestinal afflictions, while all of them (including TSST) show superantigen properties, i.e. activation of T- cell proliferation by binding to MHC class II leading to massive cytokine release [14].

Emerengence of MRSA & PVL positive strains in the community

Methicillin resistant *S. aureus* (MRSA) is a consequence of antibiotic usage. MRSA were first described in the 1960ies, but continuous usage of antibiotics has led to constantly rising incidences of these strains over the last decades [15, 16]. Most MRSA isolates are confined to hospitals, and these strains are usually of low virulence and associated with nosocomial infections. Only recently, a number of new MRSA strains in the community have been discovered, and were named community-associated (CA)-MRSA. Quite alarmingly, these CA-MRSA clones rapidly spread around the globe and epidemiological data indicate that CA-MRSA strains are far more virulent than their hospital-confined relatives [17, 18].

Resistance to Methicillin is mediated by the acquisition of penicillin-binding protein (PBP2a) encoded by the *mecA* gene and is characterized by low affinity to β-lactam based antibiotics. While the four native peptidoglycan synthethases (penicillin-binding proteins 1 to 4) are inactivated by their high affinity to β-lactam antibiotics, the PBP2a is still able to perform cell wall synthesis in the presence of β-lactams [19]. Nosocomial MRSA isolates usually carry large staphylococcal chromosomal cassette mec (SCCmec) of I, II or III of sizes ranging from 34 to 67kb [20]. In contrast to nosocomial MRSA, CA-MRSA strains carry the smaller (21 to 36kb) SCCmec IV, V or VII [21]. It has been speculated that the smaller sizes of SCCmec types found in CA-MRSA facilitate the acquisition by Methicillin sensitive *S. aureus* (MSSA) [22].

Adding to the danger of CA-MRSA, many of these strains carry genes for the pore-forming toxin PVL [15]. Recent studies have shown that PVL positive *S. aureus* strains were related to hemorrhagic, necrotizing pneumonia in otherwise immunocompetent healthy children and young adults [23]. Of great clinical importance, high mortality rates of up to 75% were described in these patients [24]. Although PVL has been shown to be associated with these serious lung infections,

and also skin infections, the causal link has never been clearly demonstrated. A series of recent investigations provided both prove and doubt in the role of PVL as a critical factor for CA-MRSA-associated pneumonia [12, 25-32].

PVL is a beta barrel octamer pore-forming toxin comprised of two subunits (LukF-PV and LukS-PV) that was recently found to be associated with necrotizing pneumonia and skin infections in humans (Fig.1&2) [33, 34].

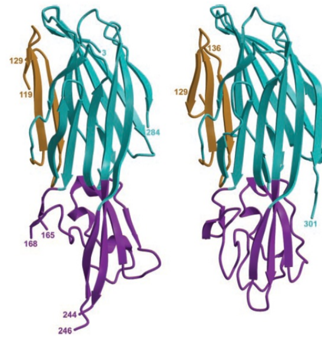

Figure 1: Three dimensional structure of LukS-PV (left) and lukF-PV (right) [33].

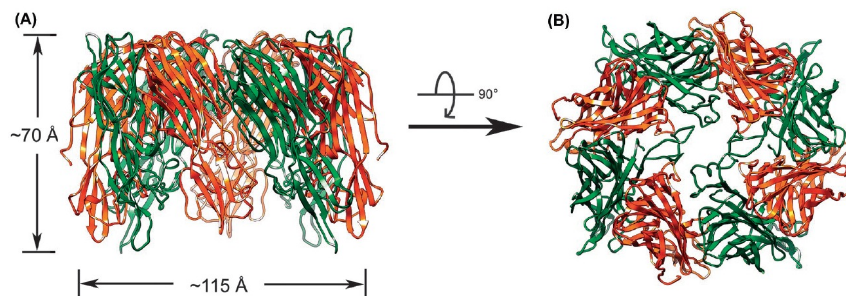

Figure 2: (A) Side view and (B) top view of fully assembled PVL octamer. The octamer is comprised of lukS-PV and lukF-PV in a 1:1 stoichiometry [34].

The molecular mechanism as to how PVL is able to cause necrosis of cells and tissues is poorly understood and still contradictory [31]. Previously it was shown that high doses of PVL have the ability to kill neutrophils and low doses to mediate apoptosis of neutrophils [12, 35].

PVL is integrated into the chromosome by a lysogenic bacteriophage (phiSLT) in a region distinct from SCCmec (Fig.3). The relatively small SCCmec IV & V cassettes seem to have no negative effect on the genetic fitness of *S. aureus*. The bigger SCCmec types I, II and III, which are carrying resistance genes for a wide range of antibiotics, seem to be not fit enough to survive within the community and are

therefore only distributed in nosocomial *S. aureus* strains [15]. The increased presence of PVL has been attributed by some authors to the fact that PVL-positive bacteria are frequently found in necrotic skin lesions, which allows for easy spread via skin contact [36, 37].

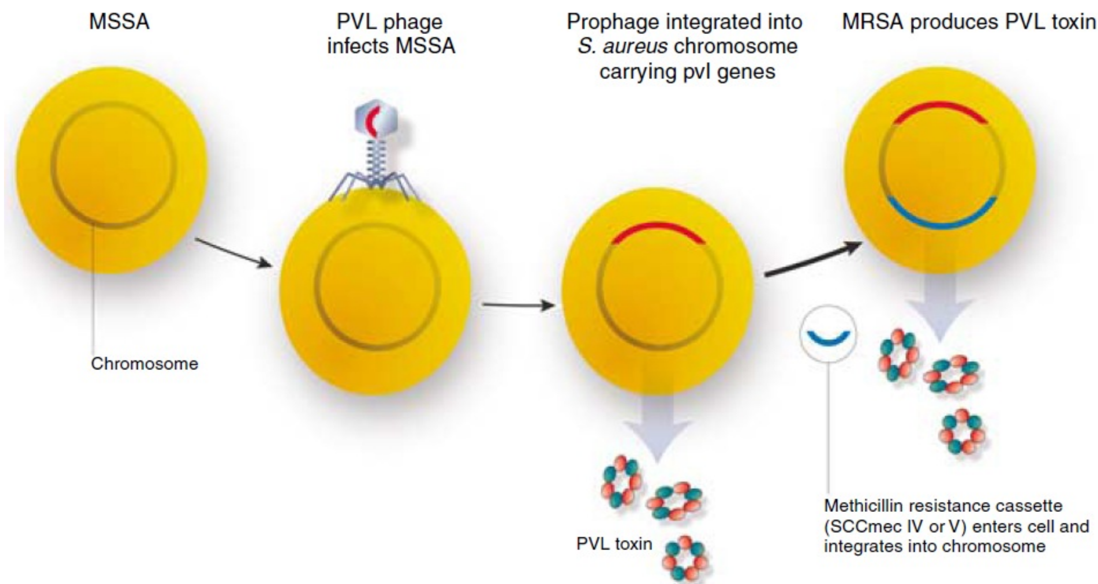

Figure 3: Suggested model for the emergence of PVL producing CA-MRSA; A methicillin susceptible strain (MSSA) is infected and lysogenized by a phage (phiSLT) carrying lukS-PV and lukF-PV. Independently a SCCmec cassette of type IV or V is subsequently integrated into the chrosmosome by horizontal gene transfer [15].

Respiratory tract infections and pathogen encountering lung cells

The lungs are exposed to a large array of air borne microbes and particles and hence are exceptionally sensitive to potential bacterial infections. While the upper respiratory tract is colonized by a large number of microorganisms that usually don't cause any strong inflammatory response, the lower respiratory tract remains relatively 'sterile'. Even though, it was recently shown that also the lower respiratory tract can host microorganisms [38]. With an incidence of more than 429 million cases of lower respiratory tract infections in 2004, respiratory tract infections are the 3rd leading cause of death worldwide [39]. The innate immune system provides the first line of defense against inhaled pathogens and particles and consists of cellular and

soluble factors. Soluble factors also include plasma components, which are translocated to the site of infection by pericellular and transcellular transport mechanisms via endothelial and epithelial cells and include natural antibodies, complement proteins, C-reactive protein and pentraxin 3. All of these factors support host defense via opsonization or their bacteriostatic and microbicidial properties [40-42]. Alveolar macrophages and lung-epithelial cells, which are located at the air tissue interphase, are the first cells to encounter pathogens. Both above mentioned cells types express an array of pattern recognition receptors (PRR). Among those PRRs are Toll-like receptors (TLRs), scavenger receptors as well as cytosolic receptors like NOD like receptors (NLR's), RIG-I like receptors (RLR's) and recently found intracellular DNA and RNA sensing receptors [43, 44]. To attract other effector cells, macrophages and epithelial cells secrete an array of inflammatory mediators upon recognition of bacteria [43]. These inflammatory mediators (i.e. cytokines and chemokines) lead to the recruitment of PMNs and monocytes [45, 46]. To eliminate microbes, PMNs are equipped with a set of mechanisms to phagocytose and kill bacteria via reactive oxygen species (ROS), bactericidal permeability inducing protein, lactoferrin and degradative enzymes like elastase [47]. Once neutrophils fulfilled their task, they rapidly undergo apoptosis and are then eliminated via phagocytosis by alveolar macrophages [48, 49].

Receptors and signaling pathways of the immune system

Pattern recognition receptor signaling

The first step of the immune response is the detection of invading microbes. This detection system is comprised of a panel of receptors, termed pattern recognition receptors (PRRs), which all have the ability to recognize common structural moieties of microbes, also known as pathogen associated molecular patterns (PAMP) (Fig.4). These PAMPs are evolutionary conserved and are recognized by the innate immune system of many species. In contrast to the adaptive immune system, which is highly adaptable to conserved, as well as to new pathogenic protein structures, the innate immune system, recognizes these conserved structures via a repertoire of PRRs, which is then considered the first and immediate line of defense against infections.

The recognition of PAMPs is not a random process but a well-defined mechanism where distinct PRRs recognize distinct PAMPs, which will result in well-defined pathway activation.[50].

PRRs	PAMPs	Signaling pathway activated
TLRs[1]		
TLR1-TLR2	Triacylated lipopeptide	MyD88-dependent activation of NF-κB
TLR2-TLR6	Diacylated lipopeptide	MyD88-dependent activation of NF-κB
TLR4	LPS	MyD88-dependent activation of NF-κB and TRIF-dependent activation of NF-κB and IRF3
TLR3	Poly(I:C)	TRIF-dependent activation of NF-κB and IRF3
TLR7	ssRNA	MyD88-dependent activation of NF-κB and IRFs
TLR9	CpG-DNA	MyD88-dependent activation of NF-κB and IRFs
RLRs[21]		
RIG-I	Paramyxoviridae, short blunt dsRNA bearing a 5′ triphosphate (<50 bp),[24] and short poly(I:C) (<300 bp)[23]	IPS-1–dependent activation of NF-κB and IRFs and inflammasome activation
MDA5	Picornaviridae, long dsRNA, and long poly(I:C)[23]	IPS-1–dependent activation of NF-κB and IRFs
LGP2	?	Positively regulating RLR signaling
NLRs[1, 25]		
NOD2	Muramyl dipeptide	RIP2-dependent activation of NF-κB
NALP3	Uric acid crystal, silica, asbestos, hemozoin, zymosan, *C albicans*, influenza virus, *L monocytogenes*, *S aureus*	Inflammasome activation
IPAF	Flagellin, *Salmonella typhimurium*, *Legionella pneumophila*, *Shigella flexneri*	Inflammasome activation
AIM2	dsDNA	Inflammasome activation
CLRs[30]		
Mincle	SAP130 nuclear protein,[26] *Malassezia* species,[28] trehalose dimycolate[29]	Syk-dependent signaling
Clec9a/DNGR-1	Necrotic cells[27]	Syk-dependent signaling

Figure 4: Pattern recognition receptors, their respective pathogen-associated molecular patterns (PAMPs; i.e. ligand), and activated pathways [51].

TLR receptor signaling

The most important and best-characterized family of PRRs, which play a tremendous role in innate immunity, are TLRs. These receptors engage in the recognition of bacteria, their fragments or endogenous molecules, respectively (Fig.4). By now 12 TLRs have been identified in mammals, and various TLRs recognize lipids, proteins or nucleic acids. TLRs are type I transmembrane proteins and are either located in endosomal compartments (TLRs 3, 7, 8, 9), or expressed on the cell surface (TLRs 1, 2, 4, 5, 6) (Fig.5) [50].

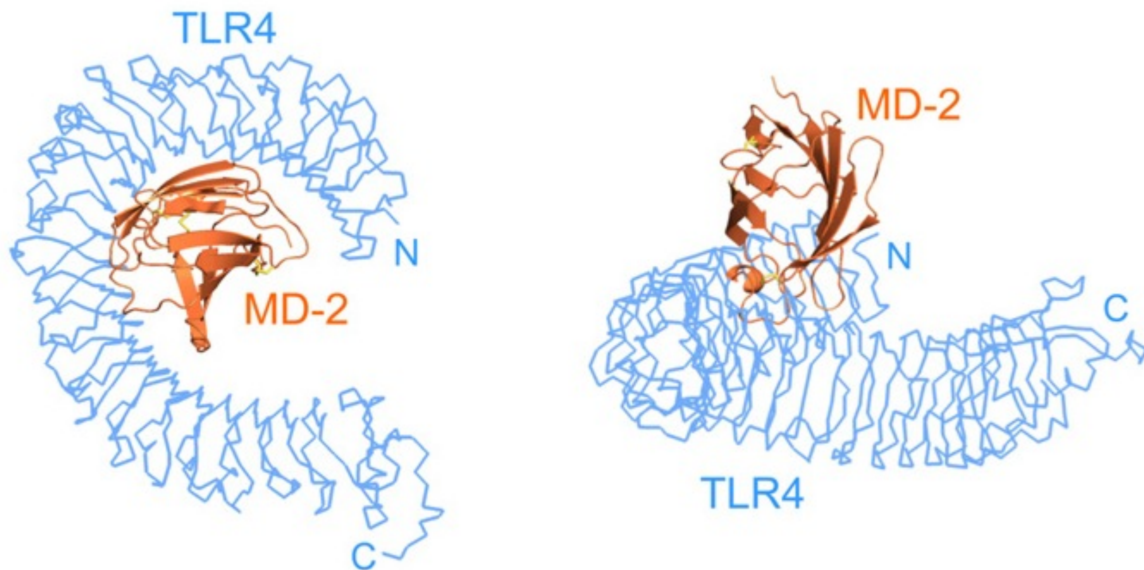

Figure 5: MD-2 bound to its receptor TLR4 (left: top view & right: side view) [52]

Signaling downstream of TLRs unequivocally results in the activation of several transcription factors, most importantly nuclear factor-kB (NF-κB) (Fig.6) [53]. Since its discovery in 1986, NF-κB has been shown to mediate a vast variety of cellular response patterns while at the same time being activated by a huge number of stimuli. There is growing evidence that both enhanced and impaired activation of the NF-κB pathway may play a role during the development of human diseases. In resting cells, NF-κB dimers are sequestered in the cytoplasm thanks to their association with kB inhibitors (IkBs, e.g. IkBα, IkBβ, and others) [54]. The IkB proteins are composed of a central ankyrin repeat region that interacts with the

nuclear localizing signals of the Rel homology domain, which prevents nuclear translocation of NF-κB, and an N-terminal regulatory domain responsible for their inducible degradation. Specific (e.g. LPS, cytokines such as TNF-α or IL-1) as well as unspecific (e.g. γ and UV radiation, oxidative stress) activating signals can trigger the transduction pathways leading to the dissociation of NF-κB from IkB proteins. The first step of this process involves activation of the IkB kinases (IKK). The IKK complex consists of two catalytic subunits: IKKα (IKK1) and IKKβ (IKK2) as well as a regulatory subunit: NEMO (NF-κB essential modulator, IKKγ). IKKβ is crucial for IkBα phosphorylation triggered by such stimuli as TNF-α and IL-1. Engagement of TNF-α and IL-1 receptors on the cell surface lead to the activation of downstream kinases, which are responsible for direct phosphorylation of the IKKβ activation loop. Subsequently, IKKβ phosphorylates serine 32 and 36 residues in the N-terminal part of the IkBα protein, thus creating a binding site for the subunits of the ubiquitin ligase complex, which results in the rapid polyubiquitination of IkBα followed by its degradation in the 26S proteasome. Dissociation of IkB exposes the nuclear localization signal in NF-κB proteins and leads to their nuclear translocation and binding to the promoters of target genes [55-57].

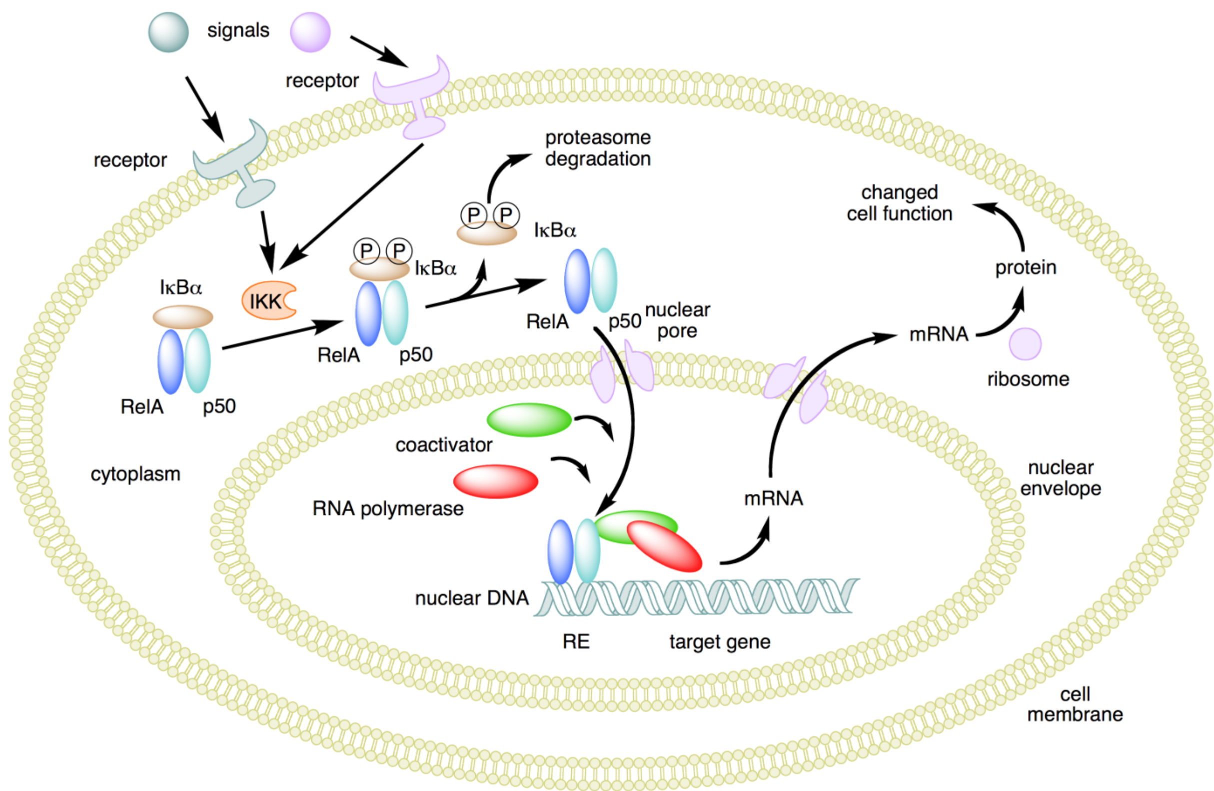

Figure 6: NFκB signaling pathway [58]

TNF-R1 signaling

The TNF receptor superfamily, also termed as death receptors, consists of six known human receptors and regulates important key roles in immune modulation via transcription of proinflammatory genes, cell survival, apoptosis and proliferation [59]. These receptors can be anchored by being type I transmembrane proteins itself, associated at the membrane via glycophospholipids or can even be secreted. While the extracellular domain is responsible for different ligand binding, the cytoplasmic domain is highly [60] conserved and serves as binding site for intercellular adaptor proteins, i.e. Fas-associated death domain (FADD) and TNF-receptor associated death domain (TRADD) (Fig.7) [61].

Receptor	HUGO[a]	Ligand	HUGO	Pivotal adaptor
TNFR1	TNFRSF1A	TNF, LTα	TNF, TNFB	TRADD
CD95 (Fas)	FAS	CD95L	FASLG	FADD
DR3	TNFRSF25	TL1A	TNFSF15	TRADD
DR4 (TRAILR1)	TNFRSF10A	Apo2L/TRAIL	TNFSF10	FADD
DR5 (TRAILR2)	TNFRSF10B	Apo2L/TRAIL	TNFSF10	FADD
DR6	TNFRSF21	?		TRADD?

Figure 7: Death Domain receptors and their respective ligand [62]

While FADD is able to regulate survival or apoptosis via activation of caspase 8 and 10, which are known to be pro-apoptotic enzymes, TRADD controls the non apoptotic function via recruitment of receptor-interacting protein-1 (RIP1), TNF-receptor associated kinase-2 (TRAF2) and cellular inhibitor of apoptosis proteins (cIAPs). These proteins can stimulate phosphorylation of IKK, an important trigger for activation of the NFκB pathway, as well as mitogen-activated protein kinases (MAPK), c-Jun-terminal kinase (JNK) and p38 which leads to different transcriptional responses necessary for modulation of cellular function and fate (Fig.8) [62].

Hence, while FADD and his upstream associated receptors like CD95 (Fas), DR4 and DR5 activates mainly proapoptotic pathways, TRADD and his associated receptors TNF-R1 and DR3 mediate proinflammatory and immune stimulatory effects [63].
Ligand binding to CD95 drives receptor clustering and binding of FADD to the intracellular domain of the death receptor. Binding of FADD leads to the recruitment and activation of caspase 8 and 10, which are initiator caspases. This complex, also called death-inducing signaling complex (DISC), leads to the autocatalytic activation of, caspase 8 and 10, which are released in the cytoplasm where they are able to activate their targets caspase 3, 6 and 7 by proteolytic cleavage. These caspases, also known as executioner caspases have various protein targets which are then leading to cellular apoptosis.

As mentioned before, TRADD dependent signaling is required for TNF-R1 and DR3 mediated responses. Similar to the formation of the DISC complex in FADD

dependent signaling, an intracellular complex is formed to fulfill TRADD signaling. Complex I is comprised of RIP-1, TRAF2 & 5, cIAP's and the intracellular TRADD domain [64]. Complex I, in contrast to DISC, is able to stimulate NF-κB, JNK and p38 downstream signaling. During formation of Complex I, cIAP's mediates polyubiquitination of RIP-1. The ubiquitination process of RIP-1 can also be negatively regulated by A20 and caspase 8/10–associated ring protein 2 (CARP2) by virtue of their deubiquitinating activity [65]. Activation of RIP-1 results in the interaction with transforming growth factor-β-activated kinase 1 (TAK1), via TAK1 binding proteins 1 and 2 (TAB1,2). TAK1 is furthermore able to activate NF-κB via interaction with the upstream IKK complex of NF-κB signaling [66]. While NF-κB transcriptionally activates many genes, like those encoding for proinflammatory cytokines and chemokines, it is also responsible for the transcription of cIAP's and c-FLIP's which have mainly antiapoptotic properties. Moreover Complex I has the capacity to also directly activate JNK and p38 signaling pathways [67]. An additional complex was found formed following TNF-R1 dependent signaling, and called complex IIA.

After internalization of the TNF-R1 the associated proteins RIP-1 and TRAF2 undergo conformational changes, leading to the dissociation from the TRADD domain. The unbound TRADD domain gains the ability to bind to FADD, which activates caspase 8. This complex is called IIA and drives mainly proapoptotic mechanisms. C-FLIP serves as a negative regulator of complex IIA (i.e. caspase 8) and is activated by JNK and NF-κB itself [68, 69]. In addition, cIAP's can inhibit the self oligomerization of deubiquitinylated RIP-1 which would lead to TRADD binding and further caspase 8 activation. This complex is known as complex IIB and is negatively regulated by the IAP antagonists Smac and Smac mimetics, which are able to cause autoubiquitylation of cIAP and their further degradation by the proteasome [70].

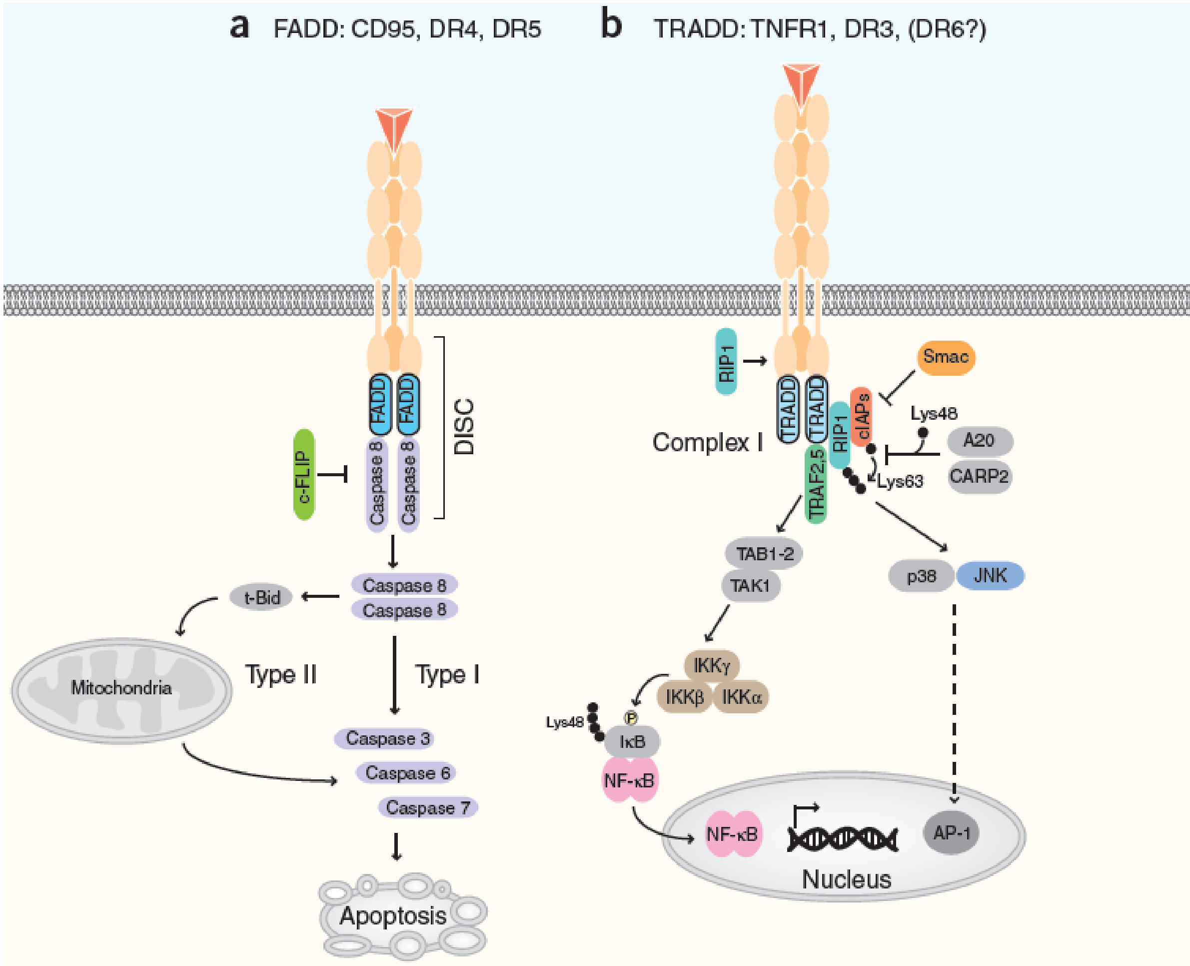

Figure 8: Signaling of FADD and TRADD dependant death receptor signaling

Cells involved in lung immune response

Respiratory epithelial cells

Airway epithelial cells encounter many different airborne compounds, like viruses, bacteria, chemicals and hence play an important role in host defense and maintenance of lung homeostasis. Over the last decades, our knowledge about the role of airway epithelial cells has increased significantly, and extended from their known function as physical barriers to a complex regulatory role in host defense. Airway epithelial cells are able to regulate innate and adaptive immune responses by production of functional molecules like antimicrobial and antiviral proteins, or proinflammatory cytokines and chemokines. These mediators in turn activate other mucosal cells and contribute to the attraction of immune cells from the circulation [71].

The anatomical structure of the alveolus consists of several cell types with distinct functions such as gas exchange, physical stability and immunological functions. Epithelial cells of the airway tract have a heterogeneous morphology depending on their allocation and function. While epithelial cells of the upper airway tract are ciliated (also known as ciliated epithelium) they do not serve primarily for gas exchange, but play a tremendous role in elevating small particles like dust, dead cells and mucus.

There are two types of alveolar epithelial cells and they mediate different roles in the alveolus. Alveolar epithelial cells type I are covering the capillary networks underneath, fulfilling gas exchange, and are tightly connected to surrounding type I cells. Another type of alveolar epithelial cells, called type II cell, resides next to type I cells (Fig.9). These alveolar epithelial cells of type II have many organelles and lamellae corpi and secrete a thin aqueous layer, containing mainly of phospholipids and proteins, which is called surfactant and covers the whole alveolus. The surfactant layer is of extreme importance as it prevents the collapse of the alveolus and serves as an aqueous layer for antimicrobial peptides and immunomodulatory agents. As type II cells harbor many organelles, they appear bigger than type I cells. Type II cells can replicate locally and also replace type I epithelial cells. Another cell type, called Clara cells, resides in the bronchioli respiratorii and don't generate cilia,

but produce short microvilli. Clara cells fulfill an important protection role, by secretion of a variety of products, which include Clara cell secretory protein and detoxifying compounds like cytochrome P450 enzymes, similar to surfactant. In addition they serve as progenitor cells for type II epithelial cells [72-74]

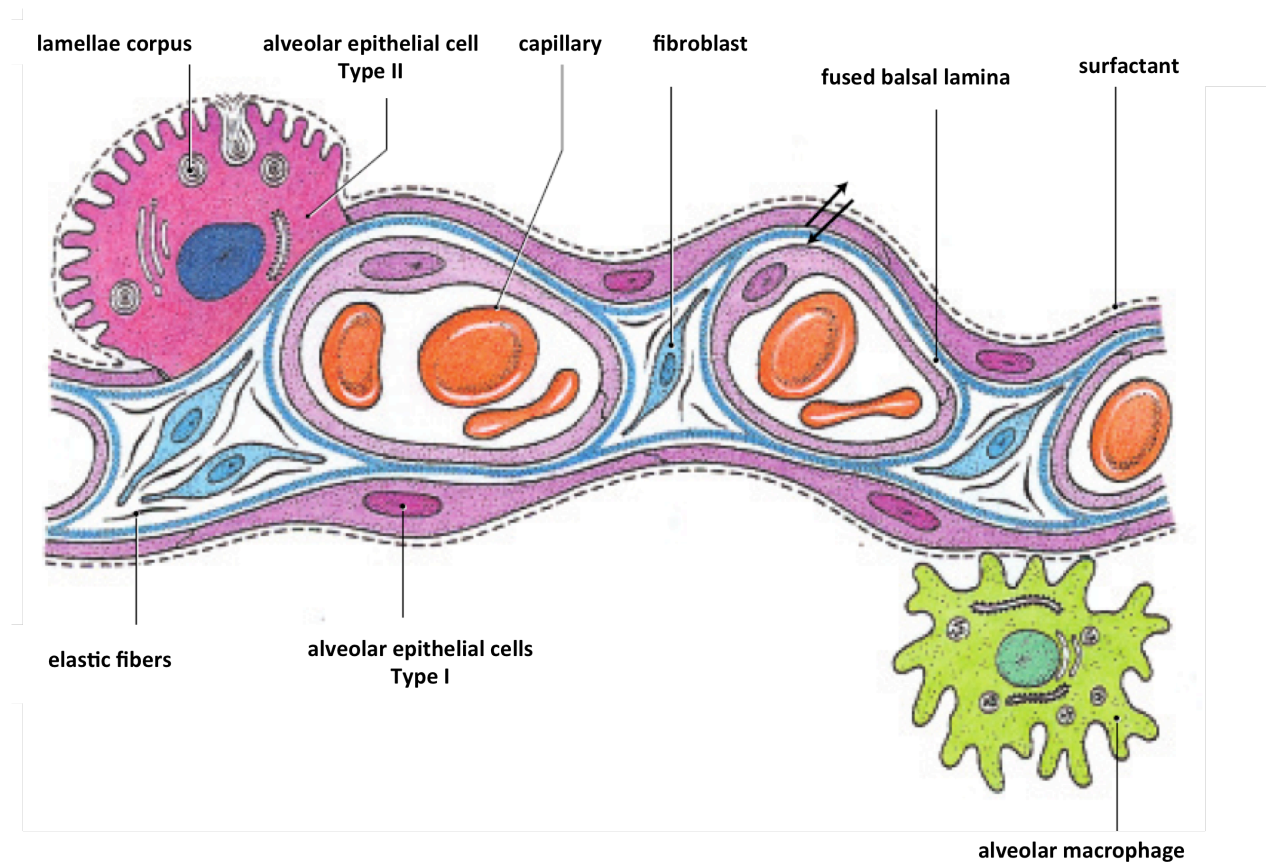

Figure 9: Alveolar structure, arrows indicate gas exchange between alveolus and capillar [73]

Alveolar Macrophages

Macrophages (Mφ) are able to perform phagocytosis and are found in lymphoid as well as non-lymphoid tissues. In their differentiated status macrophages have distinct names which are determined by the location in tissues, where they are encountered. Macrophages are important in innate, as well as adaptive immunity and in maintaining homeostasis by degradation of debris [75]. Just like some DC subsets, macrophages originate from myeloid precursor cells in the bone marrow and are repopulated locally by monocytes that then differentiate to tissue macrophages. On

their surface macrophages express several PRRs such as TLRs, which allows these cells to signal the presence of pathogens [76].

In the lung, resident macrophages are present in alveoli and are called alveolar macrophages. This localization allows close contact to alveolar epithelial cells of type I and II, and was considered important in keeping macrophages inactive via local IL-10 releases [77]. Binding of PAMPs to PRRs present on the surface of macrophages causes loss of binding to epithelial cells, which terminates the release of anti inflammatory cytokines. This in turn allows macrophages to release proinflammatory cytokines and to attract other cells of the immune system [78].

Questions and Objectives

- **Has PVL the ability to inflame lung epithelial cells?**
  As we could recently show that PVL is able to inflame the lung via a TLR2 dependent activation of alveolar macrophages (Zivkovic et al. in press), we asked whether PVL was also able to activate epithelial cells. In addition, as PVL is a pore forming toxin [33], we speculated that the association with necrotizing pneumonia [12] could depend on PVL-induced necrosis of lung epithelial cells.

- **Is there a synergism between PVL and other virulence factors of *S. aureus* in causing lung inflammation?**
  S. aureus produces several toxins and virulence factors [11] and it was recently shown, that the presence of PVL itself is upregulating the expression of the virulence factor protein A [12]. Therefore we wanted to address the question if protein A and PVL can synergistically increase the release of proinflammatory cytokines by resident lung cells.

- **Is TNF-R1 playing an essential role in PVL dependent signaling and activation of resident lung cells?**
  Recent publications showed that protein A is able to inflame lung epithelial cells via a TNF-R1 dependent mechanism [79]. We suggested that the synergistic action of protein A and PVL could be directly, or indirectly dependent on TNF-R1. To address the role of TNF-R1 *in vivo* we created a chimeric mouse model using bone marrow transplantation to study the cell-type specific role of TNF-R1 in PVL/protein A induced lung inflammation.

Results

PVL is able to inflame lung-epithelial cells

It was shown by the Knapp Lab that PVL is able to inflame the lung via TLR2-dependent activation of alveolar macrophages (Zivkovic et al. in press). Because TLR2 is also expressed on respiratory epithelial cells, we hypothesized that this cell type might also signal the presence of PVL within the lung. To study this hypothesis we first investigated the presence of TLR2 on MLE12 & MLE15 cells, which are alveolar epithelial cell lines (Fig.10). Even though the levels of TLR2 mRNA were low, we could find that PVL was stimulating the release of the proinflammatory chemokines KC, also known as CXCL-1, *in vitro* and by primary epithelial cells *ex vivo* (Fig.11A). Furthermore while administration of PVL to lungs of mice did not reveal a significant KC release in BALF, we discovered a significant increase of MCP-1 in lungs of PVL treated mice (Fig.11B). Because we could show earlier that lukF-PV is not inducing lung inflammation, it is tempting to speculate that lukS-PV alone is sufficient for inducing the MCP-1 release.

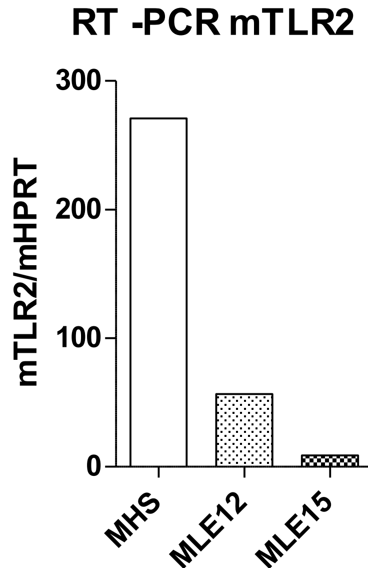

Figure 10: Real- time PCR for expression levels of murine TLR2 on indicated cell types normalized to HPRT.

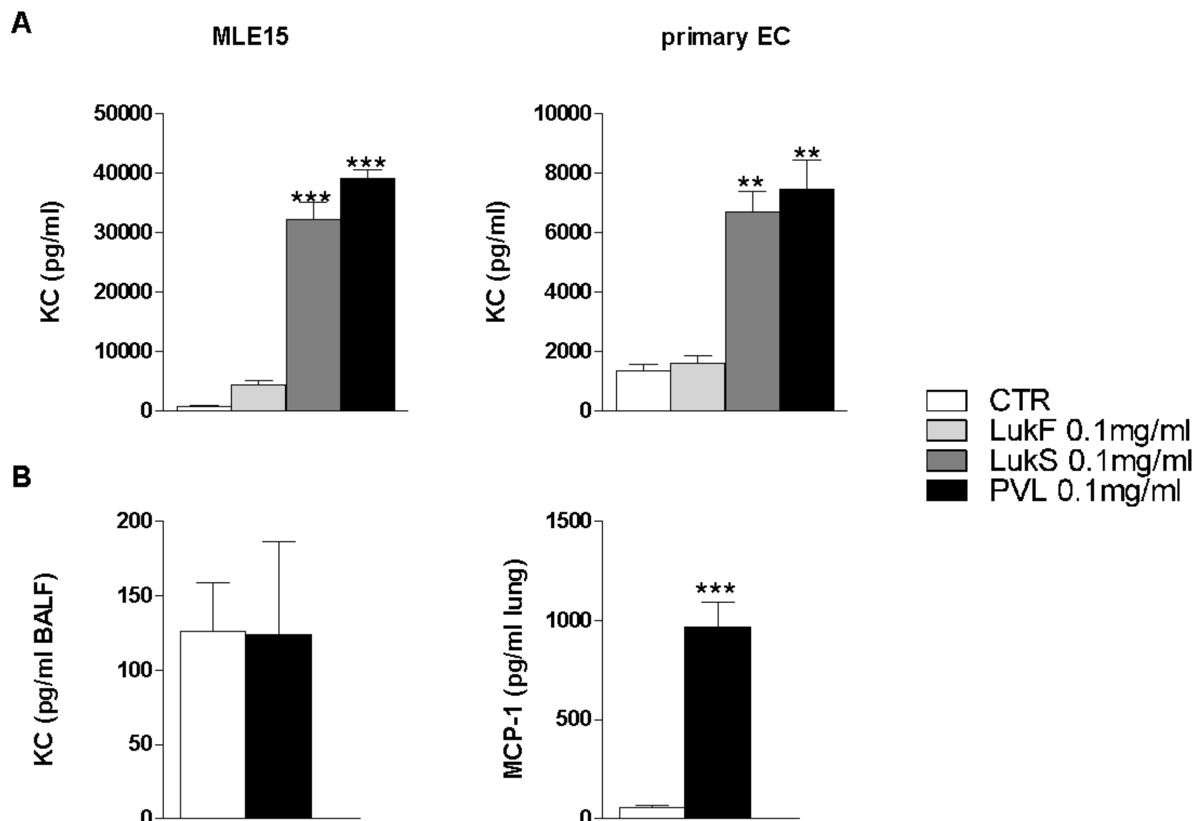

Figure 11: PVL is stimulating the secretion of proinflammatory chemokines (KC) by lung epithelial cells. (A) Immortalized murine type II lung epithelial cells (MLE15) were incubated for 16h with LukF-PV, LukS-PV and PVL. Primary mouse airway epithelial cells of C57BL/6 mice were extracted as described in the materials and methods and stimulated for 16h with LukF-PV, LukS-PV and PVL. KC was determined by ELISA. (B) Intranasal administration of saline (CTR) or PVL to C57BL/6 mice, BAL and lung were removed after 6h. KC was quantified in BALF and MCP-1 in lung homogenates by ELISA.

PVL is not able to induce apoptosis or necrosis of lung-epithelial cells

Because we and others could show that PVL has a potent role in lysing PMNs, we hypothesized that excessive necrosis during PVL-associated pneumonia might be a result of pore-formation on lung-epithelial cells. To address this question we performed several assays to investigate apoptosis and necrosis upon PVL incubation of respiratory epithelial cells.

First we measured the release of lactat dehydrogenase (LDH), an enzyme which is located in the cytosol and only released during late apoptosis or necrosis of cells. The obtained data indicate that there is no elevated LDH release upon PVL

stimulation at prolonged incubation, i.e. murine type II epithelial cells do not die in response to PVL (Fig.12A).

In a second step we analyzed DNA fragmentation upon incubation of epithelial cells with PVL. DNA fragmentation is a consequence of apoptosis, as genomic DNA is cut into small pieces by endonucleases. These small DNA pieces of apoptotic cells as well as the genomic DNA of intact cells can be extracted and visualized on an agarose gel. While the absence of apoptotic cells would results in few large bands, apoptotic or necrotic cells are reflected by many bands of various sizes that might lead to a smear. However, we could not obtain DNA fragmentation upon PVL stimulation whereas staurosporine treated cells displayed the typical smear of DNA-laddering (Fig.12C).

Finally we verified these results by using a FACS based assay using propidium iodide and Annexin V. Propidium iodide is binding to DNA, which can only happen when the cell membrane is fragmented during necrosis. During apoptosis phosphatidylserines flip to the outside of the cell membrane and serve as markers for phagocytosis. Annexin V is able to selectively bind to phosphatidylserine and both, propidium iodide and Annexin V can be detected using FACS. We could show that when treating MLE15 cells with PVL they do not undergo apoptosis or necrosis (Fig.12D).

To exclude the possibility that the immortalized nature of used cell lines interferes with the sensitivity of cells undergoing apoptosis, we also obtained primary mouse airway epithelial cells and tested for apoptosis upon stimulation with PVL. In addition to the data obtained with MLE cells, we could show that PVL is not able to cause apoptosis in *ex vivo* primary epithelial cells by an LDH release assay (Fig.12B).

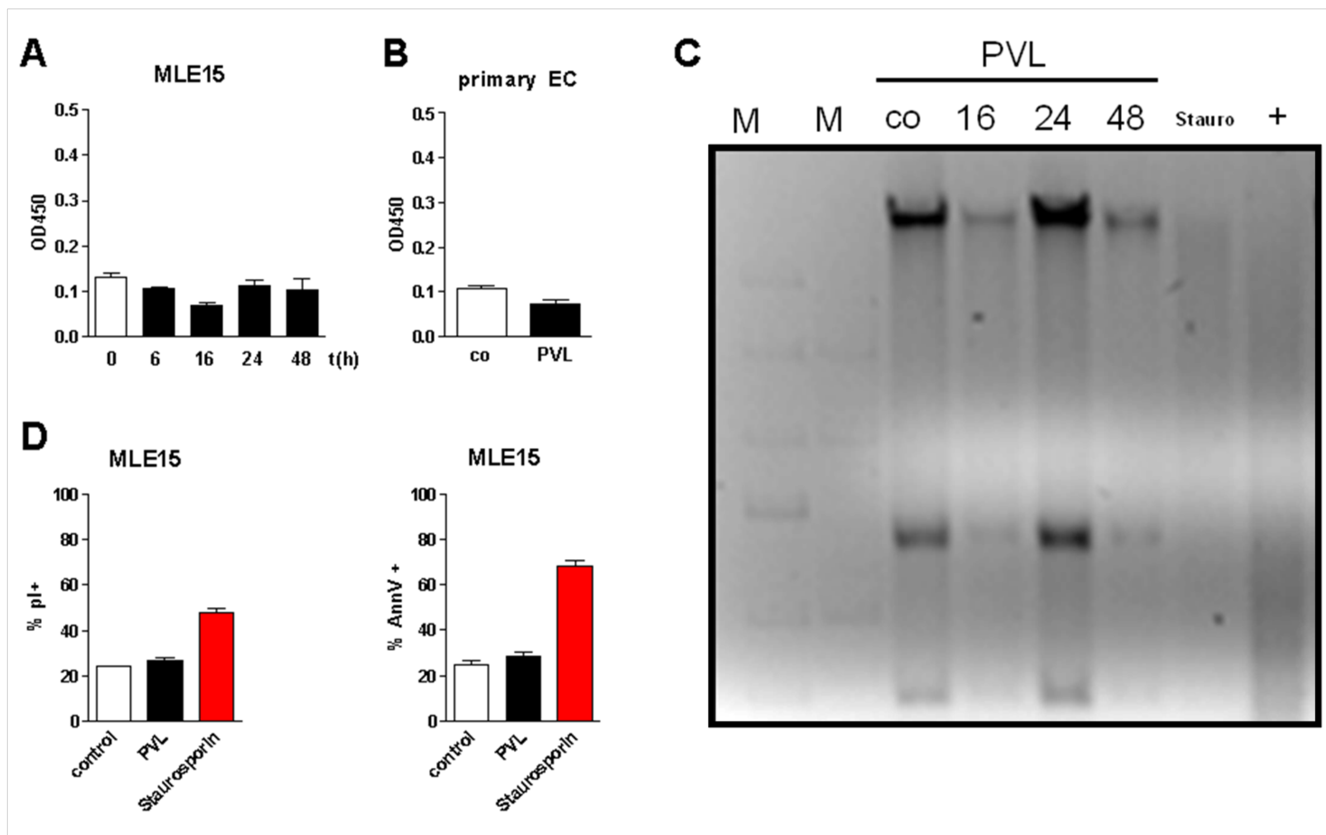

Figure 12: PVL is not causing apoptosis or necrosis of lung epithelial cells. (A) LDH release assay for PVL treated murine type II alveolar epithelial cells (MLE15) at indicated timepoints. (B) LDH release assay for primary mouse airway epithelial cells treated with PVL for 16h**.** (C) MLE15 cells treated with PVL for indicated timepoints and staurosporine for 16h; positive control was supplied by the kit used. (D) MLE15 cells were treated with PVL and Staurosporine for 16h and stained for propidium iodide & Annexin V and measured using FACS.

PVL and Protein A together enhance the inflammatory responses *in vivo*

Previous experiments could not explain tissue necrosis in the lung, because PVL alone is obviously not able to cause lung necrosis. Therefore we thought about combining PVL with other virulence factors of *S. aureus*, which might play a role in causing an overwhelming cytokine release, leading to a destructive environment and/or to enhance PVL's cytotoxic effects. As mentioned above, protein A is an important virulence factor of *S. aureus* and able to inflame lungs via epithelial TNF-R1 [79]. In addition it was reported recently, that PVL itself is upregulating the expression of protein A by *S. aureus* [12]. This led us hypothesize, that there might be an additive or synergistic effect of protein A and PVL. To study this idea we first

characterized MLE-cells and a murine alveolar macrophages cell line (MH-S) for the presence of TNF-R1 by FACS and RT-PCR (Fig.13). We could verify the presence of TNF-R1for all cell lines.

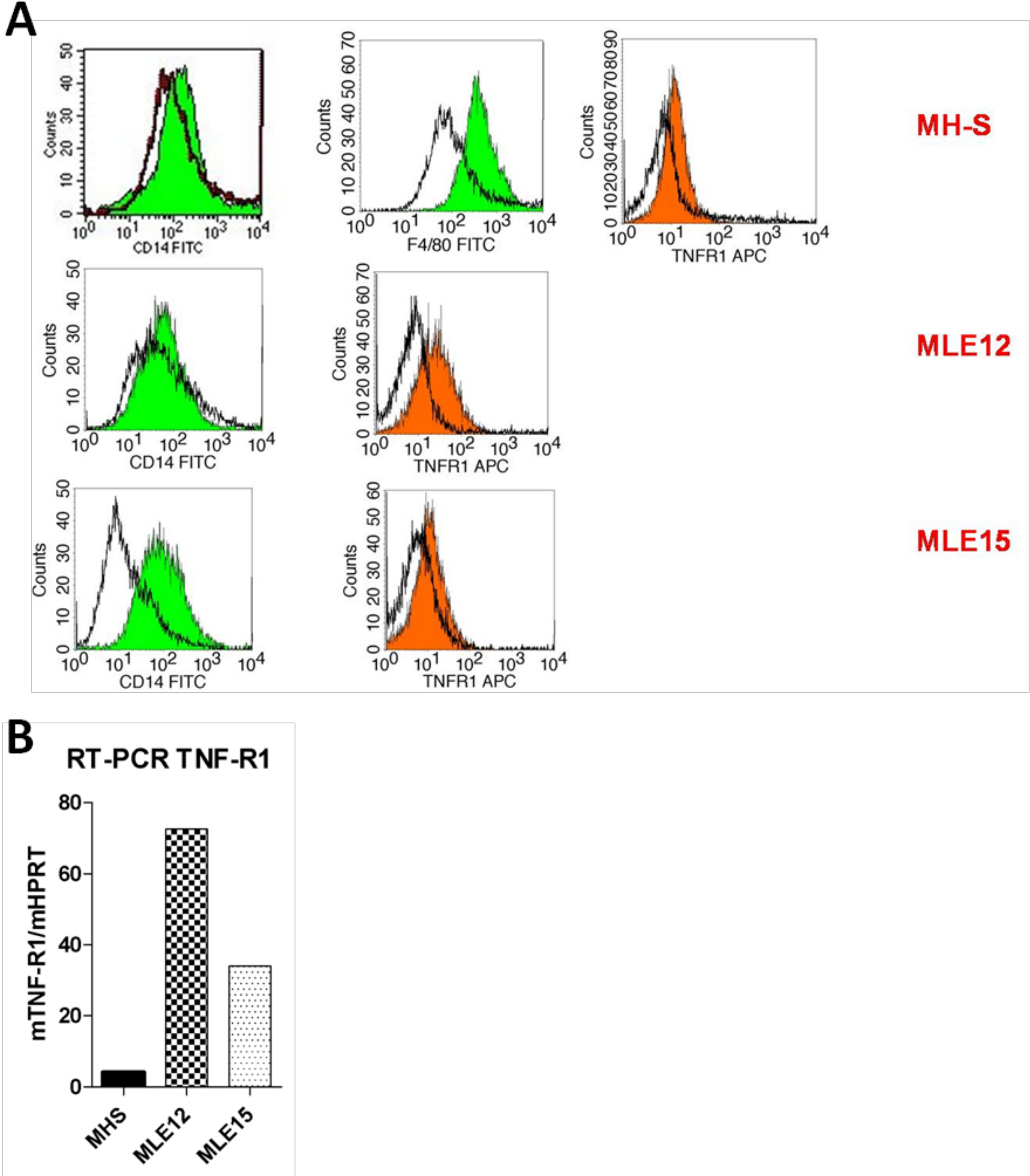

Figure 13: (A) Expression of TNF-R1 on murine alveolar macrophages (MH-S) and murine alveolar epithelial cells (MLE12 & 15) obtained by FACS. (B) RT-PCR for cDNA of MH-S, MLE12 & 15 cells for the expression of murine TNF-R1, normalized to HPRT.

Knowing that these cells express TNF-R1, we were able to plan further experiments to prove our hypothesis of synergism between protein A and PVL. To study this idea we investigated if protein A alone, or together with PVL might have any cytotoxic effect on murine alveolar epithelial cells. Testing this by LDH assay we could not find any evidence for cytotoxic effects of protein A and protein A / PVL on mouse alveolar epithelial cells (Fig.14).

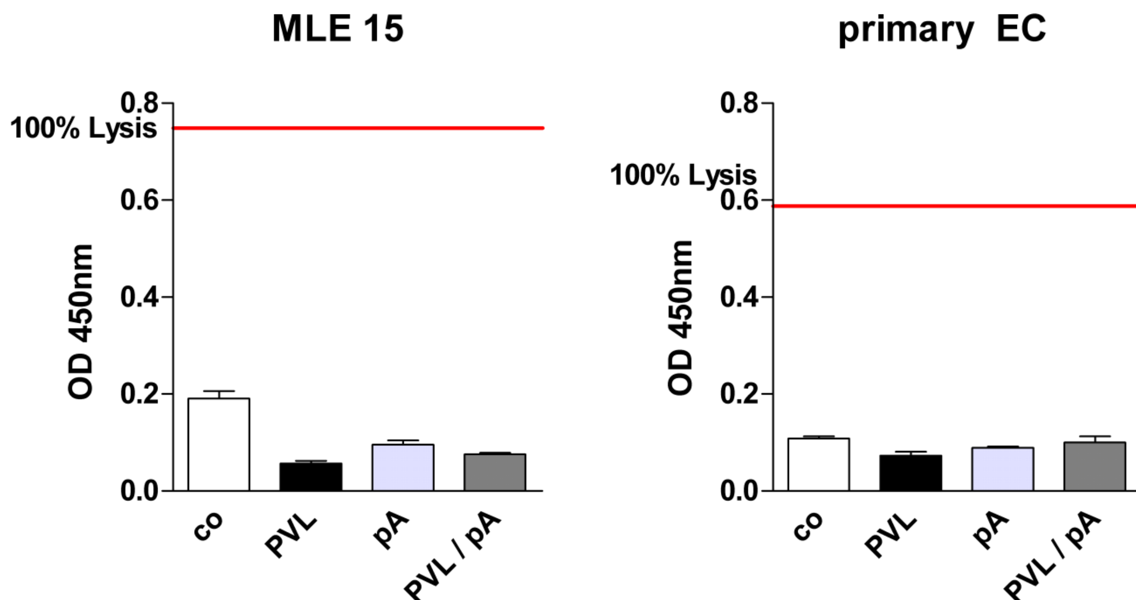

Figure 14: MLE15 cells stimulated with indicated substances for 24h. Primary murine alveolar epithelial cells stimulated for 16h. Cell death was assayed by LDH release into supernatants.

To assay for synergistic proinflammatory effects we next stimulated MLE15 and primary mouse airway epithelial cells with both PVL and protein A and quantified KC releases (Fig.15 A & B). Indeed, the combined stimulation of cells with PVL and protein A resulted in an enhanced chemokine release when compared to single stimuli, which suggests an additive effect of both virulence factors.

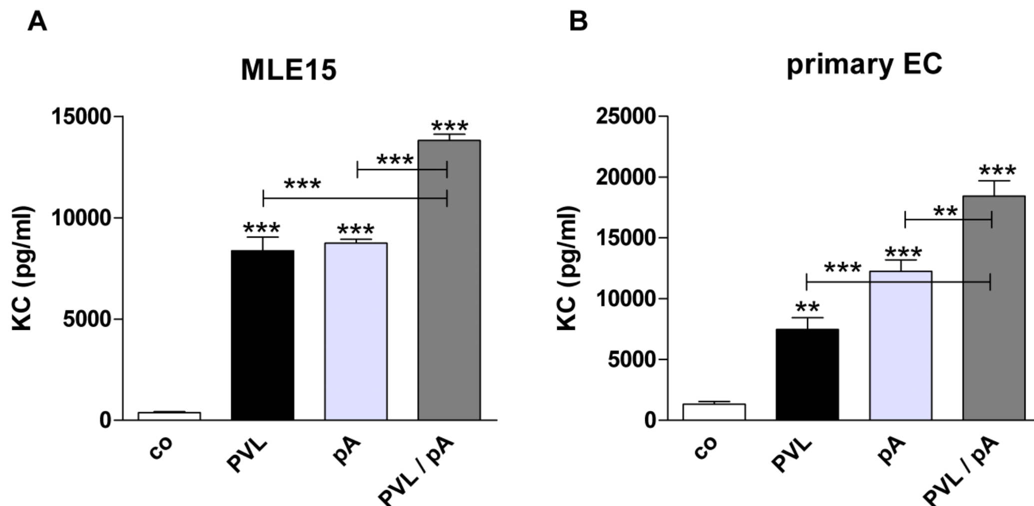

Figure 15: PVL and protein A synergistically enhance the inflammatory response. (A) Murine alveolar epithelial cells (MLE15) were stimulated with PVL (0.1mg/ml), protein A (200µg/ml) or both stimuli for 6h and chemokine concentrations were quantified by ELISA. (B) Primary mouse airway epithelial cells were treated with PVL, protein A or both stimuli. KC was measured in supernatants after 16h by ELISA.

In an additional *in vivo* experiment we challenged mice intranasally with PVL and/or protein A, and again obtained significantly higher chemokine levels in BALF and lung when both stimuli were administered (Fig.16A). Counting of PMNs in BALF revealed in addition a significant influx of PMNs when treated with both stimuli together, as compared to single stimuli (Fig.16B).

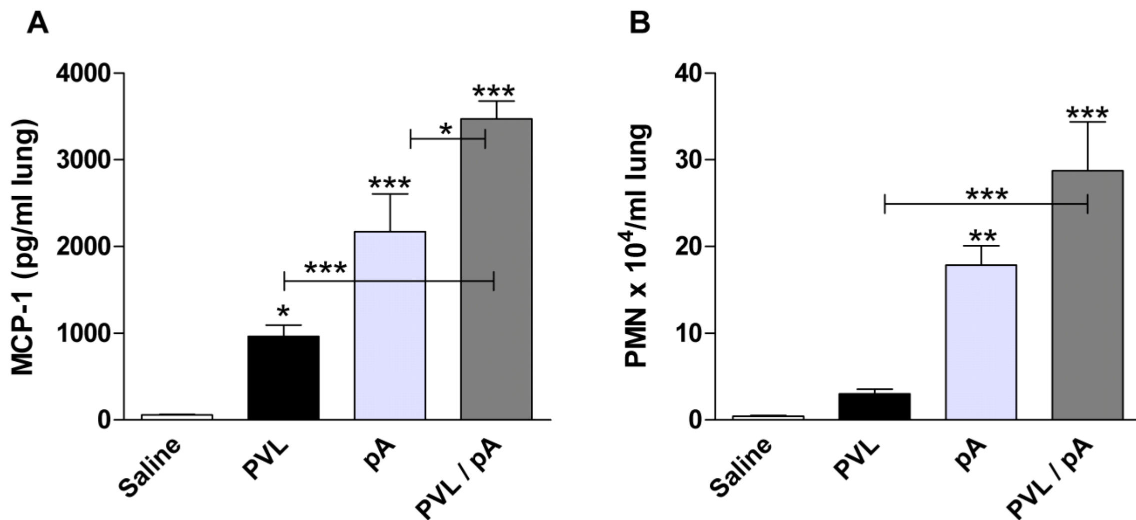

Figure 16: PVL and protein A synergistically enhance the inflammatory response *in vivo* (A) Intranasal administration of protein A, PVL or both stimuli to mice. After 6h, mice were sacrificed and BAL was performed. Chemokine was measured in BALF and lungs by ELISA. (B) PMN count of BALF.

TNF-R1 has an essential role in secretion of cytokines and neutrophil influx *in vivo*

It was shown earlier that protein A has the ability to signal via TNF-R1 expressed on lung epithelial cells [79]. Therefore, we hypothesized that TNF-R1 might play a role in the synergistic effects we observed between protein A and PVL. To address this question, we performed an *ex vivo* stimulation on primary mouse airway epithelial cells of WT and TNF-R1 KO, to enlighten the role of TNF-R1 signaling in epithelial cells (Fig.17). For WT primary murine alveolar epithelial cells we could verify the previous finding of a synergistically increased secretion of KC when both PVL and protein A were provided, as compared to PVL alone. Testing primary cells derived from TNF-R1 KO and WT mice showed that protein A responses are reduced in KO cells and synergism was no longer present. However, PVL induced inflammation was also reduced in the absence of TNF-R1 (Fig.17). To study the in vivo role of TNF-R1 we performed an additional *in vivo* experiment where we challenged WT and TNF-R1 KO mice with protein A and/or PVL intranasally and studied the inflammatory response. Surprisingly the number of recruited cells was significantly reduced in TNF-R1 KO mice when compared to WT animals irrespective if PVL was given alone or together with protein A (Fig.18A). While this response is not unexpected for protein

A, we did not anticipate diminished cell recruitment in response to PVL alone. In addition, BALF analysis revealed a significant increase of MCP-1 in WT mice as compared to KO animals. In contrast, TNF-α levels did not differ between the mouse strains (Fig.18B). We therefore concluded that the efficient attraction of cells into the pulmonary department depended on TNF-R1, suggesting a role for TNF-α in secondarily boosting the inflammatory response to PVL *in vivo*. The fact that protein A administration did not result in consistently abolished inflammation in TNF-R1 KO mice is in agreement with previous publications, showing that protein A is not exclusively recognized by TNF-R1 in the lung [80].

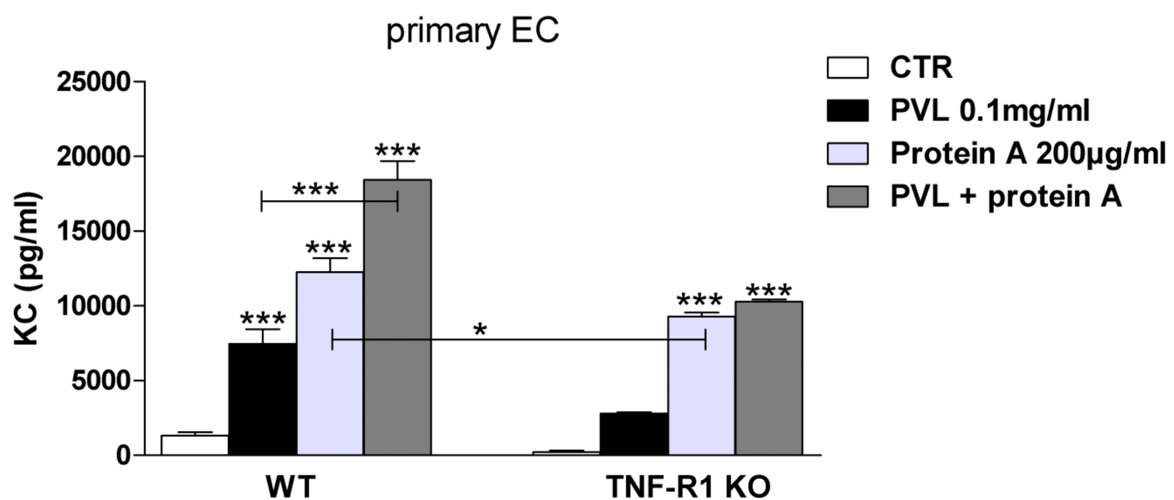

Figure 17: TNF-R1 plays an important role in protein A / PVL dependent signaling *ex vivo*. Primary mouse airway epithelial cells were stimulated for 16h with the indicated compounds, and KC was quantified by ELISA.

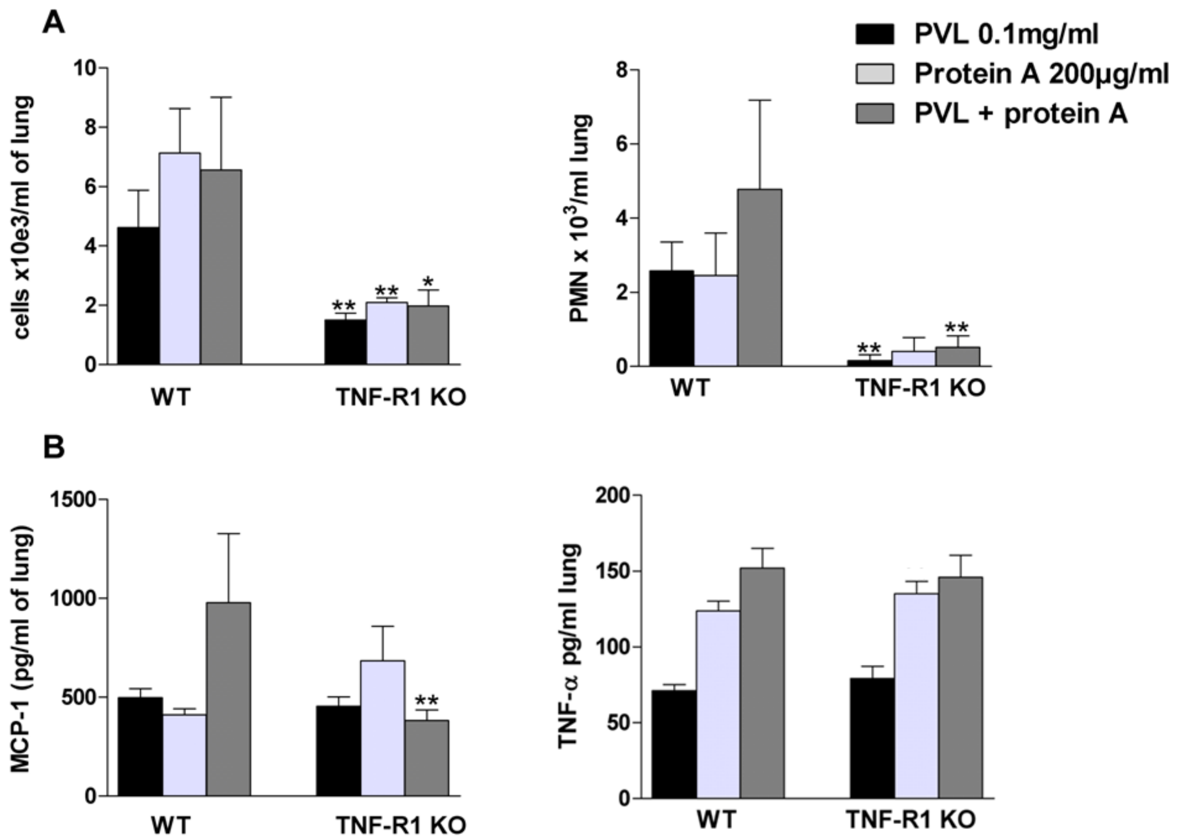

Figure 18: TNF-R1 plays an important role in protein A / PVL dependent signaling *in vivo*. (A)WT and TNF-R1 KO mice were inoculated intranasally with the indicated stimuli for 6h. After this BALF was collected, total cells / PMNs were enumerated (B) and cytokines were measured in BALF and lung homogenates.

In addition, stimulations of primary alveolar macrophages (AM) and primary peritoneal macrophages (PM) were performed to study the role of TNF-R1 in response to PVL treatment. KO macrophages showed significantly decreased levels of proinflammatory cytokines as compared to WT cells (Fig.19).

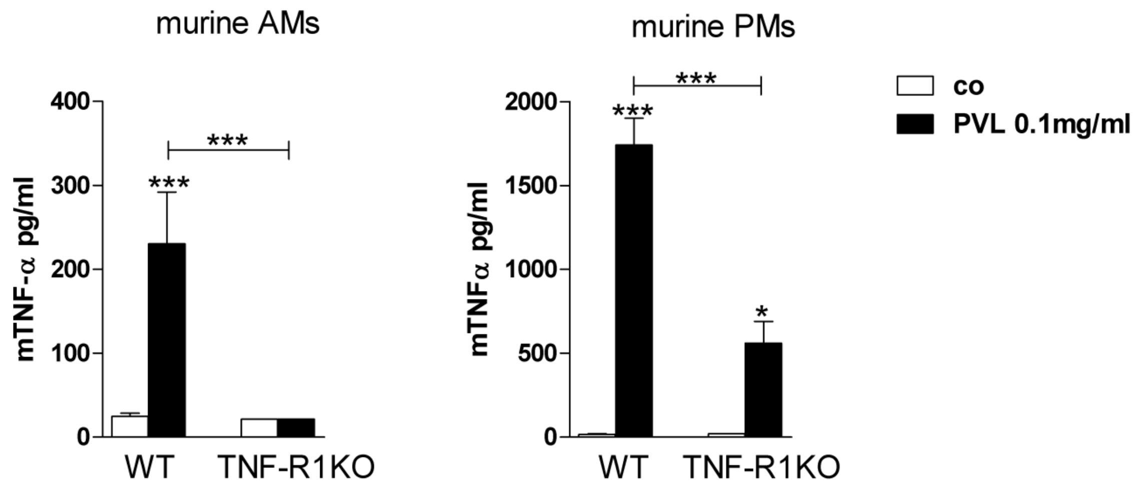

Figure 19: Murine AMs and PMs derived from WT and TNF-R1 KO mice were stimulated with PVL for 6h and TNF-α was measured in supernatants by ELISA.

To exclude the possibility that PVL might signal directly via TNF-R1, we stimulated WT and TNF-R1 KO AMs with PVL and assayed for TNF induction by RT-PCR (Fig.20). TNF-α stimulations were used as control. Because there was no significant difference in the induction of TNF-α gene expression in PVL treated WT and KO cells, we concluded that PVL is not signaling directly via TNF-R1.

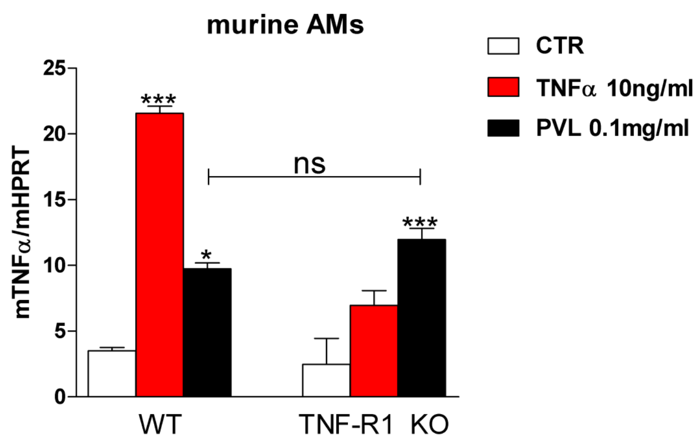

Figure 20: Alveolar macrophages of WT and TNF-R1 KO mice were extracted via BAL. The cells were stimulated for 1h with the respective stimuli RT-PCR was performed (TNF-α normalized to HPRT).

A bone marrow transplant experiment strengthens the importance of TNF-R1 *in vivo* and reveals an important role for lung alveolar macrophages

Because previous *in vivo* and *in vitro* experiments could not clearly explain the role of TNF-R1 in PVL induced lung inflammation, and the contributing role of various cell subsets in the lung, we decided to generate chimeric mice and study the inflammatory response to PVL. We used 4 groups of mice, where WT recipients received WT or TNF-R1 KO bone marrow cells and TNF-R1 KO recipients received WT or TNF-R1 KO cells. This way, we anticipated to understand the respective contribution of TNF-R1 to lung inflammation (Table 1).

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

Table 1: The four created groups of mice according to their genotype in alveolar macrophages and airway epithelial cells.

Six weeks after BMT blood was drawn and AMs were isolated to ensure reconstitution with donor cells (Fig.21 A & B).

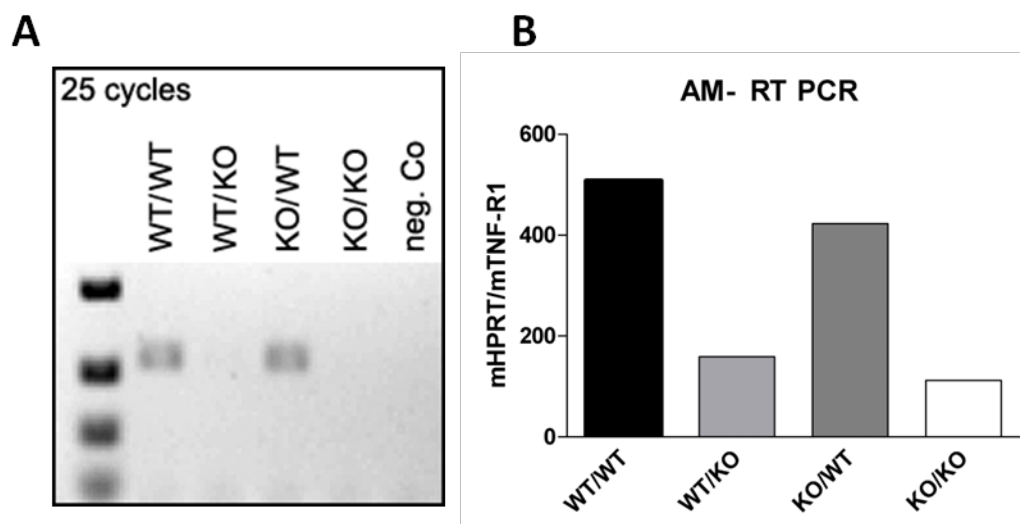

Figure 21: (A) PCR for TNF-R1 of whole blood DNA (25 cycles) and (B) real time PCR for TNF-R1 normalized to HPRT of AM cDNA 6 weeks after bone marrow transplantation.

After ensuring proper reconstitution of alveolar macrophages, mice were intranasally inoculated with PVL and a BAL was performed after 6h. PMN influx was enumerated and data clearly disclosed that TNF-R1 plays an important role in PVL-induced

inflammation (Fig.22A). While WT/TNF-R1 KO and TNF-R1 KO/WT chimeric mice showed modestly reduced PMN numbers, TNF-R1-KO/TNF-R1-KO had strongly reduced neutrophil counts in their lavages (Fig.22A.). Accordingly, KC as well as MCP-1 levels were found lowest in TNF-R1 KO/TNF-R1 KO animals (Fig.22B). These data illustrate that TNF-R1 on either macrophages or epithelial cells contributes to PVL-induced lung inflammation *in vivo*. Since we excluded the possibility that PVL signals via TNF-R1, we hypothesize that PVL induces TNF-α that in turn boosts the local inflammatory response via a paracrine mechanism.

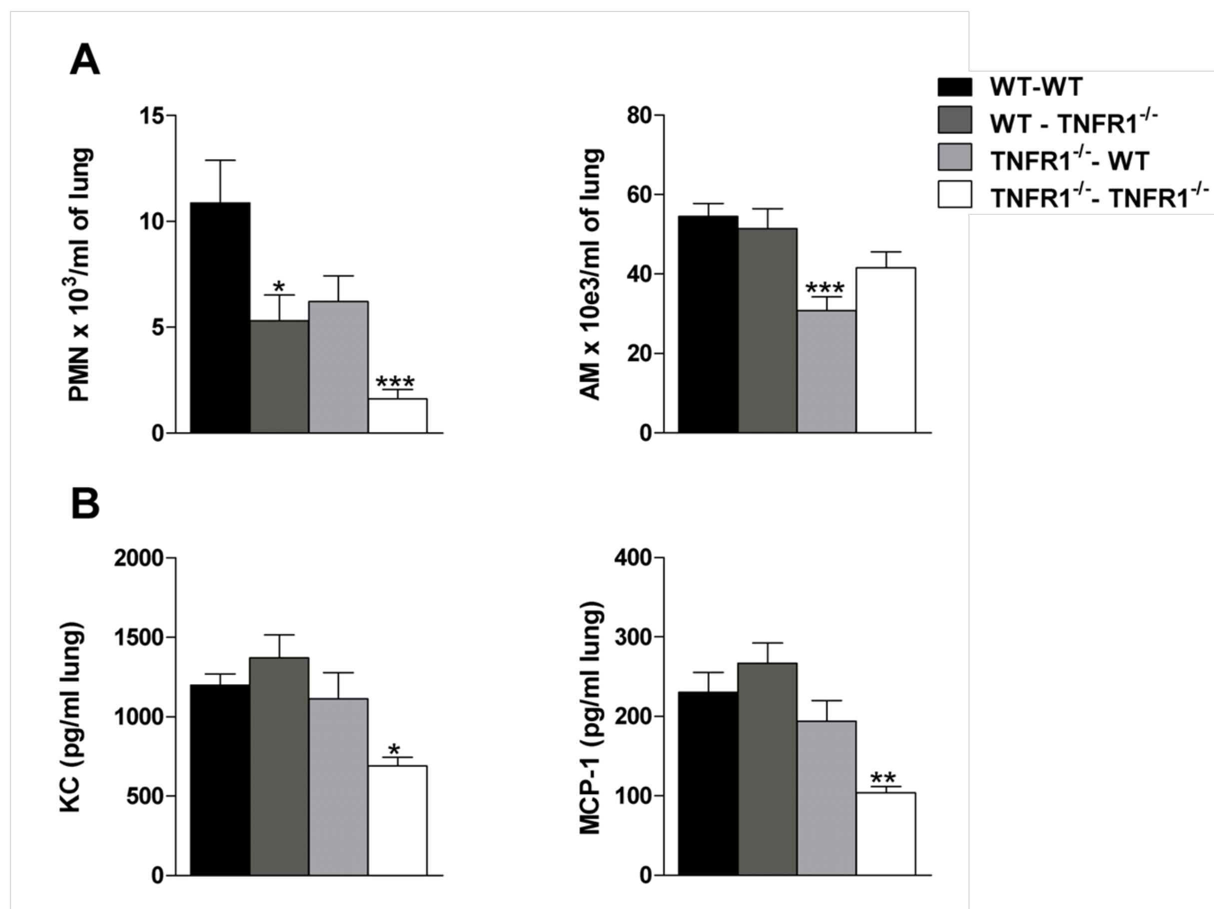

Figure 22: TNF-R1 is important for the recruitment of PMNs and the secretion of proinflammatory cytokines *in vivo*. Mice were intranasally inoculated with PVL and (A) BALF was taken enumerate PMN and determine (B) chemokine concentrations by ELISA.

Conclusion

In this work we could show that PVL is a potent inducer of inflammatory responses by lung epithelial cells. Based on the earlier notion that TLR2 mediates the inflammatory response by AMs in response to PVL, we now show that TLR2 is ubiquitously expressed on mouse alveolar epithelial cells, which most likely explains the responsiveness of these cells.

Epidemiologic studies link PVL to necrotizing pneumonia in humans [12, 24], which led us to determine the possibility that PVL might directly affect lung epithelial cells. Testing the effect of PVL on epithelial cell lines as well as primary murine respiratory epithelial cells did not enable us to show any direct toxic effect by PVL. Hence, PVL can only cause pores and necrosis of myeloid cells, and the clinical observation of lung necrosis cannot be imitated in a murine lung inflammation model.

PVL itself has been suggested as an important virulence factor of CA-MRSA pneumonia, but several recent reports challenged this hypothesis, because direct proof of PVL's toxic properties could not be shown in various animal models tested so far. Because CA-MRSA exerts more virulent traits, when compared to HA-MRSA strains, several groups attempted to discover potential other virulence factors. Among others, higher protein A expression has been shown to be associated with PVL-carrying bacterial strains. Knowing about the importance of protein A as an important virulence factor during *S. aureus* pneumonia [79], we hypothesized that PVL and protein A might synergize in inducing lung inflammation. We therefore studied this idea and indeed found some degree of synergism when testing epithelial cell lines and after infecting mice *in vivo*.

When studying the role of TNF-R1, which has been reported to signal the presence of protein A in lungs [79], we surprisingly discovered, that the absence of TNF-R1 significantly diminished lung inflammation in response to PVL alone. Further tests allowed us to illustrate that TNF-R1 is not a receptor directly signalling the presence of PVL. Rather, PVL is a potent inducer of TNF-α by alveolar macrophages, and bone marrow experiments enabled us to show that TNF-R1 present on both epithelial cells and macrophages mediates the inflammatory response to PVL-induced TNF-α.

Materials and Methods

Cell culture media

MLE media: RPMI 1640 (Gibco) supplemented with 2% FCS and 1% Penicillin/ Streptomycin, additionally 2.1mg Insulin- Transferrin-.Sodium Selenite (Sigma), 5mg Transferrin (Sigma), 1.8mg Hydrocortisone (Sigma), 1.4mg β-Estradiol (Sigma)

RAW media: RPMI 1640 (Gibco), supplemented with 10% FCS and 1% Penicillin/ Streptomycin; used for RAW cell line and primary alveolar macrophages

BMDM media: RPMI 1640 (Gibco), supplemented with 10% FCS, 10% supernatant of L929 cells and 1% Penicillin/ Streptomycin

MH-S media: RPMI 1640 (Gibco), supplemented with 10% FCS and 1% Penicillin/ Streptomycin and 0.01 % β-mercaptoethanol

Cell lines

MLE 12 murine type-II- lung- epithelial cells

MLE 15 murine type-II- lung- epithelial cells

Murine lung epithelial (MLE) cell lines with bronchiolar and alveolar origin were originally isolated out of lung tumors of transgenic mice, containing the viral oncogene simian virus 40 (SV40) large tumor antigen, under control of the human surfactant protein (SP-C) gene. [72]

Cell lines were kindly provided by Jeffrey Whitsett

Chemicals and Buffers

Annexin V Binding Buffer: 0.1 M Hepes (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$. Store at 4°C

PBS pH 7,4 10x Concentrate

ELISA washing Buffer: 0.05% Tween 20 in PBS

ELISA blocking Buffer: 1%BSA in PBS

TMB Microwell Peroxidase Substrate System

Protein production and purification

To generate PVL we used pETM11 Vectors containing LukF and LukS (Zivkovic et al., in press). Respective LukF-pETM11 and LukS-pETM11 constructs were transformed into competent DH5-α cells. 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 French-Press; His-tagged proteins were isolated using Ni-NTA resins (Qiagen) and desalted using ZEBA columns (Thermo Scientific). Finally, proteins were subjected to LPS removal using Detoxi Gel Endotoxin Removing Columns (Thermo Scientific) until a final LPS concentration of <0.02 EU/ml was ensured (Charles River).

LDH release assay.

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 LDH release was determined at indicated times at OD450nm.

Apoptotic DNA ladder assay

$2x10^6$/ml MLE12 cells were treated with 0.1mg PVL or 1µM staurosporine (St; positive control [81]) 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.

Propidium iodide & Annexin V staining

$1x10^6$/ml MLE12 & MLE15 cells were plated in 12 well plates and left to adhere for 4 hours. Stimulations were done in duplicates and performed with 10µM Staurosporine or 0.1mg/ml PVL over 16h. Then cells were harvested in 1ml PBS/EDTA solution and placed in micronic tubes. Cells were washed in PBS twice and centrifuged for 5min at 1250rpm. The resulting cell pellet was resuspended in 100µl Annexin binding buffer and 5µl of Annexin V and incubated for 10 minutes at room temperature. Afterwards 4µl of a 10µg/ml propidium iodide stock solution was added, the cells were vortexed gently and incubated for 15 min at room temperature in the dark. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. After incubation time the cells were immediately analyzed using FACS.

ELISA

For measurement of cytokines DuoSet ELISAs (R&D) was used. 96 well flat-bottom high binding plates (Greiner) were coated with capture antibody (KC 2µg/ml, MCP-1 0.2µg/ml, TNF-α 0.8µg/ml) over night. The next day, plates were washed (0.05% Tween 20 in PBS) and blocked (1%BSA in PBS) for 1h. The standard dilution was plated together with the blank in duplicates on the plate (highest standards: TNF-α 2000pg/ml, KC 1000pg/ml, MCP-1 250pg/ml) together with the samples in their desired dilution and incubated for 2h. After washing the detection antibody (TNF-α & KC 200ng/ml, MCP-1 50ng/ml) was incubated on the plate for 2h. Following washing and incubation with Streptavidin HRP (1:200 dilution) for 20 minutes, the plates were washed and the TMB substrate mix (1:1) was added on for approx. 10-20 min. The color reaction was stopped with 2N sulfuric acid and the color is quantified with an Anthos Zenyth 340 Photometer.

Real time RT-PCR

After treatment with the stimuli according to the experimental setup, RNA was extracted using a RNA isolation Kit (Marchery-Nagel). After isolation of the RNA, the concentration of RNA was measured using the NanoDrop spectrophotometer (Thermo Scientific). The amount of RNA was normalized to equal amount for the

following reverse transcription to cDNA using the iScript cDNA synthesis kit (Biorad). The cDNA was used for real time PCR (Roche Diagnostics) and the obtained CT values were normalized to HPRT.

FACS Analysis

MH-S cells were detached with lidocain, and MLE12 /15 cells using 10mM EDTA/PBS solution. Harvested cells were washed twice with PBS and dissolved in 10ml of FACS Buffer (PBS + 0.5%BSA) before counting. $5x10^6$cells/ml were transferred to micronic tubes cells were stained with anti-TNF-R1 antibody (1:50; Cell Signaling), anti-CD14 (1:50 for MH-S & 1:10 for MLE12/15; BD Pharmingen) and F4/80 (1:50 MH-S only; Serotec) and left to incubate for 30 min. The cells are washed twice with 2ml of FACS buffer before the secondary antibody was added and incubated for 30 minutes in the dark to prevent degradation of the fluorophores. Cells were washed and subjected to FACS analysis on a FACScalibur (BD Bioscience).

Isolation of primary epithelial cells from mice

Tissue culture petri-dishes (10cm) were coated with 20µg/plate of each, anti-mouse CD45 and CD16/32 antibodies (ebioscience) diluted in 5ml PBS, and 96-well plates were coated with 200µl of 10µg/ml collagen type IV (Sigma) diluted from stock 1:100 with 0.25% glacial acetic acid in dH_2O. Both plates are covered with foil and kept overnight at 4°C. The vena cava of the mice was cut to drain the blood and the lung was flushed with saline, which was injected into the right ventricle to remove blood cells. After this, 3ml of dispase I (Sigma) was injected into the lung, immediately followed by 500µl of 1% w/v low melting agarose (Sigma). After solidification of the agarose the lung was extracted and kept in 1ml of dispase for 45 min at room temperature. Then the lung was dissected using forceps in DMEM containing 0.01% DNAse I (Sigma). The cell isolate was filtered through 70µm and 40µm filters, spun down and cells were resuspended in DMEM containing 10% heat inactivated sterile filtered FCS and incubated on the CD45/CD16/CD32 precoated petri dishes at 37°C for 2h. The supernatant of non-adherent cells was collected and the cells were counted using a Türck hemocytometer. Next, $5x10^5$ cells per well were plated on the

collagen coated 96 well plates at 37°C in HITES medium. The next day stimulations were performed.

Bone marrow transplantation experiments

All animal experiments were approved by the Animal Review committee of the Medical University of Vienna and the Ministry of Sciences. For bone marrow transplant experiments we used 9 week old female C57BL/6 and TNF-R1 -/- mice. Four mice of each strain were used to isolate bone marrow, which was done exactly as described above. Recipient mice were irradiated with a dose of 9Gray, after which prewarmed bone marrow cell suspensions ($2x10^6$ cells in 200µl) were injected via the retro-orbital route. Two control mice did not receive any bone marrow cells to test for efficient radiation. Mice were fed autoclaved food and water and were left for at least 6 weeks to let cells repopulate. After 6 weeks, BAL was performed and alveolar macrophages were tested for donor DNA (WT or TNF-R1 deficiency). Following verification of repopulation, mice were intranasally challenged with PVL, and the inflammatory response was evaluated after 6h by means of cell influx, cytokine and chemokine production in BALF and lung homogenates.

Retro-orbital injection in mice

(Adapted from the Institutional animal care and use committee- IACUC of the Oregon Health and Science University)

The retro- orbital injection of bone marrow is an alternative way of the tail vein injection and requires anesthesia of the mouse. This method is being used to repopulate the bone marrow after removal of resident bone marrow cells by irradiation. The volume limit for this procedure is 200µl of cell suspension. To prevent clumping of cells, filter the suspension through cell strainers before injection. For anesthetization Isoflurane is used, further 27-30 gauge needles are needed. The mouse is positioned on its side, restrained by the thumb and the middle finger of the non- dominant hand, pulling back the loose skin over the shoulder and behind the ears. The index finger of the non-dominant hand is being used to draw back the skin

above the eye and the thumb to draw back the skin below the eye. This will result in slight protrusion of the eye. Insert the needle at the side of the eye through the conjunctiva. The tip of the needle is then positioned behind the globe of the eye in a cavity called “retrobulbar sinus”, where the cell suspension is being injected. The needle is removed gently to prevent tissue damage and injury. The eyelid is being closed and mild pressure is applied on the site of injection with a gauze sponge. During the recovery process the mouse should be monitored for adverse effects of the injection site as well as other behavioural abnormalities in the following days after transplantation.

Bone marrow derived macrophages

Mice were sacrificed by isoflurane overdose following removement of both legs at the hip. It is important not to injure the bone and to remove the fur carefully to prevent contamination of the bone marrow. The feet were cut off and the extracted bones were placed in ice cold PBS. In a sterile lamina flow hood the bones were sprayed with 70% ethanol and the flesh was removed. Femur and crus are separated at the knee joint. Of both, femur and crus the bone is cut on each side to reveal the medullary cavity. The bone marrow was flushed using a 27-30G needle filled with RPMI 1640 at room temperature. The cell clumps were resuspended using a 5- or 10ml pipette. The suspension was transferred to 50ml falcons and spun down at 1250rpm for 7 minutes at room temperature. The resulting pellet was resuspended in 10ml of BMDM media and divided into two 10cm cell culture petri dishes (5ml per plate). The plates were incubated in a humidified chamber at 37°C and 5% CO_2. On day 3 another 5ml of fresh BMDM media was added. On day 7-8 the cells were harvested by trypsinization or with PBS/Lidocain solution. The cells were counted and plated in the desired density for the experiment.

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Curriculum Vitae

Mario Biaggio

Köstlergasse 16/7

A-1060 Vienna

+43 699 17141872

A0401860@unet.univie.ac.at

Personal data

Date of Birth	14.6.1985
Place of Birth	Voitsberg
Nationality	Österreich
Marital status	single

Academic

09/2009 – to date	Diploma thesis at the Laboratory of Univ. Prof. Dr. Sylvia Knapp, PhD at the Medical University of Vienna Department of Infectious Diseases & Tropical Medicine & Ce-M-M Center for Molecular Medicine of the Austrian Academy of Sciences Title: „ The role of lung epithelial cells and TNF-α in Panton-Valentine Leukocidin induced lung inflammation “
10/2004 – to date	Study of Molecular Biology at the University of Vienna Focus on: Immunology, Molecular medicine & Cell biology
09/1995 – 07/2003	Secondary School Köflach

Scholarship & Publications

2010	Co- authorship "Toll- like receptor 2 mediates innate Immunity and lung inflammation to Staphylococcal Panton Valentine Leukocidin in vivo" Zivkovic et. al. In press, Journal of Immunology
2009	Scholarship ("Leistungsstipendium")

Work Experience

2008-2009	Freelancer, Apple Computer Inc., Vienna
2007-2009	Freelancer, Siemens AG, Vienna
2004	Internship, Stölzle Oberglas AG, Köflach
2003	Internship, ESTAG/ FHKW, Graz Internship, Stölzle Oberglas AG, Köflach Waiter, Schlossbad Bärnbach
2002	Internship, Steweag-Steg GmbH Rosental a.d. Kainach
2001	Internship, Raiffeisenbank Voitsberg