Modulation of dendritic cell functions by *Staphylococcus aureus* phenol-soluble modulin peptides

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Abbreviations

Agr	accessory gene regulator
AP-1	activator protein-1
APC	antigen-presenting cell
BM-DCs	bone marrow-derived dendritic cells
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
CD	cluster of differentiation
CREB	cAMP response element binding protein
DC	dendritic cell
ERK	extracellular signal-regulated kinase
Foxp3	forkhead box p3
FPR2	formyl-peptide receptor 2
IL	interleukin
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen- and stress-activated protein kinase
MHC	major histocompatibility complex
MRSA	methicillin-resistant *Staphylococcus aureus*
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen associated molecular pattern
PFT	pore forming toxin
PRR	pattern recognition receptor
PSM	phenol-soluble modulin
PVL	Panton-Valentine Leukocidin

S. aureus	*Staphylococcus aureus*
SSTI	skin and soft tissue infection
TCR	T-cell receptor
TGF-β	transforming growth factor-β
T_h	T helper cell
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
T_{reg}	regulatory T cell

Abstract

Staphylococcus aureus (*S. aureus*) is an important human pathogen that causes severe diseases ranging from local to systemic infections. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains are highly resistant to antibiotic treatment and are the most dangerous and pathogenic strains due to their secretion of a variety of virulence factors, enabling efficient evasion of the host immune response. Phenol-soluble modulin (PSM) peptides comprise one group of secreted virulence factors that contribute to the pathogenicity of CA-MRSA. These peptides modulate various types of immune cells, including dendritic cells (DCs), which are a class of professional antigen-presenting cells that link innate and adaptive immunity. Our group previously showed that PSMs in combination with a TLR2 ligand induce tolerogenic DCs, as identified by diminished clathrin-mediated endocytosis and a modulated cytokine secretion profile characterized by anti-inflammatory IL-10, ultimately leading to impaired T cell differentiation. PSMs induce regulatory T cell (T_{reg}) priming by DCs and, in contrast, inhibit T helper 1 cell development. However, the underlying molecular mechanisms remained elusive.

Within this work, we addressed the following questions: (1) Which signaling pathways are modulated by PSMs leading to the increased production of IL-10 by DCs upon TLR2 ligand treatment? (2) Are the activated signaling pathways involved in the priming of T_{regs} by PSM-treated DCs? (3) Are PSMs actively internalized by DCs or are they acting by binding to the formyl-peptide receptor 2 (FPR2)? (4) Do PSMs in general affect DC functions, including maturation, cytokine production and T cell priming, upon treatment with various TLR ligands?

(1) In this study, we demonstrated that mouse bone marrow-derived DCs stimulated with PSMα3 and *S. aureus* cell lysate (a TLR2 ligand) had increased levels of phosphorylated ERK, p38, CREB and NF-κB. However, only the inhibition of phosphorylated p38 and downstream MSK1 prevented the secretion of IL-10 in a concentration-dependent manner.

(2) In DCs, the PSM-modulated p38-CREB pathway was also responsible for the altered differentiation of T cells. Inhibition of this axis also prevented the increased priming of T_{regs} by PSM- and TLR2-treated DCs.

(3) PSMα3 peptides modulated the p38-CREB signaling pathway independent of their receptor FPR2. PSMα peptides penetrate DCs independent of macropinocytosis or receptor-mediated endocytosis, most likely through transient pore formation in the DC membrane. Furthermore, we observed that PSMα peptides co-localized with p38 as well as phosphorylated p38 in the cytosol of DCs.

(4) PSM peptides induced a tolerogenic DC phenotype independent of the activated TLR receptor. The tolerogenic phenotyp was characterized by reduced production of the pro-inflammatory cytokines IL-12, TNF and IL-6 but increased IL-10. Moreover, the tolerogenic DCs displayed increased co-stimulatory molecule expression and an enhanced activation of T_{regs} by stimulation of extracellular as well as intracellular TLRs.

The new scientific knowledge gained in this thesis describing the ability of secreted *S. aureus* PSMs to induce tolerogenic DCs by direct modulation of the p38 MAPK contributes to basic insights into the immune evasion strategies of *S. aureus* and to the development of possible therapeutic strategies against CA-MRSA infections in the future.

Zusammenfassung

Staphylococcus aureus (*S. aureus*), ist ein bedeutender humaner Krankheitserreger, der dafür bekannt ist lokale als auch systemische Infektionen zu verursachen. Die Methicillin-resistenten *Staphylococcus aureus* (MRSA) Stämme, welche in der Lage sind auch nicht immunsupprimierte Menschen zu infizieren, tragen daher den Namen „community-associated“ (CA-MRSA). Sie sind die gefährlichsten Stämme, da sie eine Vielzahl an Virulenzfaktoren produzieren, die zum einen Antibiotikaresistenz vermitteln aber auch Immunzellen beeinträchtigen. Die phenollöslichen Modulin (PSM) Peptide sind eine Gruppe von Virulenzfaktoren, die zur Pathogenität der CA-MRSA Stämme beitragen. PSMs beeinträchtigen die Funktion verschiedener Immunzellen, beispielsweise die der Neutrophilen und der Dendritischen Zellen (DCs). DCs sind professionelle Antigen präsentierende Zellen, welche die angeborene mit der adaptiven Immunität verknüpfen. Unsere Gruppe konnte bereits zeigen, dass DCs nach Behandlung mit PSMs und TLR2 Liganden einen tolerogenen Phänotyp besitzen. Dieser ist durch eine verringerte Clathrin-vermittelte Endozytose und eine veränderte Zytokin-Sekretion, charakterisiert durch den Anstieg des anti-inflammatorischen Zytokins IL-10, gekennzeichnet. Diese DCs aktivieren vermehrt regulatorische T-Zellen wohingegen die Differenzierung zu T-Helfer 1-Zellen inhibiert ist. Die zu Grunde liegenden molekularen Mechanismen waren bisher unbekannt.

Im Rahmen dieser Arbeit wurden folgende Fragestellungen untersucht: (1) Welche Signalwege werden von PSM Peptiden reguliert, die zu einem Anstieg der IL-10 Produktion in TLR2 stimulierten DCs führen? (2) Sind die durch PSM Peptide aktivierten Signalwege in DCs verantwortlich für die Aktivierung regulatorischer T-Zellen? (3) Werden PSMs von DCs aktiv aufgenommen oder wirken diese über die Bindung an ihren Formylpeptid Rezeptor 2 (FPR2)? (4) Beeinflussen PSMs im Allgemeinen die Funktionen von DCs nach Behandlung mit verschiedenen TLR Ligaden, wie zum Beispiel die Maturation, Zytokinproduktion und T-Zellentwicklung?

(1) In dieser Arbeit konnte gezeigt werden, dass Knochenmark generierte DCs, welche mit PSMα3 und dem TLR2 Ligand *S. aureus* Zelllysat stimuliert wurden, eine erhöhte Phosphorylierung der Signalmoleküle ERK, p38, CREB und NF-κB aufweisen. Jedoch beeinträchtigte nur die Inhibition der p38 Phosphorylierung und der nachgeschalteten Kinase MSK1 konzentrationsabhängig die IL-10 Ausschüttung der DCs.

(2) Der durch PSMs modulierte p38-CREB Signalweg in TLR2 stimulierten DCs ist für eine veränderte T-Zelldifferenzierung verantwortlich. Die Hemmung dieses Signalweges unterband zum Großteil die Differenzierung zu regulatorischen T-Zellen.

(3) PSMα3 Peptide sind in der Lage unabhängig von der Bindung an ihren Rezeptor FPR2 den p38-CREB Signalweg zu modulieren. PSMα Peptide dringen unabhängig von Makropinozytose oder rezeptorvermittelter Endozytose in DCs ein, höchstwahrscheinlich durch vorübergehende Porenbildung in die Zellmembran der DCs. Des Weiteren konnten wir beobachten, dass PSMα Peptide im Zytosol der DCs mit p38 ebenso wie auch phosphoryliertem p38 kolokalisieren.

(4) Unabhängig davon, ob ein intrazellulärer oder extrazellulärer TLR Rezeptor aktiviert wird, induzieren PSMs einen tolerogenen DC Phänotyp, der einerseits durch eine verringerte Produktion der pro-inflammatorischen Zytokine IL-12, TNF und IL-6 und andererseits durch einen Anstieg der IL-10 Sekretion charakterisiert ist. Außerdem weisen diese tolerogenen DCs eine gesteigerte Expression kostimulatorischer Moleküle auf und induzieren verstärkt die Differenzierung zu regulatorischen T-Zellen.

Die neuen wissenschaftlichen Erkenntnisse, die wir im Rahmen dieser Dissertation über PSMs und deren direkte Modulierung der MAPK p38 in DCs erlangt haben, tragen zum grundlegenden Verständnis der Immunevasionsstrategien von *S. aureus* bei und in der Zukunft zur Entwicklung neuer Therapieansätze gegen CA-MRSA Infektionen.

1 General introduction

1.1 *Staphylococcus aureus*

The gram-positive spherical bacterium *Staphylococcus aureus* (*S.* aureus) belongs to the genus staphylococci, which consists of approximately 50 species and subspecies (Mathema et al., 2009). *S. aureus* is a commensal bacterium as well as a major human pathogen (Coates et al., 2014). Approximately 20% of the human population is continuously colonized, mainly in the nares but also on the skin and in the gastrointestinal tract, while 30% are colonized intermittently (van Belkum et al., 2009; Wertheim et al., 2005). However, *S. aureus* as a pathogen is a serious public health threat and causes severe diseases, ranging from skin and soft tissue infections (SSTIs) to life-threatening bloodstream infections (e.g., endocarditis or septic shock syndrome) (Fraunholz and Sinha, 2012). A hallmark of *S. aureus* is its frequent recurrence in approximately 8-33% of SSTIs or systemic infections (Kallen et al., 2010). The wide range of diseases associated with *S. aureus* is due to the variety and abundance of its virulence factors as well as its high-level antibiotic resistance (Morikawa et al., 2012). Resistance began with the incidence of penicillin-resistant *S. aureus* strains after the discovery of penicillin as a useful antibiotic for the treatment of bacterial infections. Later, methicillin was discovered; however, shortly thereafter, methicillin-resistant *Staphylococcus aureus* (MRSA) strains arose, representing a therapeutic challenge. Initially, MRSA infections occurred solely in hospital settings, especially in patients with permanent catheters, low-birth-weight infants, the elderly and in immunosuppressed individuals (David and Daum, 2010). However, in the mid-1990s, a shift to community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections was observed in the general population without these risk factors (David and Daum, 2010). CA-MRSA strains are found all over the world, but the most prominent isolate, USA300, is responsible for more than 97% of MRSA infections in the United States. USA300 is also present in Europe, but ST80 is the most prevalent strain in this region (Mediavilla et al., 2012; Moran et al., 2006). CA-

MRSA strains have epidemic characteristics and are able to cause serious infections, especially in healthy individuals (R. Wang et al., 2007). The severity and pathogenicity of these strains is due to increased virulence that has been associated with a large repertoire of immune evasion factors and cytolytic toxins, such as Panton-Valentine Leukocidin (PVL), α-Toxin and phenol-soluble modulin (PSM) peptides, which permit *S. aureus* to survive within the host or to evade the immune system (Diep et al., 2006; Foster, 2005; R. Wang et al., 2007). PVL is a pore-forming toxin consisting of the subunits LukS-PV and LukF-PV and targets leukocytes (Prévost et al., 1995). Initially, the PVL virulence factor was thought to be the driving force for CA-MRSA epidemics. However, PVL-negative strains appeared to be as virulent as PVL-positive strains, and further studies showed that the virulence differences are dependent on the experimental model (Diep et al., 2008). α-Toxin is a small β-barrel pore-forming toxin that causes lysis of different cell types, including erythrocytes, monocytes, neutrophils, T cells, keratinocytes and endothelial cells, depending on the concentration of the toxin (Bhakdi and Tranum-Jensen, 1991). The cellular receptor of α-Toxin is a disintegrin and metallopeptidase referred to as ADAM10. Binding to this receptor leads to the disruption of host cell membrane junctions (Wilke and Bubeck Wardenburg, 2010). In an *S. aureus* pneumonia mouse model, anti-α-Toxin antibodies protected mice from disease (Wardenburg and Schneewind, 2008).

1.2 **Phenol-soluble modulin peptides**

PSMs, which are secreted by all pathogenic staphylococci (Rautenberg et al., 2011; R. Wang et al., 2007), are surfactant-like peptides that were originally discovered in *Staphylococcus epidermidis* (Mehlin et al., 1999). They consist of two subgroups, the α-type and the longer β-type (R. Wang et al., 2007).

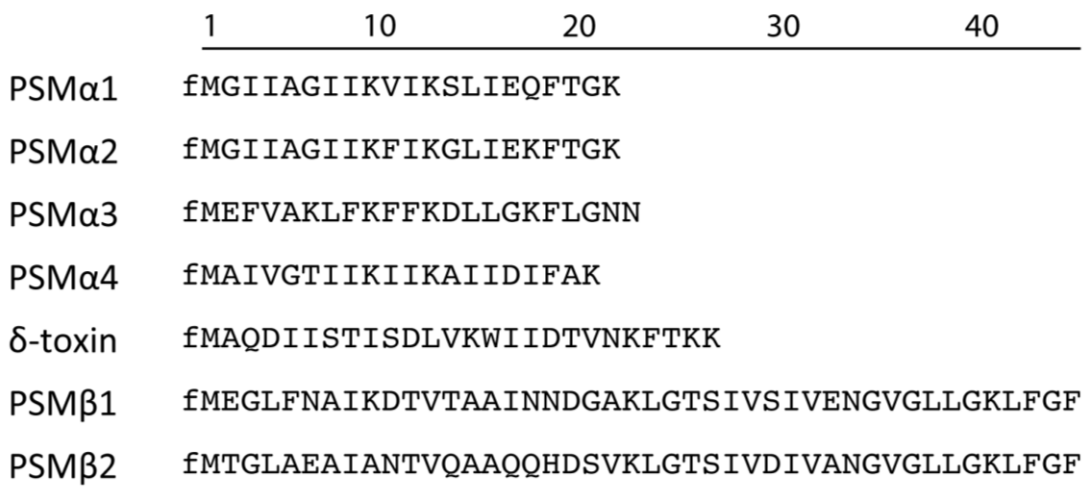

Figure 1: Amino acid sequences of phenol-soluble modulin peptides (R. Wang et al., 2007)

PSMs are α-helical amphipathic toxins that are 20 to 44 amino acids in length. PSMs carry an N-formyl methionine at their N-terminus, as they are secreted without a signal peptide. *S. aureus* secretes 4 α PSMs, 2 β PSMs and δ-toxin, which are limited in sequence similarity (Figure 1). α PSMs and δ-toxin are considered the most toxic PSMs (L. D. Wang and Wagers, 2011). PSM-mec, is similar to the α-type PSMs; however in contrast to all other PSMs, PSM-mec is located on the mobile genetic element SCC*mec*, not the chromosome (Qin et al., 2016). In *S. aureus*, more than 50% of the protein mass secreted into the media is PSMs. PSMs are exported by the ATP-binding cassette transporter Pmt, which is essential for bacterial growth and plays a key role in virulence phenotypes (Chatterjee et al., 2013). The PSM genes are organized into subgroup specific clusters in the core genome. In CA-MRSA, PSM expression is elevated facilitating bacterial spread and increased severity of infection compared to less virulent hospital-associated MRSA strains (R. Wang et al., 2007). PSMs are regulated by the quorum-sensing accessory gene regulator (Agr) system, especially by the response regulator protein AgrA. Agr ensures that PSMs are only expressed when a high cell density is present (Queck et al., 2008). PSMs have multiple roles in *S. aureus.* Originally PSMs were identified as having a role in the colonization of epithelial surfaces (Periasamy et al., 2012). According to the National Institutes of Health in the United States, 80% of microbial infections, including *S. aureus* infections, are directly connected

with biofilm formation (Römling and Balsalobre, 2012). On one hand, PSMs disrupt cell-cell connections between bacterial molecules, leading to biofilm dispersal and dissemination to secondary infection sites (Otto, 2013). Conversely, PSMα1 and PSMα4 are the main drivers of biofilm formation in *S. aureus* and mediate resistance to biofilm matrix degrading enzymes (e.g., DNAses and proteases). PSMα1 and PSMα4 are able to form extracellular fibril structures, but they show less cytotoxicity in comparison to PSMα2, PSMα3 and δ-toxin (Marinelli et al., 2016; Schwartz et al., 2012). The cytolytic activity of PSMs is dependent on the hydrophobicity of the α-helix, while the hydrophobic residues are responsible for PSM aggregation and thus for their amyloid potential (Marinelli et al., 2016). The target receptor of PSMs is the formyl-peptide receptor 2 (FPR2) expressed on human and mouse neutrophils, dendritic cells and microglial cells (Kretschmer et al., 2010; Migeotte et al., 2006; Schreiner et al., 2013). Binding to the FPR2 receptor leads to a massive influx of human neutrophils into the infection site and the release of a specific cytokine repertoire (Kretschmer et al., 2010; R. Wang et al., 2007). Furthermore, PSMs can bind to the FPR1 receptor with lower affinity (Kretschmer et al., 2010). In addition, proteolytically processed products of PSMα1 and PSMα2 display antimicrobial activity against *Streptococcus pyogenes*, contributing to competition against colonizing pathogens (Joo et al., 2011). Moreover, PSMs demonstrate receptor-independent lysis as determined by their capacity to lyse artificial phospholipid vesicles (Chatterjee et al., 2013).

1.3 **Dendritic cells**

Dendritic cells (DCs), discovered in 1973 by Ralph Steinman and Zanvil Cohn (Steinman and Cohn, 1973), are central coordinators of the immune system. This cell type links innate and adaptive immunity. In addition to monocytes, macrophages and B-lymphocytes, DCs are the most potent and efficient specialized antigen-presenting cells (APCs) that control the immune response by regulating the equilibrium between protective immunity to pathogens and self-tolerance (Volkmann et al., 1997). DCs acquire, process and present antigens to T cells and have the unique ability to prime naïve T cell immune responses. After antigen uptake via macropinocytosis, receptor-mediated

endocytosis or phagocytosis, DCs migrate from the peripheral tissues to lymphoid organs and phenotypically mature, which is characterized by the up-regulation of major histocompatibility complexes (MHCs), co-stimulatory molecules and adhesins. In the lymphoid tissues, DCs present peptide-MHC complexes in combination with co-stimulatory molecules to antigen-specific lymphocytes and produce inflammatory cytokines to initiate immune responses against various pathogens. However, DCs also play a major role in inducing and maintaining immune tolerance against self-antigens (Banchereau et al., 2000; Banchereau and Steinman, 1998). Mature DCs can induce tolerance by priming naïve T cells into regulatory T cells (T_{regs}), which are characterized by the secretion of the anti-inflammatory cytokine interleukin (IL)-10. The maturation of tolerogenic DCs is often caused by pathogens in a disease-related background to prime T_{regs} instead of effector T cells (Maldonado and Andrian, 2010). Various pathogen-associated molecular patterns (PAMPs) are expressed by pathogens, and cells of the immune system sense these PAMPs using pattern recognition receptors (PRRs) (Saraiva and O'Garra, 2010). The most well-known PRRs are the Toll-like receptors (TLRs) (Merad et al., 2013).

DCs are a heterogeneous cell population in regards to their phenotypes and functions and can therefore be classified into different subsets depending on their location and distinct marker expression. There are two main DC populations that arise from the same hematopoietic lineage: Steinman's DCs, which have been renamed classical DCs and plasmacytoid DCs. These lineages differ in their morphology, phenotype and function (Colonna et al., 2004; Shortman and Liu, 2002). Furthermore, there are differences in surface marker expression of the various DC subgroups in the mouse and in humans, differences in their location in lymphoid versus non-lymphoid organs and differences in function. In the mouse, classical DCs can be further divided into cluster of differentiation (CD)103^+CD11b^- and $CD11b^+$ migratory DCs in the non-lymphoid tissues and resident DCs in the lymphoid tissues. For example, in the spleen and lymph nodes, $CD11b^+CD8\alpha^-$ and $CD11b^-CD8\alpha^+$ DCs are distinct (Guilliams et al., 2010), and both subsets are able to sense pathogens. In the steady state, $CD11b^+CD8\alpha^-$ DCs are more efficient at presenting MHC class II peptide complexes to $CD4^+$ T cells than $CD11b^-CD8\alpha^+$ DCs, which have an

enhanced capacity to cross-present antigens on MHC class I molecules to $CD8^+$ cytotoxic T cells (Dudziak et al., 2007; Haan et al., 2000). This is also the case in an inflammatory setting, where $CD11b^-CD8\alpha^+$ DCs play a key role during influenza virus infection, and $CD11b^+CD8\alpha^-$ DCs play a key role during infection with the parasite *Plasmodium* (T. S. Kim and Braciale, 2009; Lundie et al., 2008). TLR expression differs between the DC subsets and depending on the species under study, 11-13 different TLRs have been identified. Various PAMPs can be detected by different TLRs (Figure 2).

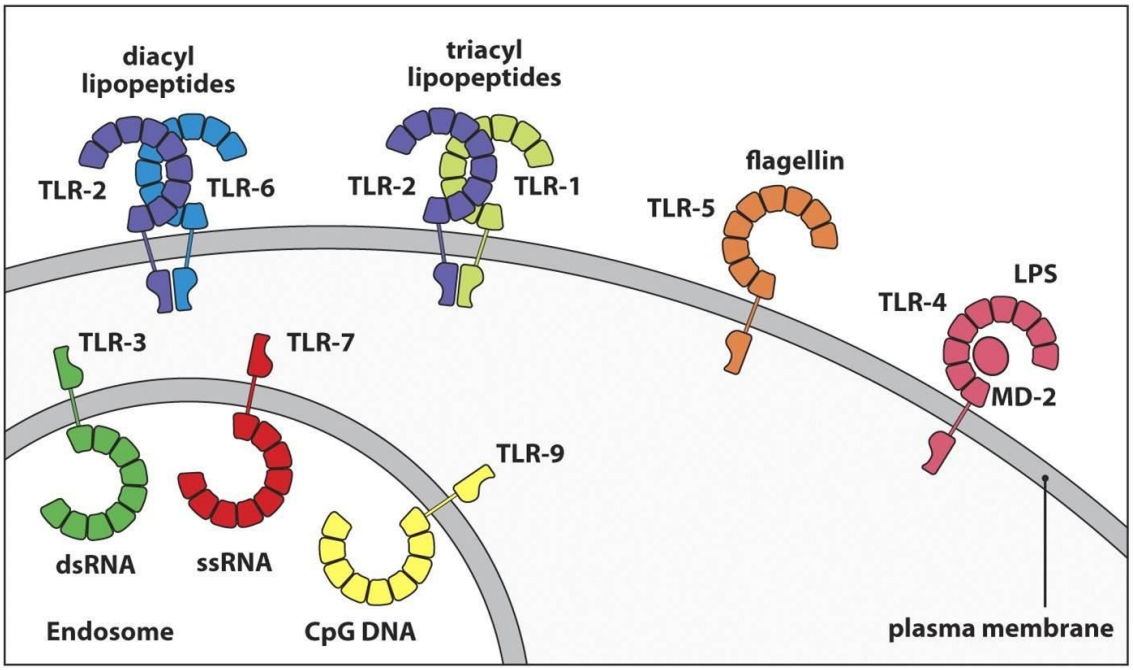

Figure 2: Toll-like receptors and their ligands (Murphy and Weaver, 2016)

TLR2 recognizes lipopeptides together with TLR1 or TLR6 by forming a heterodimer. Lipopolysaccharides (LPS) are TLR4 ligands, single strain RNAs or imiquimod are recognized by TLR7 or TLR8, and unmethylated CpG DNA is a TLR9 ligand. Some TLRs are expressed on the cell surface to detect bacterial components, whereas others are expressed in intracellular endosomes and detect viral products. TLR stimulation leads to the activation of different transcription factors that initiate the transcription of type 1 interferons and pro-inflammatory cytokines (Kawai and Akira, 2006). Furthermore, in DCs and macrophages, TLR2 signaling can result in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the mitogen- and

stress-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase (ERK) 1 and 2. These factors are able to initiate the transcription of the anti-inflammatory cytokine IL-10 via cAMP response element binding protein (CREB) and activator protein 1 (AP-1) (Saraiva and O'Garra, 2010).

1.4 **T cells**

T cells are lymphocytes that mature in the thymus, and each T cell expresses a unique T cell receptor (TCR) on its cell surface. T cells play a key role in immunity against viral, bacterial and fungal infections but are also important for the control of malignant cells. Based on their glycoprotein expression type, T cells can be divided into $CD4^+$ T helper (T_h) cells or $CD8^+$ cytotoxic T cells. Antigen-presenting cells, such as DCs, present pathogenic antigens to naïve T cells that become activated, differentiate into effector cells and migrate to the infection site to clear the pathogen. Naïve $CD4^+$ T cells differentiate into effector T_h1, T_h2, T_h17, T_{regs} (Figure 3) or follicular T helper cells depending on different cytokines (Bettelli et al., 2006; Mosmann and Coffman, 1989; Nakae et al., 2007).

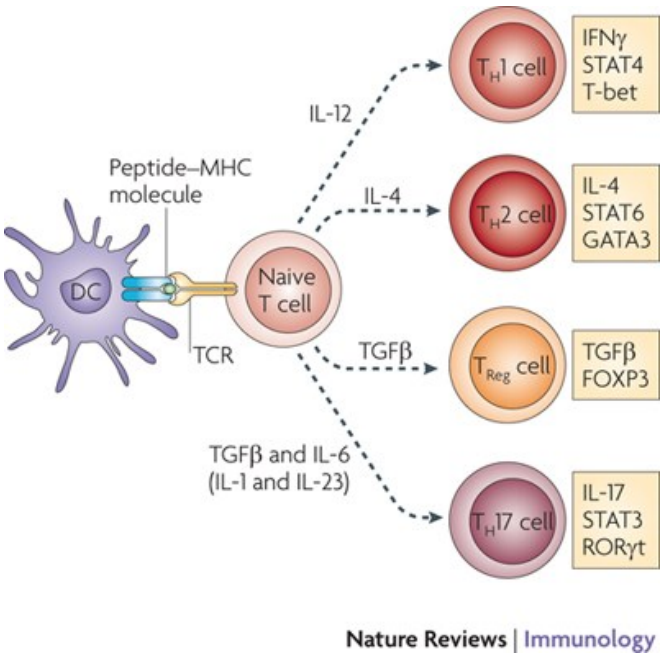

Figure 3: Differentiated effector T cell subsets with their associated transcription factors and cytokine profiles (Zou and Restifo, 2010)

Each subset is characterized by the production of specific pro- or anti-inflammatory cytokines that play critical roles in the differentiation of immune cells or directing effector responses. Naïve $CD8^+$ T cells differentiate into cytotoxic T lymphocytes that proliferate in the presence of IL-2 to expand their numbers for effective target cell killing. Naïve T cells can differentiate into short-lived effector cells or long-lived memory cells (Weaver et al., 2006).

T_{regs} have several functions, including the maintenance of immunological tolerance in the periphery, down-regulation or suppression of other immune responses (e.g., against pathogens) or prevention of autoimmune diseases. T_{regs} are characterized by expression of the transcription factor forkhead box p3 (Foxp3) and the interleukin-2 receptor CD25 (Sakaguchi et al., 1995) and are divided into natural T_{regs} derived from the thymus and induced T_{regs} that develop from $Foxp3^-CD4^+$ T cells in the periphery (Bluestone and Abbas, 2003). T_{regs} secrete the immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β) to prevent the production of inflammatory cytokines or the proliferation of effector T cells and to induce cell-cell mediated immunosuppression (McGuirk et al., 2002; Nakamura et al., 2001). It is important to consider that T_{regs} can be beneficial to the host by preventing infection-induced immunopathologies but can also benefit the pathogen through the suppression of protective T_h1 responses, leading to evasion of the host immune response (Maloy et al., 2003; Suvas et al., 2004).

1.5 **Dendritic cells and *Staphylococcus aureus***

DCs play an important role in host defense against various pathogens and are therefore required in the fight against *S. aureus* infections. However, depending on the experimental model, including host organism and *S. aureus* strain, various DC subsets are responsible for different immune reactions. Mice infected intravenously with *S. aureus* display a fast recruitment of functional DCs to the infection site. However, DC-depleted mice show a higher bacterial load in the lungs and kidneys, higher mortality, increased inflammation and inhibited IL-12 secretion, which can be recovered by the application of exogenous recombinant IL-12 (Queck et al., 2008; Schindler et al., 2012).

Additionally, DC-depleted mice infected intranasally with *S. aureus* USA300 have an increased bacterial load in the lungs (Martin et al., 2011; Periasamy et al., 2012). Stimulation of human and mouse DCs and Langerhans cells with the *S. aureus* strain Cowan I results in increased production of the pro-inflammatory cytokine IL-12. Furthermore, epidermal DCs reduce their CCL7 production, which is partially responsible for the T_h2-oriented immune response (Heufler et al., 1996; Mitsui et al., 2003; Römling and Balsalobre, 2012). Furthermore, some virulence factors of *S. aureus* initiate a T_h1 immune reaction. In bone marrow-derived DCs (BM-DCs), the leukocidin LukF induces the secretion of tumor necrosis factor-alpha (TNF-α) and IL-12 (Inden et al., 2009; Otto, 2013). Monocyte-derived DCs stimulated with the TLR2 ligand peptidoglycan, a component of the *S. aureus* cell wall*,* demonstrate a pro-inflammatory immune response through the production of IL-12 and IL-23, thereby activating a T_h1/T_h17 response (Frodermann et al., 2011; Marinelli et al., 2016; Schwartz et al., 2012). Lipoteichoic acid and muramyl dipeptide stimulate TNF-α and IL-12 production in mature human DCs (H. J. Kim et al., 2007; Marinelli et al., 2016). Hence, DCs play a unique and key role in the immune responses against *S. aureus* by inducing T_h1 effector cells, likely through the production of the pro-inflammatory cytokine IL-12.

1.6 **Influence of phenol-soluble modulins on immune cells**

PSMs manipulate various immune cell responses to *S. aureus*. The α-helical amphipathic structure enables their cell lytic capacity, likely through cell membrane disruption. PSMα3 attracts and activates human neutrophils at nanomolar concentrations by binding to FPR2, whereas, at micromolar concentrations, PSMs lyse neutrophils in a receptor-independent manner and induce innate immune cell killing. CA-MRSA circumvents neutrophil-mediated killing after phagocytosis, thereby evading the innate immune response through the up-regulation of virulence factor genes, genes encoding capsule synthesis components and oxidative stress. This capability is a central feature of the highly virulent CA-MRSA pathogenicity (Kretschmer et al., 2010; Voyich et al., 2005; R. Wang et al., 2007). Confirming the *in vitro* studies that PSMs contribute to leukocyte killing, a mouse peritonitis model showed that α PSMs

are responsible for increased chemotaxis and the downstream killing of neutrophils and monocytes (R. Wang et al., 2007). In addition, PSMs are able to lyse human erythrocytes, which play an important role in infection progression. PSMα1-3 mediate direct lysis, whereas PSMβ peptides need to synergize with α PSMs to cause hemolysis (Cheung et al., 2012). Another immune cell type influenced by PSMs is osteoblasts. For this cell type, α PSMs act as intracellular toxins; they enter the osteoblasts and kill the cells, leading to extensive bone damage (Rasigade et al., 2013). Moreover, BM-DCs display an impaired cytokine secretion profile after treatment with PSMs. Combined stimulation of TLR2 is characterized by reduced production of the pro-inflammatory cytokines TNF, IL-6 and IL-12 but increased anti-inflammatory IL-10 secretion *in vitro*. In addition, clathrin-mediated endocytosis is impaired in these DCs, and their T cell priming properties towards T_h1 are inhibited. In contrast, the higher production of IL-10 by PSM-treated DCs increases the priming of T_{regs} (Schreiner et al., 2013).

1.7 **Aims of the thesis**

The major human pathogen *S. aureus* has become an increasing clinical challenge, especially due to the prevalence of multi-drug resistant strains. These highly pathogenic CA-MRSA strains cause severe diseases and produce a variety of virulence factors to efficiently subvert the host immune response. PSM peptides comprise one group of these factors that are likely responsible for the virulence properties of these strains. PSMs modulate different innate immune cells, including DCs. In general, DCs sense pathogens with their PRRs and therefore play a protective role in the host by activating the immune system to clear the *S. aureus* infection. However, PSMs affect the endocytic capacity and cytokine secretion profile of DCs, which is characterized by increased production of the anti-inflammatory cytokine IL-10 but reduced TNF, IL-12 and IL-6 pro-inflammatory cytokine secretion. Furthermore, PSMs alter the priming of naïve T cells by DCs towards the direction of T_{regs}. However, the underlying molecular mechanisms of PSM-mediated DC modulation remain poorly understood. To further investigate the mode of action of PSMs, this thesis addressed the following questions:

(1) Which signaling pathways are modulated by PSMs resulting in increased production of IL-10 by DCs upon TLR2 ligand treatment?
(2) Are the activated signaling pathways involved in the priming of T_{regs} by PSM-treated DCs?
(3) Are PSMs actively internalized by DCs or do they act through binding to their receptor FPR2?
(4) Do PSMs in general affect DC functions, including maturation, cytokine production and T cell priming, upon treatment with various TLR ligands?

To address these questions, BM-DCs were generated, and the phosphorylation of different signaling molecules was determined. A variety of inhibitors was tested for their ability to reduce the IL-10 production induced in DCs upon PSM treatment and to reverse the increased priming of T_{regs}. Furthermore, BM-DCs from wild-type and $FPR2^{-/-}$ mice were treated with fluorescently labeled PSMs to

investigate the possible mode of action of PSMs in DCs by multispectral imaging flow cytometry. Finally, we analyzed co-stimulatory and adhesion molecule expression, cytokine production, antigen uptake and T cell priming of BM-DCs treated with PSMs in combination with various TLR ligands to address the generality of PSMs to modulate TLR-treated DCs.

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1.9 List of figures

2 PSM Peptides of *Staphylococcus aureus* Activate the p38-CREB Pathway in Dendritic Cells, Thereby Modulating Cytokine Production and T cell Priming

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Running title: PSMs modulate p38-CREB-IL-10 axis

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[3]Abbreviations used in this article: CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; PSM, phenol-soluble modulin; DC, dendritic cells; BM-DCs, bone marrow-derived DCs; HA, hospital-associated; FPR, formyl peptide receptor; T_{regs}, regulatory T cells; PFT, pore forming toxin.

Abstract

The challenging human pathogen *Staphylococcus aureus* has very efficient immune evasion strategies causing a wide range of diseases from skin and soft tissue to life-threatening infections. Phenol-soluble modulin (PSM) peptides are major pathogenicity factors of community-associated methicillin-resistant *S. aureus* strains. In previous work, we demonstrated that PSMs in combination with TLR2 ligand from *S. aureus* induce tolerogenic dendritic cells (DCs) characterized by the production of high amounts of IL-10, but no pro-inflammatory cytokines. This in turn promotes the activation of regulatory T cells while impairing Th1 response. However, the signaling pathways modulated by PSMs remain elusive. Here, we analyzed the impact of PSMs on signaling pathway modulation downstream of TLR2. TLR2 stimulation in combination with PSMα3 lead to increased and prolonged phosphorylation of NF-κB, ERK, p38 and CREB in mouse bone marrow-derived DCs compared to single TLR2 activation. Furthermore, inhibition of p38 and downstream MSK1 prevented IL-10 production, which in turn reduced the capacity of DCs to activate regulatory T cells. Interestingly, the modulation of the signaling pathways by PSMs was independent of the known receptor for PSMs as shown by experiments with DCs lacking the formyl peptide receptor 2. Instead, PSMs penetrate the cell membrane most likely by transient pore formation. Moreover, co-localization of PSMs and p38 was observed near the plasma membrane in the cytosol, indicating a direct interaction. Thus, PSMs from *S. aureus* directly modulate the signaling pathway p38-CREB in DCs thereby impairing cytokine production and in consequence T-cell priming to increase the tolerance towards the pathogen.

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is an opportunistic pathogen that can causes soft-tissue and systemic infections. Twenty percent of the population is permanently colonized with *S. aureus* and antibiotic treatment is often ineffective because the strains develop resistance. Methicillin-resistant

S. aureus (MRSA) occurs frequently in hospital-associated (HA) infections as well as in community-associated (CA) diseases (1, 2). CA-MRSA strains cause mainly skin and soft tissue infections in healthy individuals with USA 300 as the most prominent strain worldwide. CA-MRSA strains express a wide range of virulence factors consisting of Panton-Valentine Leukocidin, α-toxin and phenol-soluble modulin (PSM) peptide toxins (1, 2). PSM peptides are secreted by CA-MRSA strains in much higher concentrations than by HA-MRSA strains and are essential virulence factors in mouse models of sepsis and soft tissue infection (3).

PSM peptides comprise seven different members, all arranged into an amphipathic α-helix. These include five α-peptides (PSMα1-4 and δ-toxin) with 20-25 amino acids length and two β-peptides (PSMβ1-2) with 44 amino acids (3, 4). PSM peptides can affect the generation of bacterial biofilms because of their physical and chemical characteristics and their detergent activities (4). They can attract and activate human neutrophils at nanomolar concentrations; whereas at micromolar concentrations, they induce neutrophil lysis with their ability to form transient pores (3, 5). Nanomolar concentrations of PSM peptides are recognized by the human formyl peptide receptor 2 (FPR2) on neutrophils. Furthermore, this receptor recognizes the pathogenicity status of bacteria and adapts the immune reaction (6). There is a substantial body of research on the impact of PSM peptides on innate immune cells; however, little is known about cells of the adaptive immune system.

Dendritic cells (DCs) are the most important antigen-presenting cells that trigger immune responses. They link the innate and adaptive immune system by their ability to recognize pathogens and to activate B and T cells (7). Moreover DCs are mediators of anti-inflammatory immune responses inducing tolerance (8). Mouse DCs express mFPR2 and are also attracted by PSM peptides like neutrophils, although higher concentrations are needed (9). In contrast to neutrophils, DCs are not lysed by micromolar PSM concentrations (9). Their phenotype changes by PSM peptide treatment showing decreased endocytosis and increased TLR2 ligand-induced secretion of IL-10 whereas TNF, IL-12 and IL-6 secretion is abrogated. Consequently, DCs treated with PSM peptides

demonstrate a decreased priming ability for T helper 1 cells but enhanced induction of $FoxP3^+$ regulatory T cells (T_{regs}). However, the tolerogenic phenotype of DCs caused by PSM peptides is mFPR2 independent (9). A possible explanation is the ability of PSM peptides to generate transient pores into the cell membrane (5), thereby enabling access to the cytosol. However, the signaling pathway involved in the induction of tolerogenic DCs remains elusive.

DCs detect pathogens via microbial products by pattern recognition receptors (10), which include e.g. Toll-like receptors (TLRs) and NOD-like receptors (11, 12). TLRs are important regulators of the immune response as they initiate the production of different cytokines and chemokines (13). In myeloid DCs, TLR2 agonists induce the expression of anti-inflammatory IL-10 via activation of nuclear factor-κB (NF-κB), p38 and extracellular signal-regulated kinases (ERKs) (10).

Here we show that TLR2 ligand-stimulation of DCs in combination with PSMα3 induces the phosphorylation of p38-cAMP response element binding-protein (CREB) pathway, independently of FPR2. Inhibition of the p38-CREB pathway reduced IL-10 secretion and induction of T_{regs} by DCs. Furthermore, our results point towards a specific interaction of cytosolic PSMs with p38 thereby potentially increasing the tolerance towards the pathogen.

Materials and Methods

Mice

Female C57BL/6JolaHsd mice were purchased from Janvier (St. Berthevin Cedex, France). $FPR2^{-/-}$ mice (14) with a genetic C57BL/6 background were bred in the animal facilities of the University Clinic of Tübingen. All mice were held under specific pathogen-free conditions, were provided food and water ad libitum and were used for experiments between 6-12 weeks of age. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations

(FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Anzeige 09.01.2014).

Generation of bone marrow-derived DCs (BM-DCs)

RPMI-1640 medium (Merck) supplemented with 10% fetal calf serum (FBS; Sigma-Adrich), 2 mM glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol (Roth), 1mM sodium pyruvate (Merck) and 1x non essential amino acids (Merck) was used in all cell culture experiments. BM-DCs were prepared using granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described (9, 15, 16). Briefly, 2 x 10^6 bone marrow cells, flushed from the femurs and tibias of C57BL/6 and FPR2$^{-/-}$ mice, were seeded in 100 mm dishes in 10 ml medium containing 200 U/ml GM-CSF. After 3 days, an additional 10 ml of fresh medium containing 200 U/ml GM-CSF was added to the cultures. On day 6 half of the culture supernatant was replaced by fresh medium containing GM-CSF. At day 7-8, the slightly attached cells were used for the experiments described in this report.

Reagents

Formylated PSMα3 and δ-toxin peptides with the recently published sequence (Wang et al. 2007) and Fluorescein isothiocyanate (FITC) labeled PSMα2 and PSMα3 and $OVA_{323-339}$ were synthesized in house. BM-DCs were treated with *S. aureus* cell lysates specifically activating TLR2 (9). Treatment of BM-DCs with 3 µg/ml *S. aureus* cell lysates was done simultaneously in combination with PSMα3 peptide (10 µM). Where indicated, BM-DCs were pretreated with the following inhibitors in different concentrations for 1h: BAY11-7082 (NF-κB inhibitor, Selleckchem), PD98059 (p-ERK inhibitor, Merck), SB203580 (p-p38 MAPK inhibitor, Merck) and Gö6976 (MSK1 inhibitor, Cell Signaling).

Cytokine production by BM-DCs

BM-DCs (2,5 x 10^5) were seeded in 96-well plates, pretreated with inhibitors and following incubated with *S. aureus* cell lysate and PSM peptides. Supernatants were collected after 24 h for IL-10 (BD Biosciences) and ELISAs were performed according to the manufacturer's instructions.

Flow cytometry

5 x 10^5 BM-DCs were seeded in 48-well plates and treated as described above. Cells were removed from the plate using Accutase (Sigma-Aldrich) and stained with 7-AAD (Biomol) or Aqua Life/Dead (Invitrogen) according to the manufacturer's instructions to exclude dead cells. Cells were stained for 20 min at 4°C with extracellular antibodies against CD11c-PE (N418) (eBioscience) and MHC class II-FITC (M5/114.15.2) (Miltenyi). For p-CREB staining cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioscience) and stained with primary antibody phospho-CREB mAb (Ser133; clone 87G3) (Cell Signaling) for 30 min in the dark at room temperature followed by secondary goat anti-rabbit IgG-DyLight™649 (Jackson ImmunoResearch) for 15 min at 4°C. To detect intracellular p-ERK, p-p38 and p-NF-κB BM-DCs were fixed with 2% paraformaldehyde (VWR) in PBS, permeabilized with 90% freezing methanol (Applichem) and stained with the primary antibodies to phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; clone 197G2), phospho-p38 MAPK (Thr180/Tyr182; clone 12F8) and phospho-NF-κB p65 (93H1) (all from Cell Signaling) for 60 min in the dark at room temperature followed by goat anti-rabbit IgG-PE-Cy7 (Santa Cruz Biotechnology) for 15 min at 4°C. PBS with 0,5% bovine serum albumin (Biomol) was used for all incubations and washing steps. At least 50,000 cells were acquired using a Canto-II or LSRFortessa flow cytometer (BD Biosciences) with DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.0.7r2 software (Tree Star).

Multispectral imaging flow cytometry (MIFC)

1 x 10^6 BM-DCs were seeded in 1.5 ml Eppendorf tubes and stimulated for different times with FITC-labeled PSMα2 and PSMα3 (0.5 µM) alone or in combination with OVA-Alexa647 (0.5 µM). Cells were washed 3 times and stained with Zombie NIR (Biolegend) according to the manufacturer's protocol to exclude dead cells. Cells were stained with CD11c-PE (N418, eBioscience) and MHC class II-eFluor 450 (M5/114.15.2, eBioscience) for 15 min at 4°C. Then cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience). For localization analysis cells were incubated with a primary antibody against p38 (clone 27, BD Biosciences) or phospho-p38 (T180/Y182 12F8 Rabbit mAb Lot #9, Cell Signaling) for 30 min at room temperature. 5% NGS in PBS was used to block unspecific binding sites. Goat anti-mouse IgG-DyLight594 (Abcam) or Goat anti-rabbit IgG-PE/Cy7 (Santa Cruz Biotechnology) were used for 15 min at 4°C as secondary antibodies to stain p38 and phospho-p38, respectively. Images of up to 100,000 BM-DCs were then acquired with multispectral imaging flow cytometry (MIFC) using the ImageStreamx mkII with the INSPIRE instrument controller software. The data were analyzed using the IDEAS analysis software (Amnis, EMD Millipore), which allows an objective and unbiased analysis of thousands of images per sample on the single cell level. The same range of pixel intensity was set for all samples within an experiment and all samples were gated on CD11c$^+$MHC II$^+$ cells as shown in Fig S4B.

T-cell assay

5 x 10^4 BM-DCs were seeded in 96-well U-bottom plates and treated as described above. Splenic CD4$^+$ T cells from C57BL/6 mice were purified using CD4$^+$ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. 2 x 10^5 CD4$^+$ T cells were added to the BM-DCs and cultured in RPMI-1640 medium (Merck) supplemented with 20% fetal calf serum (FBS; Sigma-Adrich), 2 mM glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol (Roth), 1mM sodium pyruvate (Merck), 10

mM HEPES-Buffer (Biochrom AG) and 1x non essential amino acids (Merck). 96 h later T cells were stained first with Zombie NIR (Biolegend) according to the manufacturer's protocol to exclude dead cells followed by CD4-BrilliantViolet510 (V4), CD3e-PerCP/Cy5.5 (BM10-37), CD25-PE-Cy7 (B6.1) and Foxp3-APC (FJK-16s) (Biolegend) (Foxp3 staining buffer set, eBioscience). 100,000 cells were acquired using LSRFortessa flow cytometer (BD Biosciences) with DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.0.7r2 software (Tree Star).

LDH release assay

2 x 10^5 BM-DCs were seeded in 96-well U-bottom plates and treated with 10 µM PSMα2, PSMα3, δ-toxin or OVA for 10 min. Lactate dehydrogenase (LDH) was analyzed in the supernatant using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's protocol. Absorbance was measured at 490 nm using an ELISA reader.

Statistical analysis

Statistical analysis was performed with the GraphPadPrism 6 software (GraphPad, San Diego, CA) using one-way ANOVA with Bonferroni posttest. The differences were considered as statistically significant if $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

Results

PSMs induce a sustained NF-κB p65 phosphorylation in TLR2-stimulated DCs

It has been shown that TLR2-stimulated DCs co-incubated with PSMα-peptides increased the production of the anti-inflammatory cytokine IL-10 (9). However, the intracellular signaling pathways involved in the PSM-induced cytokine modulation have not been elucidated. It was previously shown that the TLR-dependent activation of the NF-κB subunit p65 leads to an extended and enhanced *IL-10* transcription in DCs (17). To investigate whether NF-κB signaling plays a role in the cytokine modulation by PSMs, bone marrow-derived (BM-) DCs (referred to as DCs) were treated with synthetic PSMα3, the TLR2 ligand *S. aureus* cell lysate or the combination of both. We have previously shown that *S. aureus* cell lysate specifically activates TLR2 and no other PRRs (9). Phosphorylation of NF-κB p65 (p-NF-κB) was analyzed by flow cytometry.

The level of p-NF-κB in DCs was slightly increased after 60 min of treatment with PSMα3 and 1.5 fold with *S. aureus* cell lysate compared to untreated DCs (Fig 1). DCs treated with *S. aureus* cell lysate and PSMα3 revealed a significant 2-fold increase of p-NF-κB (Fig 1). Moreover, the increased NF-κB phosphorylation was prolonged over a time period from 30 to 240 min (Fig S1A). Similar results were observed for mFPR2 deficient DCs in comparison to DCs from WT mice (Fig 1B), demonstrating an mFPR2-indepentent effect. These data show a cooperating effect of PSMα3 and the TLR2 ligand *S. aureus* leading to enhanced and prolonged NF-κB activation in DCs.

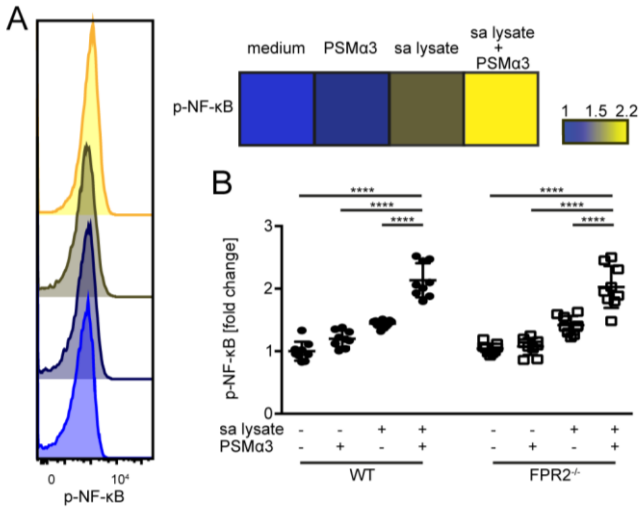

FIGURE 1. PSMs induce a sustained NF-κB p65 phosphorylation in TLR2-stimulated DCs. BM-DCs from wildtype and FPR2$^{-/-}$ mice were incubated for 60 min with *S. aureus* cell lysate (sa lysate), PSMα3 and in combination. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-NF-κB and analysis by flow cytometry. (A) Representative histogram overlays of p-NF-κB in DCs (gated on CD11c^{+}MHC II^{+} cells). The heatmap shows fold change (FC) of p-NF-κB normalized to untreated DCs (medium). Heatmap and histogram overlays are colored according to FC of phosphorylation. (B) Statistical analysis of p-NF-κB staining shown in (A). Graph shows three independent experiments performed in triplicates (mean ± SD). * indicates statistically significant differences (one-way ANOVA with Bonferroni post-test).

Enhanced MAPK phosphorylation in DCs induced by PSMs and TLR2

The virulence factor β hemolysin/cytolysin of *Group B streptococcus* was shown to induce IL-10 secretion in macrophages by activating p38 MAPK (18). Furthermore, TLR2 stimulation of DCs leads to phosphorylation of ERK1/2, which induces IL-10 production (19). To investigate whether the MAPKs play a role in the cytokine modulation by PSMs, we stimulated DCs as described above and analyzed phopho-p44/42 MAPK (p-ERK1/2) or phospho-p38 MAPK (p-p38) by flow cytometry. Treatment of DCs with PSMα3 did not affect p-ERK, whereas a strong increase for *S. aureus* cell lysate was observed 15 min post-

treatment compared to untreated DCs (Fig 2). DCs incubated with *S. aureus* cell lysate combined with PSMα3 revealed a 2.2 fold increase of ERK phosphorylation (Fig 2). Similar results were observed for mFPR2 deficient DCs (Fig 2B). These results were only visible shortly after treatment, as no increased p-ERK could be detected 30 min post-stimulation (Fig S1B), indicating a strong but short activation of the ERK pathway.

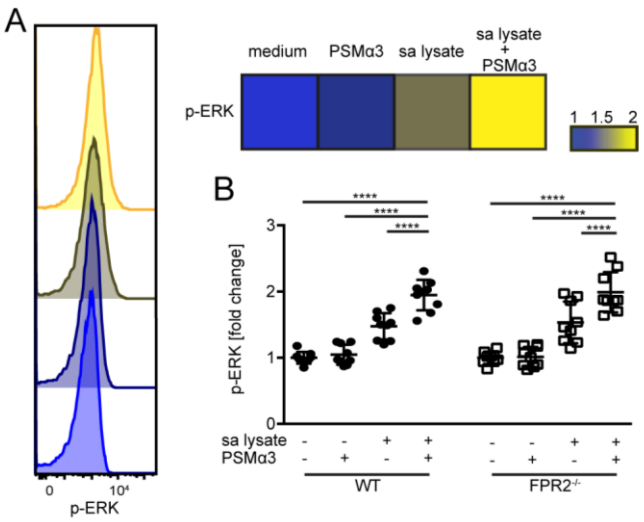

FIGURE 2. Enhanced ERK phosphorylation in DCs induced by PSMs and TLR2. BM-DCs from wildtype and FPR2$^{-/-}$ mice were incubated for 15 min with *S. aureus* cell lysate (sa lysate), PSMα3 and in combination. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-ERK and analysis by flow cytometry. (A) Representative histogram overlays of p-ERK in DCs (gated on CD11c^{+}MHC II^{+} cells). The heatmap shows FC of p-ERK normalized to untreated DCs (medium). Heatmap and histogram overlays are colored according to FC of phosphorylation. (B) Statistical analysis of p-ERK staining shown in (A). Graph shows three independent experiments performed in triplicates (mean ± SD). * indicate statistically significant differences (one-way ANOVA with Bonferroni post-test).

No significant change in the phosphorylation of p38 was observed in DCs treated with PSMα3 compared to untreated cells over time, whereas in DCs treated with *S. aureus* cell lysate phosphorylation of p38 was increased by 1.5 fold starting 30 min post treatment (Fig 3 and Fig S1C). DCs incubated with *S. aureus* cell lysate and PSMα3 for 30 min revealed a significant 2.5 fold increase

of p38 phosphorylation, which was independent of mFPR2 (Fig 3). The data likewise show a cooperating effect of PSMα3 and the TLR2 ligand *S. aureus* cell lysate for the activation of MAPKs in DCs independently of mFPR2.

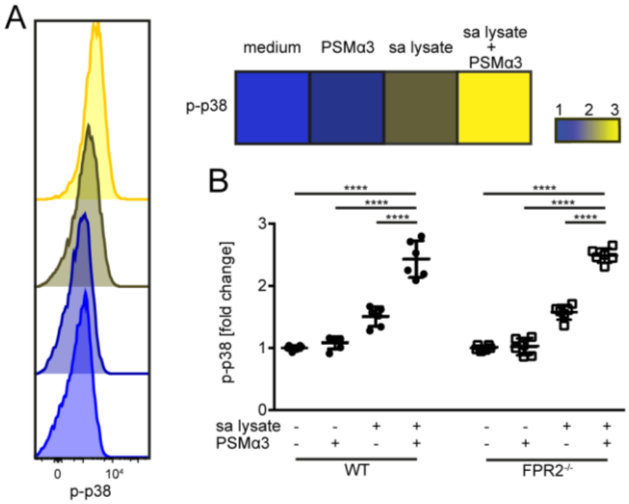

FIGURE 3. Enhanced p38 phosphorylation in DCs induced by PSMs and TLR2. BM-DCs from wildtype and FPR2$^{-/-}$ mice were incubated for 30 min with *S. aureus* cell lysate (sa lysate), PSMα3 and in combination. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-p38 and analysis by flow cytometry. (A) Representative histogram overlays of p-p38 in DCs (gated on CD11c^{+}MHC II^{+} cells). The heatmap shows FC of p-p38 normalized to untreated DCs (medium). Heatmap and histogram overlays are colored according to FC of phosphorylation. (B) Statistical analysis of p-p38 staining shown in (A). Graph shows two independent experiments out of three performed in triplicates (mean ± SD). * indicates statistically significant differences (one-way ANOVA with Bonferroni post-test).

Enhanced CREB phosphorylation in DCs induced by PSMs and TLR2

It was shown that in macrophages and myeloid DCs p38 and ERK activate MSK1/2 that directly phosphorylate CREB, which eventually binds to the IL-10 promoter (8, 20). To investigate whether CREB signaling plays a role in the cytokine modulation by PSMs, DCs were stimulated with PSMα3, *S. aureus* cell lysate and in combination and CREB phosphorylation (p-CREB) was analyzed

by flow cytometry (Fig 4). Similar results as for p-p38, p-ERK and p-NF-κB were observed for p-CREB with the highest phosphorylation levels in DCs treated with the combination of PSMα3 and *S. aureus* cell lysate for 30 and 60 min (Fig 4 and Fig S1D). Taken together PSMα3 in collaboration with TLR2 stimulation enhances the activation of various signaling pathways downstream of TLR2 involved in the production of the anti-inflammatory molecule IL-10.

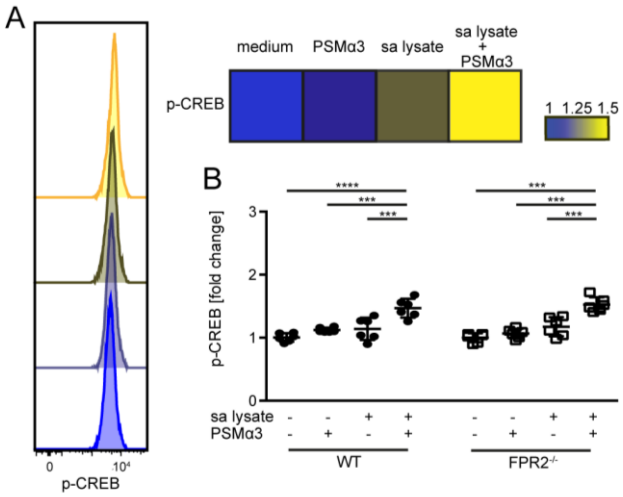

FIGURE 4. Enhanced CREB phosphorylation in DCs induced by PSMs and TLR2. BM-DCs from wildtype and FPR2-/- mice were incubated for 60 min with S. aureus cell lysate (sa lysate), PSMα3 and in combination. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-CREB and analysis by flow cytometry. (A) Representative histogram overlays of p-CREB in DCs (gated on CD11c+MHC II+ cells). The heatmap shows FC of p-CREB normalized to untreated DCs (medium). Heatmap and histogram overlays are colored according to FC of phosphorylation. (B) Statistical analysis of p-CREB staining shown in (A). Graph shows two independent experiments out of three performed in triplicates (mean ± SD). * indicate statistically significant differences (one-way ANOVA with Bonferroni post-test).

The p38-CREB axis mediates IL-10 secretion in TLR2 and PSM-treated DCs

The production of IL-10 was analyzed to address whether the enhanced activation of the signaling pathways NF-κB, p38 and ERK has an impact on the modulation of cytokine secretion by PSMα3. IL-10 is exclusively produced by

DCs stimulated with the combination of PSMα peptides and *S. aureus* cell lysate (Figure 5 and (9)).

Pretreatment of DCs with various concentrations of the NF-κB inhibitor BAY 11-7082 had no effect on IL-10 production except for the 10 µM concentration (Fig 5A). However, this concentration was cytotoxic for the cells as determined by cell viability assay using 7-AAD (Fig S2A). Thus, enhanced NF-κB activation by PSMα3 is not involved in IL-10 production by DCs.

Pre-incubation of DCs with various concentrations of the p-ERK inhibitor PD 0325901 did not affect the IL-10 production after stimulation with *S. aureus* cell lysate and PSMα3, indicating no impact of enhanced ERK phosphorylation on IL-10 production (Fig 5B). In contrast, pretreatment with the p-p38 MAPK inhibitors SB 203580 and BIRB 0796 lead to a concentration dependent inhibitory effect of IL-10 production (Fig 5C and 5D). Furthermore, inhibition of MSK1 by Gö6976, which acts downstream of p38 and ERK and upstream of CREB, revealed a concentration dependent decrease of IL-10 secretion by DCs (Fig 5E). No toxic effect was observed for the used inhibitor concentrations as well as for the vehicle (Fig S2B-F). The specificity of the inhibitors was assessed by flow cytometry for p38 or CREB phosphorylation. SB 203580 and BIRB 0796 prevented the phosphorylation of p38 and Gö6976 of CREB in a dose dependent manner (Fig S3), respectively, whereas these inhibitors had no effect on activation of other signaling pathways (data not shown). Together, our data demonstrate the involvement of the p38-CREB axis in IL-10 production induced by PSMα3.

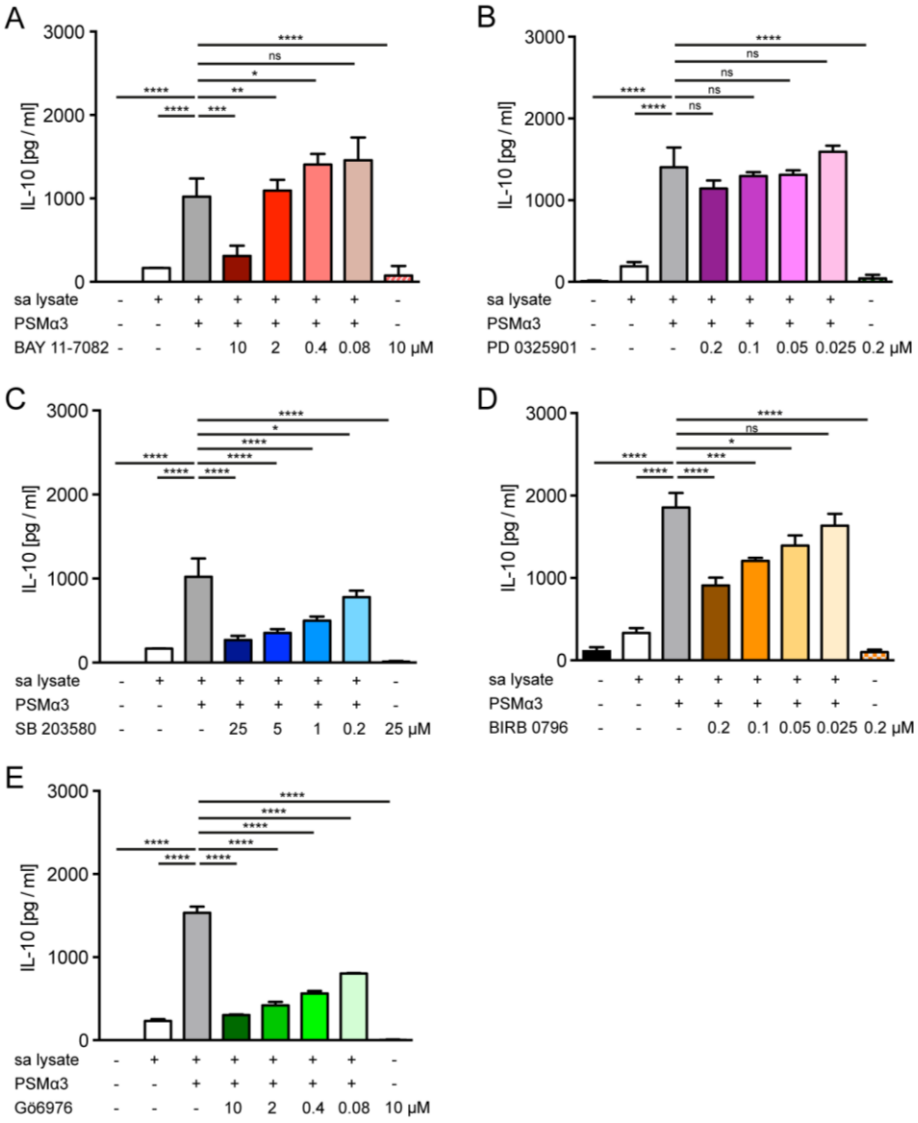

FIGURE 5. The p38 – CREB axis mediates IL-10 secretion in TLR2 and PSM-treated DCs. BM-DCs were treated for 1h with the indicated concentrations of the NF-κB inhibitor BAY 11-7082 (A), the p-ERK inhibitor PD 0325901 (B), the p-p38 inhibitors SB 203580 (C) and BIRB 0796 (D), or the MSK1 inhibitor Gö6976 (E) prior to treatment with S. aureus cell lysate (sa lysate) and PSMα3. 24 h later cell culture supernatants were collected and analyzed for IL-10 by ELISA. The graphs show one representative out of two to three (A, C-E) or pooled data from two (B) independent experiments performed in triplicates (mean ± SD). * indicate statistically significant differences (one-way ANOVA with Bonferroni post-test).

Enhanced activation of p38-CREB-IL-10 axis by PSMs in TLR2-stimulated DCs primes T_{regs}

Previously we showed that increased IL-10 production by DCs upon TLR2 and PSM stimulation primes regulatory T cells (9). To directly address whether the p38-CREB-IL-10 axis is involved in the priming of T_{regs} by PSMs, DCs were treated with p38 inhibitors prior to incubation with *S. aureus* cell lysate and PSMα3. 24h later DCs were incubated with naive $CD4^+$ T cells and T cell priming was assessed 4 days later by flow cytometry. The frequency of $CD4^+CD25^+FoxP3^+$ T cells was significantly increased when DCs were treated with *S. aureus* cell lysate and PSMα3 compared to *S. aureus* cell lysate alone (Fig 6 and Fig S4A: gating strategy). Inhibition of p38 signaling by the inhibitors SB 203580 and BIRB 0796 prevented this increase in $CD4^+CD25^+FoxP3^+$ T cells mediated by PSMα peptides in a dose-dependent manner (Fig 6). Thus, PSMs induce T_{regs} by modulating the p38-CREB-IL-10 axis in DCs.

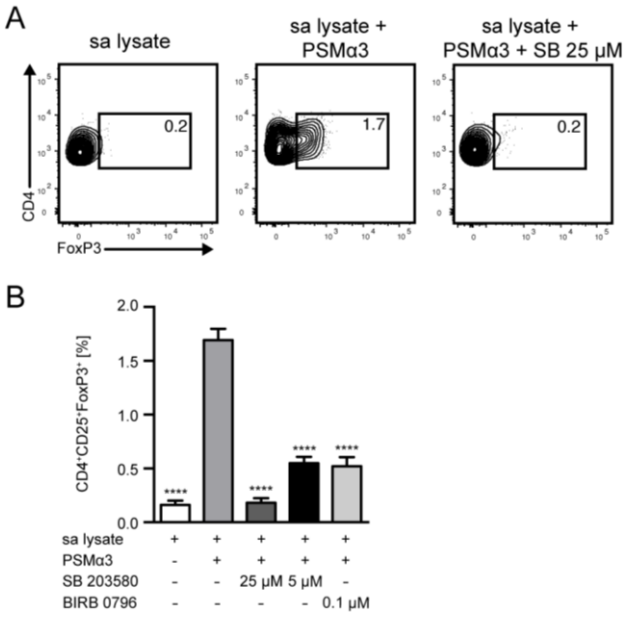

FIGURE 6. Activation of p38-CREB-IL-10 axis by PSMs in DCs primes T_{regs}. BM-DCs were pretreated with or without the indicated inhibitors for 1h and were subsequently incubated with *S. aureus* cell lysate (sa lysate) and PSMα3 for 24 h. Then splenic $CD4^+$ T cells isolated from wildtype mice were added to the culture for 96 h. (A) Representative dot plots show flow cytometry analysis of $CD4^+CD25^+FoxP3^+$ T cells (gating see Fig S4). Numbers adjacent to outlined areas indicate frequency of

$CD4^+CD25^+FoxP3^+$ T cells in the culture. (B) Statistical analysis of $CD4^+CD25^+FoxP3^+$ T cells after co-culture with BM-DCs. Data are from two to four independent experiments performed in quadruplicates (mean ± SEM). * indicate statistically significant differences (one-way ANOVA with Bonferroni post-test).

PSMs penetrate DCs by transient pore formation and directly interact with p38 MAPK in DCs

It has been reported that PSM peptides can induce an effective inflammatory immune response by binding to the FPR2 receptor, whereas their cytolytic activity is FPR2 independent (6, 9). It is assumed that PSMα peptides like δ-toxin are able to form transient pores (5). To address whether PSMs are internalized by DCs via mechanisms of antigen uptake e.g. receptor mediated endocytosis WT and $FPR2^{-/-}$ mice were incubated with fluorescently labeled PSMα peptides and analyzed by multispectral imaging flow cytometry. PSMα2 was located in the cytosol in WT as well as $FPR2^{-/-}$ DCs after 10, 30 and 60 min of incubation (Fig 7A, Figure S4B (gating strategy) and data not shown). The frequency of $PSM\alpha2^+$ DCs was comparable in WT and $FPR2^{-/-}$ DCs (Fig 7A), showing that PSMs penetrate DCs by an FPR2-independent mechanism. To address whether PSMs are actively internalized by DCs via macropinocytosis or receptor-mediated endocytosis DCs from WT mice were incubated with FITC-labeled PSMα2 on ice preventing actin-rearrangement and thereby endocytosis and compared to incubation at 37°C enabling endocytosis. Incubation of FITC-labeled PSMα2 with DCs on ice did not prevent PSM penetration into DCs (Fig 7B). Furthermore, simultaneous incubation of DCs with OVA-Alexa647, which is taken up by macropinocytosis and receptor-mediated endocytosis, and PSMα2-FITC on ice revealed intracellular PSMα2-FITC, whereas no OVA-Alexa647 was taken up by DCs (Fig. 7B). These data show that PSMs penetrate DCs independently of endocytosis most likely by pore formation.

To address the hypothesis that pore formation by PSMs is responsible for cell penetration LDH release by DCs upon PSM-treatment was analyzed. LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into the supernatant when the plasma membrane is damaged (21, 22). Indeed,

10 min after treatment with PSMα3 a significant amount of LDH was released from DCs, which was comparable with the LDH release of δ-toxin, known to induce transient pore formation (23) (Fig 7C). In contrast, nearly no LDH release was observed when DCs were treated with OVA peptide, further supporting that PSMs induce transient pore formation in DCs.

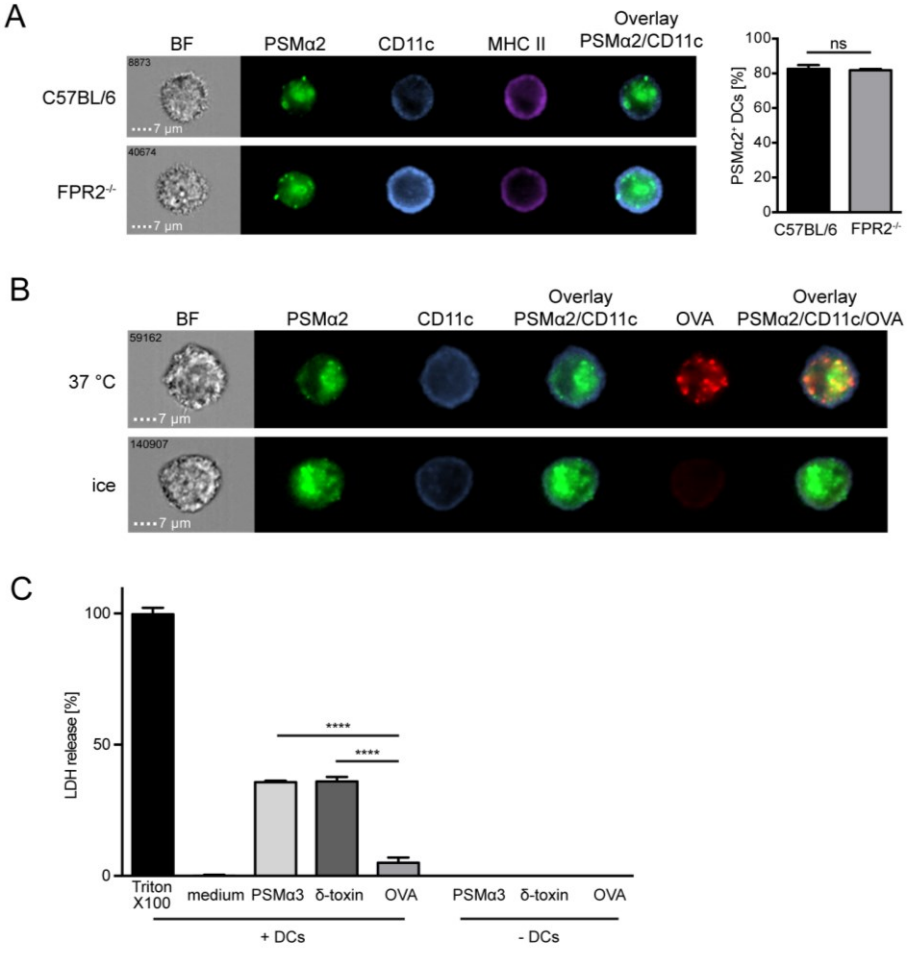

FIGURE 7. PSMs gain access to the cytosol by transient pore formation. Multispectral imaging flow cytometry analysis of PSMα-FITC^{+} DCs (gating see Fig S4B) 30 min (A) or 10 min (B) after incubation with FITC-labeled PSMα2 (37°C or ice) in the presence (B) or absence of OVA-Alexa647 (A). Representative bright field (BF) and fluorescence images of DCs are shown from two or more independent experiments with similar results performed in triplicates (A-B). The graph shows the frequency of PSMα2-FITC^{+} DCs from WT and FPR2$^{-/-}$ mice (A). (C) DCs were treated with the indicated reagents for 10 min. Graph shows the frequency of LDH release in relation to DCs treated with 1% Triton-X100 (positive control). Graph shows one out of two

independent experiments with similar results performed in triplicates (mean ± SD). * indicate statistically significant differences (one-way ANOVA with Bonferroni post-test).

PSMs were predominantly localized close to the plasma membrane in spots devoid of endosomal and lysosomal markers independently whether they where activated via TLR2-stimuation or not (Fig 8 and data not shown). Instead, PSMs co-localized with p38 (Fig 8A) and p-p38 (Fig 8B) molecules as shown by imaging flow cytometry. DCs treated with PSMs and *S. aureus* cell lysate showed an increased max pixel intensity of p-p38 gated on PSMα-FITC^{+}p-p38^{+} DCs compared to DCs treated with PSMs alone, indicating an increased phopsphorylation of p38 upon TLR2 activation (Fig 8B and 8C). These data point towards a direct interaction of PSMα peptides with the p38 MAPK signaling pathway.

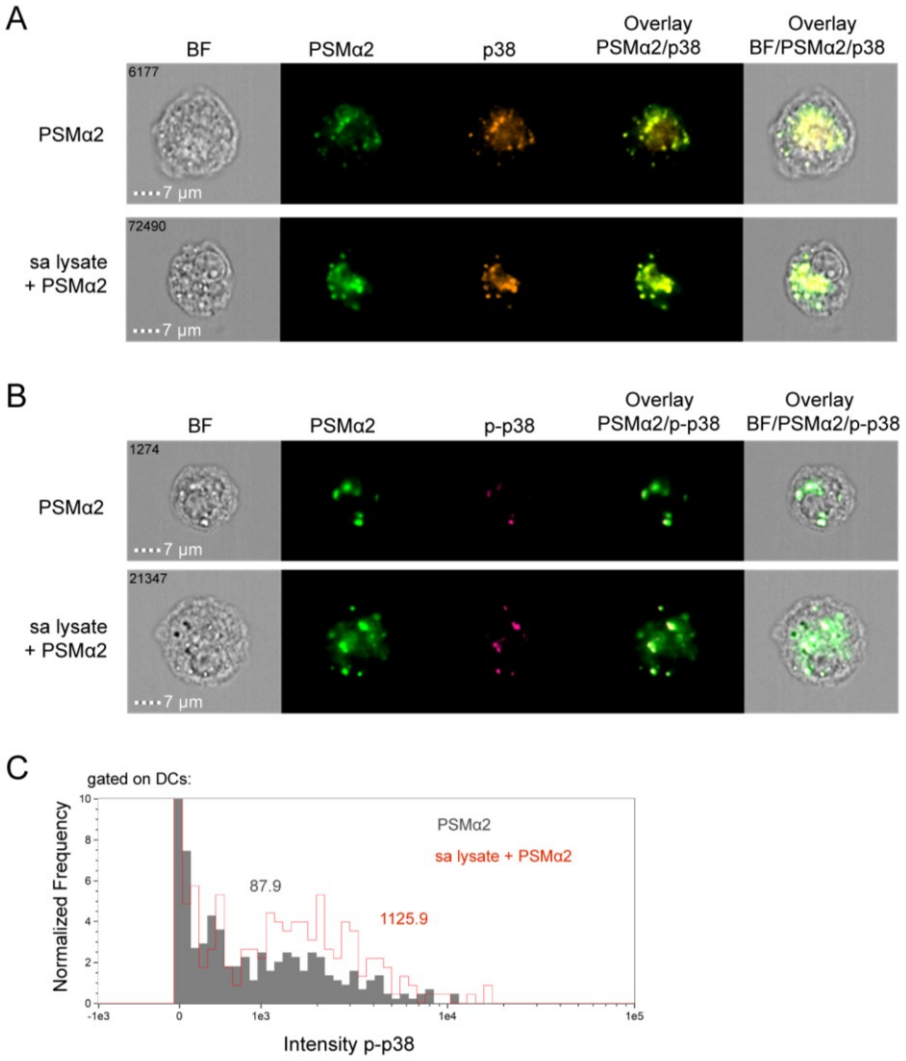

FIGURE 8. Co-localization of PSMs with p38 and p-p38. Multispectral imaging flow cytometry analysis of DCs (gating see Fig S4B) 30 min after incubation with FITC labeled PSMα2 or PSMα3 in the presence or absence of S. aureus cell lysate (sa lysate). Representative bright field (BF) and fluorescence images of PSMα-FITC+ DCs additionally stained with p38 (A) and p-p38 (B). Yellow spots indicate intracellular PSMα2 co-localized with p38 (A) or p-p38 (B). The histogram overlay shows the intensity of p-p38 in DCs treated with PSMα2-FITC (gray) and the combination of S. aureus cell lysate and PSMα2-FITC (red) (C). Numbers in histogram indicate the median intensity of p-p38. Data show representative images out of two or more independent experiments with similar results performed in triplicates.

Discussion

PSMs play a key role in the pathogenicity of CA-MRSA strains (3). They bind to human and mouse FPR2 thereby initiating chemotaxis of neutrophils and DCs (6, 9). We have previously shown that PSMα peptides induce a tolerogenic phenotype in DCs upon TLR2 stimulation, characterized by the production of IL-10 and impaired secretion of pro-inflammatory cytokines (9). Consequently, these tolerogenic DCs favored the priming of regulatory T cells (9). How PSMs induce IL-10 secretion and which signaling pathways are involved remained elusive. Here we show that PSMs penetrate DCs via transient pore formation, directly interact with p38 and upon TLR2 activation enhance its phosphorylation and downstream CREB activation. This consequently increased IL-10 production and induction of T_{regs}.

Pore-forming toxins (PFT) comprise approximately 25% of all bacterial toxins and represent the largest class of bacterial virulence factors (24, 25). The amphipathic PSMα-peptide δ-toxin was shown to form receptor-independent transient pores in solution (5, 23). Based on these experiments we hypothesize that δ-toxin and potentially other PSMs form dimers and bind to the cytoplasmic membrane at low peptide density and oligomers span the membrane and induce pore formation at high peptide density (5, 23). However, the PSM side of action in DCs remains unclear. Do PSMs act as PFTs in DCs? Can PSMs reach the cytosol via pore formation or are they internalized by DCs via macropinocytosis or receptor-mediated endocytosis? We show that PSMs are located in the cytosol independently of FPR2 (Fig 7). Although the localization of PSMs appears point-shaped, they are not in endosomes or lysosomes (data not shown) arguing against their active internalization by DCs via macropinocytosis or receptor-mediated endocytosis. In agreement with this, PSMs are similarly found in the cytosol when endocytosis (shown here with OVA) is prevented during ice incubation. Furthermore, the direct interaction of PSMs with p38 supports this conclusion, as p38 is located in the cytoplasm and not in subcellular fractions (26, 27). Moreover, FPR2 is not involved in the cytotoxic activity of PSMs on neutrophils (6) and the production of IL-10 by DCs

(9), strongly supporting the hypothesis that receptor independent processes are responsible for cytosolic localization of PSMs. Recently, Grosz et al. demonstrated that PSMα peptides are required for phagosomal escape of various cytolytic *S. aureus* strains in professional and non-professional phagocytes enabling cytoplasmic replication of these strains (28). In conclusion our data obtained by imaging flow cytometry and LDH release provide evidence that transient pore formation is mediating the transport of PSMs to the cytoplasm.

TLR2 agonists are able to induce the expression of anti-inflammatory IL-10 via activation of NF-κB, p38 and ERK in DCs (10). Furthermore, pathogens triggering the C-type lectin DC-SIGN can modify TLR signaling in DCs. Upon TLR-dependent signaling DC-SIGN activates the kinase Raf-1, which acetylates the p65 subunit of NF-κB leading to an extended and enhanced *IL-10* transcription (17). In TLR2 stimulated DCs the phosphorylation of ERK is increased, which induces the IL-10 production and represses IL-12(p70) (19). Although NF-κB and ERK signaling were increased by PSMs, blocking of these signaling pathways with chemical inhibitors had no effect on IL-10 production by PSM-treated DCs. Our data indicate a direct interaction of PSMα peptides with p38 thereby enhancing its phosphorylation and via CREB activation (20) eventually lead to high IL-10 production in DCs upon TLR2 ligand-stimulation. This is supported by Bebien et al. showing that the virulence factor β hemolysin/cytolysin of *Group B streptococcus* induces IL-10 secretion via p38 MAPK activation (18). PFTs activate MAPK signaling pathways in different eukaryotic cells - whether this is beneficial or detrimental for the pathogen is species dependent (29-36).

Besides PFTs other pathogenicity factors of Gram-negative bacteria or viruses modulate p38 signaling in host cells (29, 37). YopJ from *Yersinia pseudotuberculosis* inhibits p38 and JNK phosphorylation thereby preventing the production of TNF-α in macrophages (15, 37). Additionally, YopP from *Y. enterocolitica* (also termed YopJ) prevents IL-10 production by DCs (15, 29). Accordingly, many effector proteins of Gram-negative bacteria were shown to impair p38 MAPK activation thereby preventing pro-inflammatory cytokine

secretion (29, 38). Moreover, Leghmari et al. suggest a new immune escape mechanism for HIV-1 infection, by which the Tat protein induces IL-10 production in monocytes in a p38 MAPK dependent manner (38-41). Thus, we demonstrate a new function of the PFTs PSMα peptides acting as further pathogenicity factors by modifying p38 MAPK signaling pathway.

Their capacity to induce Tregs via production of anti-inflammatory molecules that may be secreted, membrane bound, or both define tolerogenic DCs. A variety of T_{reg} differentiation models demonstrated the necessity of IL-10 secretion by tolerogenic DCs for tolerance induction (39-42) and for the maintenance of suppressive T_{regs} upon strong inflammatory signals (9, 42-44). Like PSMα peptides, *Candida albicans*, *Cryptococcus neoformans* and *Fasciola hepatica* subvert the immune system by promoting DC tolerogenicity and T_{reg} differentiation (9, 43-45). How these pathogens impair recognition and signaling remains unknown. Here, we describe the p38-CREB-IL-10 axis as molecular mechanism for DC tolerogenicity and T_{reg} differentiation induced by PSMα peptides *in vitro* (Fig 6). Whether this holds true *in vivo* has to be shown.

For the treatment of increasing antibiotic resistant bacteria the development of new narrower-spectrum or virulence targeted antimicrobial therapeutics is necessary (45, 46). PFTs were used as live vaccines in various disease models, but successful immunization against a PFT does not always prevent disease (46). Other examples like inhibiting PFTs and using competitive inhibitors were shown to effectively prevent or cure the infection (46). Furthermore, boosting host defense e.g. by using drugs modulating MAPK signaling pathways is discussed as immunotherapy against infections. Our data point towards the use of a p38 inhibitor in the case of CA-MRSA infection possibly preventing the induction of tolerogenic DCs and thereby immune escape.

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Supporting Information

PSM Peptides of *Staphylococcus aureus* Activate the p38-CREB Pathway in Dendritic Cells thereby Modulating Cytokine Production and T-cell Priming[1]

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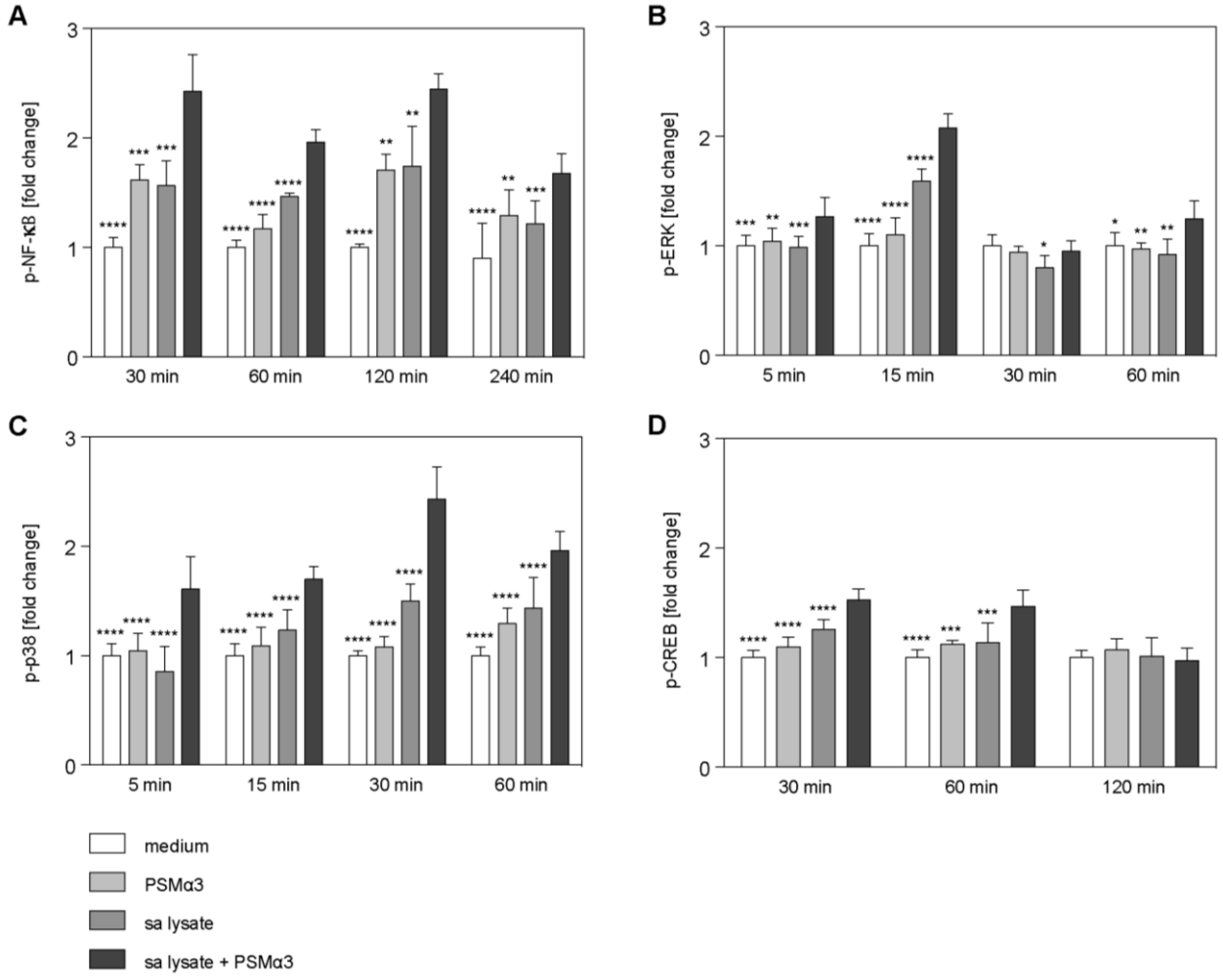

Figure S1. Phosphorylation of various signaling pathways in DCs induced by PSMs and TLR2.

BM-DCs were incubated for the indicated times with *S. aureus* cell lysate (sa lysate), PSMα3 and in combination. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-NF-κB (A), p-ERK (B), p-p38 (C) and p-CREB (D) and analysis by flow cytometry. The graphs show fold change (FC) of phosphorylation normalized to untreated DCs (medium). Data are pooled from two independent experiments out of three performed in triplicates. * indicate statistically significant differences compared to DCs treated with the combination of *S. aureus* cell lysate and PSMα3 at the respective time (one-way ANOVA with Bonferroni post-test).

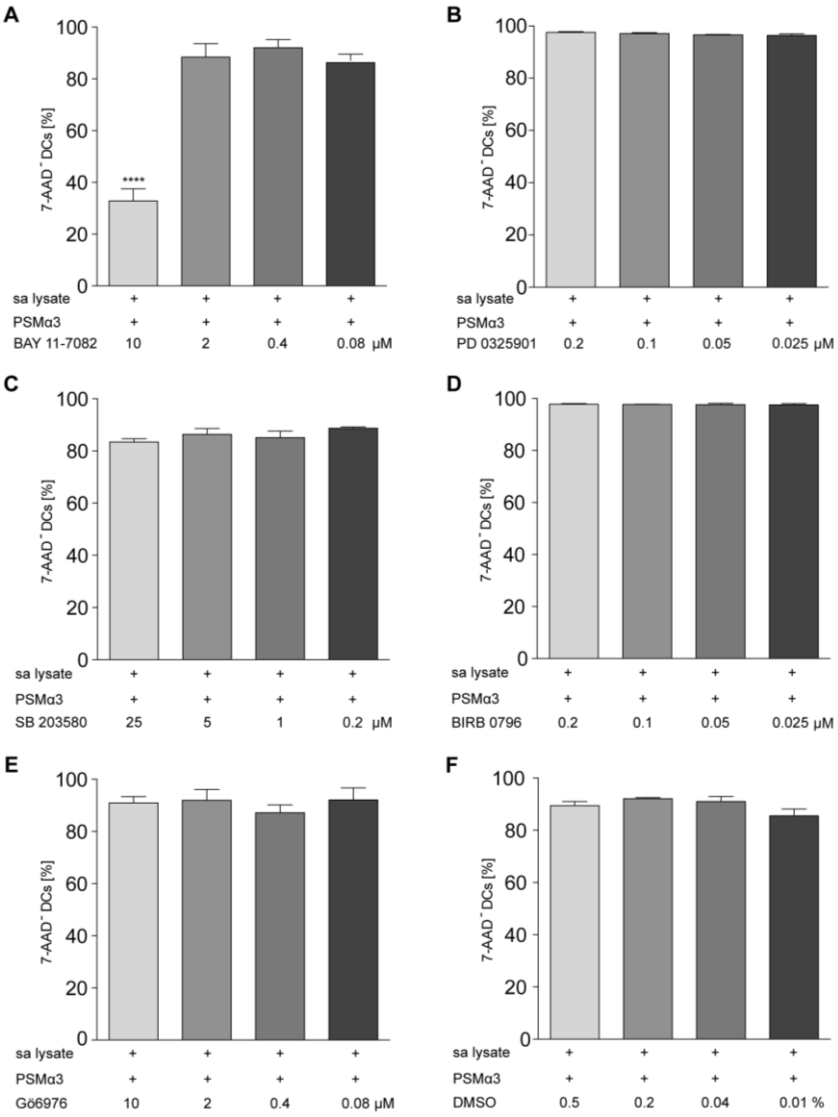

Figure S2. Cell viability assay.

BM-DCs were treated for 1h with the indicated concentrations of the NF-κB inhibitor BAY 11-7082 (A), the p-ERK inhibitor PD 0325901 (B), the p-p38 inhibitors SB 203580 (C) and BIRB 0796 (D), the MSK1 inhibitor Gö6976 (E) and the vehicle DMSO (F) prior to treatment with *S. aureus* cell lysate (sa lysate) and PSMα3 for 24h. Cells were stained with 7-AAD, CD11c and MHC II and analyzed by flow cytometry for the frequency of living (7-AAD$^-$) DCs. Graphs show one experiment performed in triplicates. * indicate statistically significant differences compared to DCs treated with the combination of *S. aureus* cell lysate and PSMα3 (one-way ANOVA with Bonferroni post-test).

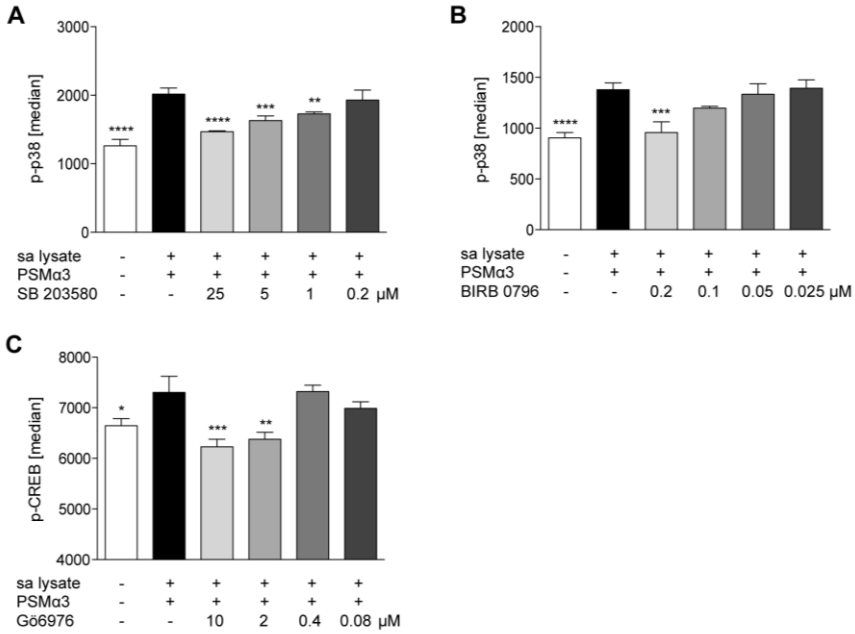

Figure S3. Determination of inhibitor specificity.

BM-DCs were treated for 1h with the indicated concentrations of the p-p38 inhibitors SB 203580 (A) and BIRB 0796 (B) and the MSK1 inhibitor Gö6976 (C) prior to treatment with *S. aureus* cell lysate (sa lysate) and PSMα3. The cells were stained with CD11c and MHC II antibodies followed by intracellular staining against p-p38 (A and B) and p-CREB (C). Graphs show the median fluorescence intensity of p-p38 (A and B) and p-CREB (C) of DCs analyzed by flow cytometry. Graphs show one out of one (B, C) or two (A) independent experiments performed in triplicates (mean ± SD). * indicate statistically significant differences compared to DCs treated with the combination of *S. aureus* cell lysate and PSMα3 (one-way ANOVA with Bonferroni post-test).

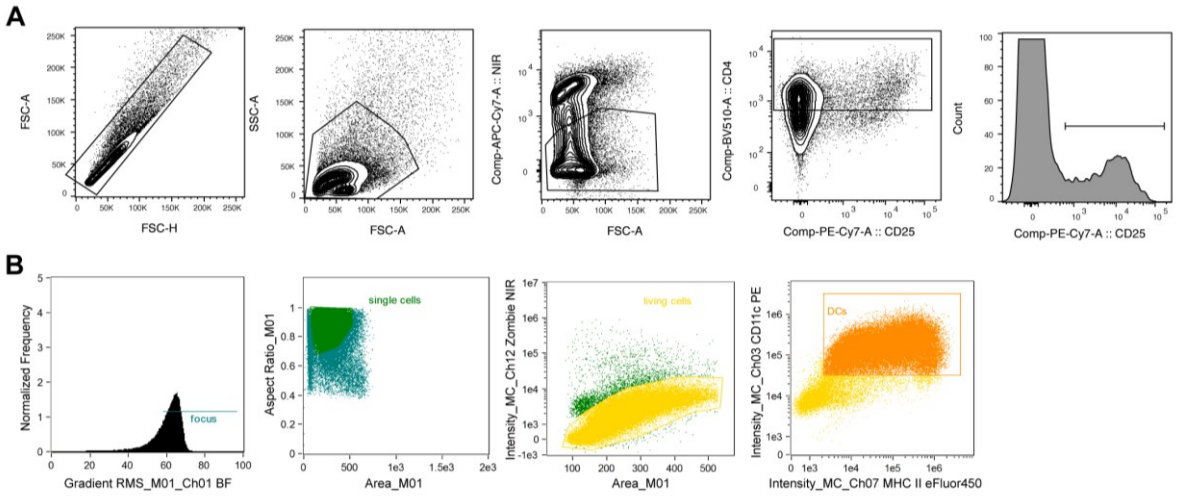

Figure S4. Gating strategies.

(A) Dot plots show the gating strategy used to define $CD4^+CD25^+$ T cells by flow cytometry 4 days after co-culture of PSM-treated DCs and naïve $CD4^+$ T cells. Cells were gated as follows: singlets/lymphocytes/living/$CD4^+$/$CD25^+$. (B) Dot plots show the gating strategy used to define $CD11c^+$MHC II^+ DCs by multispectral imaging flow cytometry. Cells were gated as follows: cells in focus/single cells/living cells/DCs.

3 *Staphylococcus aureus* PSM peptides induce tolerogenic dendritic cells upon treatment with ligands of extracellular and intracellular TLRs

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Running title: PSMs modulate TLR-activated DCs

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Abbreviations

DC, dendritic cell; PSM, phenol-soluble modulin; TLR, Toll-like receptor; T_{reg}, regulatory T cell; CA, community-associated; MRSA, methicillin-resistant *Staphylococcus aureus*; FPR2, formyl peptide receptor 2; BMDC, bone marrow-derived DC; OVA, ovalbumin.

Abstract

Dendritic cells (DCs) are key players of the immune system and thus a target for immune evasion by pathogens. We recently showed that the virulence factor phenol-soluble modulin (PSM) produced by community-associated methicillin-resistant *Staphylococcus aureus* strains induces tolerogenic DCs upon Toll-like receptor (TLR) 2 activation via the p38-CREB-IL-10 pathway. Here, we addressed the question whether this tolerogenic phenotype of DCs induced by PSMs is specific for TLR2 activation. Therefore, bone marrow-derived DCs were treated with various ligands for extracellular and intracellular TLRs simultaneously with PSMα3. We show that PSMα3 modulates antigen uptake, maturation and cytokine production of DCs activated by TLR1/2, TLR2/6, TLR4, TLR7, and TLR9. Pre-incubation of DCs with a p38 MAP kinase inhibitor prevented the PSMα3-induced IL-10 secretion, as well as MHC class II up-regulation upon TLR activation. In consequence, the tolerogenic DCs induced by PSMα3 in response to several TLR ligands promoted priming of regulatory T cells. Thus, PSMs could be useful as inducers of tolerogenic DCs upon TLR ligand stimulation for therapeutic applications.

Introduction

Dendritic cells (DCs) are the most essential antigen presenting cells that link the innate and adaptive immunity by activating T and B cells (Merad et al., 2013). Immature DCs are able to actively internalize antigens because of their high endocytic capacity. Simultaneously, inflammatory signals induce the maturation of DCs. As a result, DCs reduce antigen uptake, yet enhance antigen processing and presentation thereby enabling efficient T-cell activation (Garrett et al., 2000). DCs recognize pathogen-associated molecular patterns by their pattern recognition receptors (Saraiva and O'Garra, 2010) e.g. Toll-like receptors (TLRs) (Merad et al., 2013). TLRs respond to exogenous microbial products resulting in the activation of the adaptive immune system by downstream signaling leading to the expression of cytokines, chemokines and

interferons (Michelsen, 2001; Re and Strominger, 2004). Additionally, TLR signaling in DCs leads to the downregulation of endocytosis, up-regulation of the co-stimulatory molecules CD80 and CD86, as well as MHC class II molecules and cytokine production (Merad et al., 2013). The pro-inflammatory cytokines e.g. TNF-α, IL-6 and IL-12 recruit other immune cells for pathogen clearance and induce T helper cell differentiation (Dinarello, 2000; Elenkov and Chrousos, 2002). In contrast, the anti-inflammatory cytokine IL-10 elicits important immunoregulatory function by inhibiting IL-12 production which regulates regulatory T-cell (Treg) development (Ouyang et al., 2011).

The Gram-positive bacterium Staphylococcus aureus is the leading cause of more than fifty percent of skin and soft-tissue infections worldwide (Brown et al., 2015; Talan et al., 2011). In treatment of this disease, antibiotic abuse has led to the emergence of methicillin-resistant S. aureus (MRSA) strains. Both healthy and immunocompromised patients are susceptible to community-associated (CA) MRSA strains e.g. USA300, which is the most prevalent strain in the world (DeLeo et al., 2009; Otto, 2010). Because S. aureus possesses many virulence factors it is very effective at evading the host's innate and adaptive immune system (Brown et al., 2015; Otto, 2010). For example, CA-MRSA strains express α-toxin, Panton-Valentine Leukocidin and phenol-soluble modulin peptides (PSMs) (Otto, 2010; Thammavongsa et al., 2015). Also contributing to CA-MRSA strains pathogenicity is its ability to secrete large amounts of PSMs compared to other MRSA strains (Wang et al., 2007). PSMs contain five α-peptides (δ-toxin and PSMα1-4) and two β-peptides (PSMβ1-2) (Peschel and Otto, 2013; Wang et al., 2007). PSMs attract human neutrophils by binding to the cell surface human formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010; Wang et al., 2007). Furthermore, mouse DCs expressing the mouse FPR2 are also attracted by PSMs (Schreiner et al., 2013). The α-helical and amphipathic PSMs possess a pore forming activity (Wang et al., 2007). Previously, we showed that PSMs, most likely via pore-formation, modulate the cytokine production of DCs independently of the mouse FPR2 (expression) by increasing the TLR2 ligand-induced production of the anti-inflammatory cytokine IL-10 via specific interaction with the MAPK p38 (Armbruster et al., 2016). In contrast, PSMs inhibit the TLR2 ligand-induced pro-inflammatory cytokine

secretion of TNF-α, IL-12 and IL-6 (Armbruster et al., 2016; Dinarello, 2000; Elenkov and Chrousos, 2002; Schreiner et al., 2013). As a consequence, the PSM-treated DCs show an impaired T helper 1 cell priming capacity, but an increased induction of Foxp3+ Tregs via p38-CREB-IL-10 modulation in DCs (Armbruster et al., 2016; Schreiner et al., 2013). As p38 MAPK signaling is activated upon TLR-stimulation in general, we hypothesize that PSMs affect DC functions including maturation, cytokine production and T-cell priming upon treatment with various TLR ligands. The aim was to test whether PSMs could be useful as general inducers of tolerogenic DCs for therapeutic applications.

The major innate immune-stimulating compounds of S. aureus are lipoproteins (Hashimoto et al., 2006; Stoll et al., 2005). They induce a fast and strong cytokine release by mouse peritoneal macrophages via TLR2-MyD88 signaling leading to increased pathogenicity (Schmaler et al., 2009). Lipoproteins of S.aureus are either di- or tri-acylated, depending on the growth phase (Kurokawa et al., 2012). Therefore, we tested di- and tri-acylated lipopeptides (Pam2- and Pam3-Cys) in this study. Moreover, we used ligands for TLR4, and the intracellular TLR7 and TLR9. We show that PSM□3 generally modulates antigen uptake, maturation and cytokine production in DCs upon extracellular as well as intracellular TLR-stimulation. In consequence, these tolerogenic DCs increased priming of Tregs.

Materials and Methods

Mice

Female C57BL/6JolaHsd and BALB/cAnNRj mice were purchased from Janvier (St. Berthevin Cedex, France). FPR2$^{-/-}$ mice (Chen et al., 2010) with a genetic C57BL/6 background were bred in the animal facilities of the University Clinic of Tübingen. All mice were held under specific pathogen-free conditions, were provided with food and water ad libitum and used for experiments between 6-12 weeks of age. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science

Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Anzeige 09.01.2014).

Generation of bone marrow-derived DCs (BMDCs)

RPMI-1640 medium (Merck) supplemented with 10% FBS (Sigma-Adrich), 2 mM L-glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol (Roth), 1 mM sodium pyruvate (Merck) and 1x non essential amino acids (Merck) was used in all cell culture experiments. BMDCs were prepared using GM-CSF as previously described (Armbruster et al., 2016; Schreiner et al., 2013). Briefly, 2 x 10^6 bone marrow cells, flushed from the femurs and tibias of C57BL/6 and FPR2$^{-/-}$ mice, were seeded in 100 mm dishes in 10 ml medium containing 200 U/ml GM-CSF. After 3 days, an additional 10 ml of fresh medium containing 200 U/ml GM-CSF was added to the cultures. On day 6 half of the culture supernatant was replaced by fresh medium containing GM-CSF. At day 7-8, the slightly attached cells were used for the experiments described in this report.

Reagents for stimulation of BMDCs

Formylated PSMα3 peptides with the recently published sequence (Wang et al., 2007) and reversed PSMα4 peptides (used as control peptide) were synthesized in house. BMDCs were treated with 100 ng/ml Pam2CSK4 (InvivoGen) for TLR2/TLR6, 1 µg/ml Pam3CSK4 (InvivoGen) for TLR1/TLR2, 3 µg/ml *S. aureus* cell lysates (produced in house) for TLR2 (Schreiner et al., 2013), 100 ng/ml LPS (Sigma) for TLR4, 1 µg/ml CpG ODN 1826 (InvivoGen) for TLR9 and 5 µg/ml Imiquimod (InvivoGen) for TLR7 activation. Furthermore BMDCs were treated simultaneously in combination with 10 µM PSMα3 peptide or 10 µM control peptide. Where indicated BMDCs were pretreated with 25 µM to 0.2 µM p-p38 MAPK inhibitor SB 203580 (Merck) prior to TLR-ligand treatment.

Cytokine production by BMDCs

BMDCs (2,5 x 10^5) were seeded in 96-well plates and incubated with different TLR ligands and peptides as described above. Supernatants were collected after 6 h to determine TNF-α (eBioscience) and IL-6 (BD Biosciences) levels and after 24 h for IL-10 (BD Biosciences) and IL-12 (BioLegend) measurement. ELISAs were performed according to the manufacturer's instructions.

Flow cytometry staining of stimulated BMDCs

For maturation analysis BMDCs (2 x 10^5) were seeded in 96-well plates and incubated with different TLR ligands and peptides as described above for 3h. Extracellular staining was performed for 20 min at 4°C, using the antibodies CD11c-APC (N418; Miltenyi Biotec), MHC class II-eFluor450 (M5/114.15.2; eBioscience), CD54-FITC (3E2; BD Biosciences), CD80-PE (16-10A1; BD Biosciences) and CD86-PE (GL-1; BD Biosciences), CD40-PerCP/Cy5.5 (3/23; BioLegend). Dead cells were excluded using either 7-aminoactinomycin D (Biomol) after the extracellular staining or using Zombie Aqua (BioLegend) before extracellular staining according to the manufacturer's instructions. The cells were washed with PBS with 1% fetal calf serum (FCS; Sigma-Aldrich) and 2 mM ethylenediaminetetraacetic acid (Sigma-Aldrich) prior and acquired using a Canto-II flow cytometer (BD Biosciences) with DIVA software (BD Biosciences). Data analysis was performed using FlowJo 10.0.7r2 software (Tree Star).

OVA uptake by BMDCs after stimulation

To test the antigen uptake capability of BMDCs, 5 x 10^6 cells were seeded in 48-well plates and treated for 24 h as described above. Thereafter, cells were incubated with AlexaFluor647-labeled Ovalbumin (OVA) (10 µg/ml) for 30 min at 37°C. Then the cells were washed with ice-cold PBS with 2% FCS followed by extracellular staining with MHC class II-eFluor450 (M5/114.15.2; eBioscience)

and CD11c-PE (N418; eBioscience) antibodies and flow cytometry measurement as described above.

T-cell assay

5 x 10^4 BMDCs were seeded in 96-well U-bottom plates and treated for 20 h as described above. Splenic $CD4^+$ T cells from BALB/c mice were purified using the $CD4^+$ T-Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. 2 x 10^5 $CD4^+$ T cells were added to the BMDCs and cultured in RPMI-1640 medium (Merck) supplemented with 20% FCS (Sigma-Adrich), 2 mM L-glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol (Roth), 1mM sodium pyruvate (Merck), 10 mM HEPES-Buffer (Biochrom AG) and 1x non essential amino acids (Merck). 96 h later supernatants were collected for IL-17A (eBioscience) and IFN-γ (eBioscience) measurement and T cells were first stained with Zombie Aqua (BioLegend) according to the manufacturer's protocol to exclude dead cells, followed by extracellular staining with CD4-eFluor450 (RM4-5; eBioscience), CD3e-APC-Cy7 (145-2C11; BioLegend), CD25-APC (PC61; BioLegend), fixed and permeabilized (Foxp3 Staining Buffer Set, eBioscience) and stained intracellular with Foxp3-PE (eBioscience). 100,000 cells were acquired using Canto-II flow cytometer (BD Biosciences) as described above.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism 6 software (GraphPad, San Diego, CA) using one-way ANOVA with Bonferroni posttest. The differences were considered as statistically significant if $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

Results and Discussion

PSMs generally modulate the TLR-induced cytokine release by DCs

Previous data from our group showed that DCs treated with a TLR2 ligand (*S. aureus lysate*) in combination with PSMα peptides produce low amounts of the pro-inflammatory cytokines TNF, IL-12 and IL-6, but high amounts of the anti-inflammatory cytokine IL-10 (Schreiner et al., 2013). Here, we addressed whether PSMs generally modulate TLR-stimulated cytokine release by DCs or whether this mechanism is specific for TLR2-activation. To investigate the effect of different TLRs, BMDCs (DCs) were treated with the following TLR ligands: Pam2CSK4 for TLR2/TLR6, Pam3CSK4 for TLR1/TLR2, *S. aureus* cell lysate mainly for TLR2 (Schreiner et al., 2013), LPS for TLR4, CpG ODN 1826 for TLR9, and Imiquimod for TLR7 in combination with a synthetic PSMα3 or a control peptide. Cell culture supernatants were taken after 6 h and 24 h and analyzed by ELISA. Treatment for 24 h with PSMα3 was not cytotoxic for the DCs, determined by a cell viability assay using Zombie Aqua (Fig. S1).

IL-10 secretion by DCs treated with TLR ligands alone was comparable to DCs treated with TLR ligands in combination with the control peptide (Fig. 1A and Fig. S2A). The amount of IL-10 was significantly increased after DC treatment with TLR ligands in combination with PSMα3, with the highest amount produced after stimulation with the TLR-ligands *S. aureus* lysate, CpG and Imiquimod. Similar results although with minor differences were observed for mFPR2-deficient DCs treated under the same conditions (Fig. S3A).

The levels of the pro-inflammatory cytokines IL-6, TNF, and IL-12 were also increased 6 h and/or 24 h after DC treatment with the different TLR ligands alone or in combination with control peptide compared to the untreated DCs (Fig. 1B-D and Fig. S2B-D). Treatment of DCs with TLR ligands in combination with PSMα3 significantly impaired the production of all three cytokines tested compared to treatment with the TLR ligands alone (Fig. 1B-D and Fig. S2B-D).

Figure 1

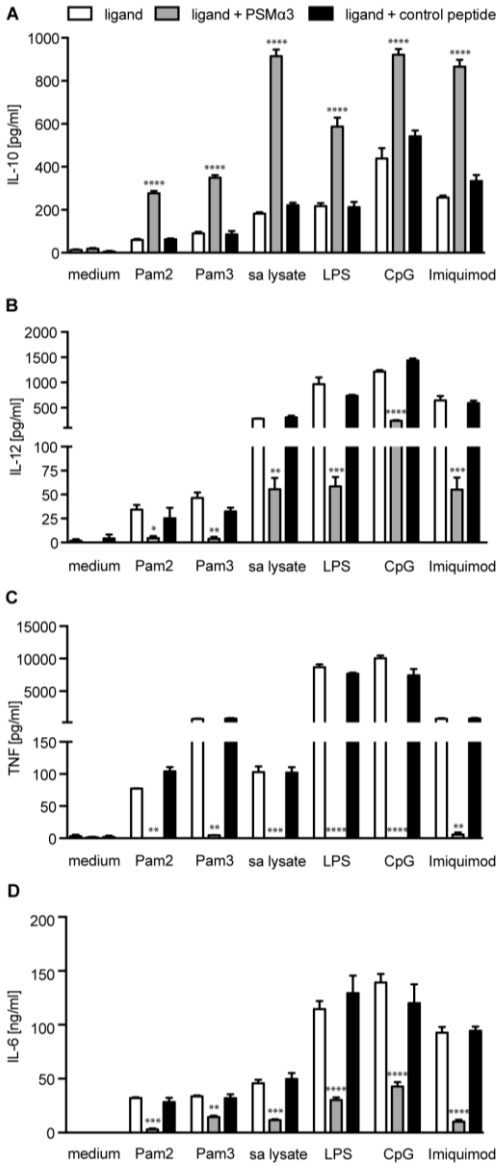

FIGURE 1. PSMs modulate the TLR-induced cytokine release of wildtype DCs. BMDCs from wildtype mice were incubated for 24 h (A-B) or 6 h (C-D) with the indicated TLR ligands alone, TLR ligands in combination with PSMα3 peptide or TLR ligands in combination with control peptide. DCs without TLR ligand +/- PSMα3 or control peptide were used as negative controls (medium). Cell culture supernatants were collected and analyzed for IL-10 (A), IL-12 (B), TNF (C) and IL-6 (D) by ELISA. Data show one representative out of three independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

Similar results were observed for mFPR2$^{-/-}$ DCs treated under the same conditions with minor significance compared to wildtype DCs (Fig. S3). Although PSMs bind to mFPR2 on the surface of DCs, our data are in accordance with previous results demonstrating an FPR2-independent modulation of cytokine production by DCs (Schreiner et al., 2013). Furthermore, the increase of the anti-inflammatory cytokine IL-10 upon PSM treatment is caused by direct interaction of intracellular PSM with p38 MAPK (Armbruster et al., 2016) and is therefore mainly receptor-independent. Together the data demonstrate a general effect of PSMα3 in modulating the cytokine production by DCs treated with various TLR ligands. The synergy between the various TLR ligands and PSMα3 in the induction of IL-10 production suggests that two signals may be required to activate or inhibit the production of certain cytokines from DC *in vitro*. The induction of a tolerogenic DC phenotype characterized by impaired secretion of pro-inflammatory cytokines (Li and Shi, 2015), but increased production of the anti-inflammatory cytokine IL-10 is described for various pathogens. For example, DCs produce high amounts of IL-10 in response to TLR4-activation and *Bordetella pertussis* infection (Higgins et al., 2003; McGuirk et al., 2002) as well as in response to *Mycobacterium bovis* activating TLR2 and TLR4 (Demangel and Britton, 2000; Uehori et al., 2003) and *Yersinia pestis* via TLR6 activation (DePaolo et al., 2008). Moreover, DC treatment with the enterotoxin cholera toxin from *Vibrio cholerae* in combination with LPS induced high IL-10 secretion (Lavelle et al., 2003) and the immunomodulatory molecule ES-62 from the parasite *Filarial nematodes* affects, dependent of TLR4 and MyD88 signaling, the IL-12 and TNF-α production by DCs (Goodridge et al., 2004). Thus, although the mechanisms are not fully understood, various bacterial and parasitic products induce DCs with a tolerogenic cytokine profile upon engagement of TLRs.

PSMs affect the TLR-induced maturation of DCs

Different TLR ligands induce maturation of DCs associated with the up-regulation of MHC class II, co-stimulatory molecules like CD40, CD80 and

CD86 and adhesion molecules e.g. intercellular adhesion molecule 1 (CD54) (Merad et al., 2013). To analyze the effect of PSMs on the maturation process, DCs were treated for 3 h or 24 h with several TLR ligands and in combination with PSMα3 or the control peptide. Maturation markers were analyzed by flow cytometry (gating strategy in Figure S4). All TLR ligands alone and in combination with the control peptide led to maturation of DCs, characterized by up-regulation MHC class II (Fig. 2A and Fig. S5A), CD86 (Fig. 2B and Fig. S5B), CD80 (Fig. 2C and Fig. S5C), CD54 (Fig. 2D and Fig. S5D), and CD40 (Fig. S5E) compared to untreated DCs. However, PSMα3 enhanced the co-stimulatory potential of DCs via up-regulation of CD86 and CD80 expression in response to TLR ligands, but impaired the up-regulation of CD54 and CD40 after 3 h and 24 h, respectively (Fig. 2D and Fig. S5D-E).

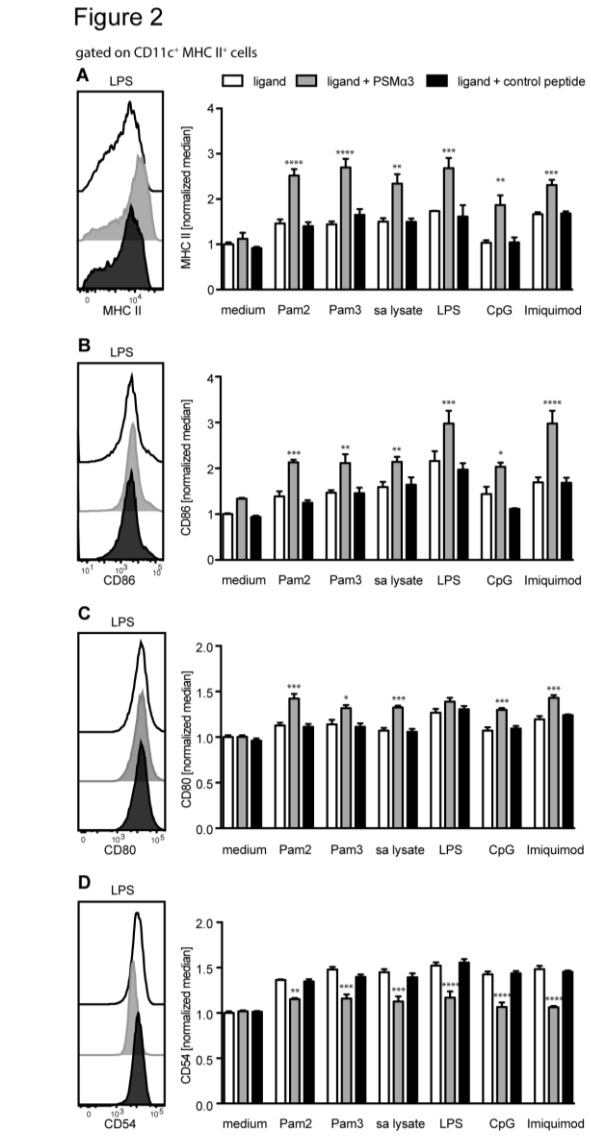

FIGURE 2. PSMs affect the TLR-induced maturation of DCs. BMDCs from wildtype mice were incubated for 3 h with the indicated TLR ligands, TLR ligands along with PSMα3 peptides or TLR ligands in combination with control peptide. The cells were stained with CD11c, MHC class II, CD86, CD80 and CD54 antibodies and analyzed by flow cytometry. Representative histogram overlays showing the median expression of MHC class II (A), CD86 (B), CD80 (C), and CD54 (D) on the surface of LPS-treated DCs (left). Graphs (right) show the statistical analysis of the depicted median normalized to untreated cells (medium) from TLR ligand-treated DCs (gated on $CD11c^{+}MHC\ II^{+}$ cells). Graphs show pooled data from three independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

Together these data show that PSMs in principle modulate TLR-induced DC maturation towards an intermediate phenotype, a prerequisite for priming of regulatory T cells. Tolerogenic DCs show, in addition to their modified cytokine pattern, high levels of MHC class II, CD80 and CD86 molecules but low expression of CD40 and CD54 (Mills, 2004).

DCs lacking CD40 expression were shown to induce IL-10 secreting T_{regs} while suppressing primery immune responses (Martin et al., 2003). Interaction of CD54 with LFA-1 promotes T_H1 priming independently of IL-12, while this T_H1 priming is blocked when DCs express high levels of CD80 and CD86, but low levels of CD40 and CD54 on their surface (McGuirk et al., 2002). Likewise DCs treated with cholera toxin and LPS showed enhanced CD80 and CD86 but reduced CD40 and CD54 expression (Lavelle et al., 2003). CD40 belongs to the TNFR superfamily (Quezada et al., 2004) and its expression is among others regulated by TNF-α. We found that synergistic treatment of DCs with PSMα3 and TLR ligands inhibits TNF-α production already within 6 h, which likely prevents up-regulation of CD40 at later times as previously shown in a setting of Crohn's disease (Danese et al., 2006).

PSM-mediated modulation of TLR-induced DC maturation and cytokine secretion is p38-dependent

Previously, we showed that PSMs directly interact with the MAPK p38, leading to its phosphorylation in DCs (Armbruster et al., 2016). To investigate the role of p38 phosphorylation in PSM-dependent maturation and cytokine secretion, DCs were pretreated with the p-p38 inhibitor SB 203580 for 1 h prior to stimulation with TLR ligands in combination with PSMα3. As described above, PSMα3 enhanced the TLR ligand-induced up-regulation of MHC class II on DCs (Fig. 2A, Fig. S5A and Fig. S6). In contrast, inhibition of p-38 signaling prevented this enhanced MHC class II up-regulation as DCs treated with SB 203580 together with TLR ligand and PSMα3 showed similar MHC class II surface expression

than DCs treated with the TLR ligand alone (Fig. S6). Moreover, inhibition of p-38 signaling prevented the PSM-induced production of IL-10 upon TLR activation with Pam2 (Fig. 3A), Pam3 (Fig. 3B), sa lysate (Fig. 3C), LPS (Fig. 3D), CpG (Fig. 3E), and Imiquimod (Fig. 3F).

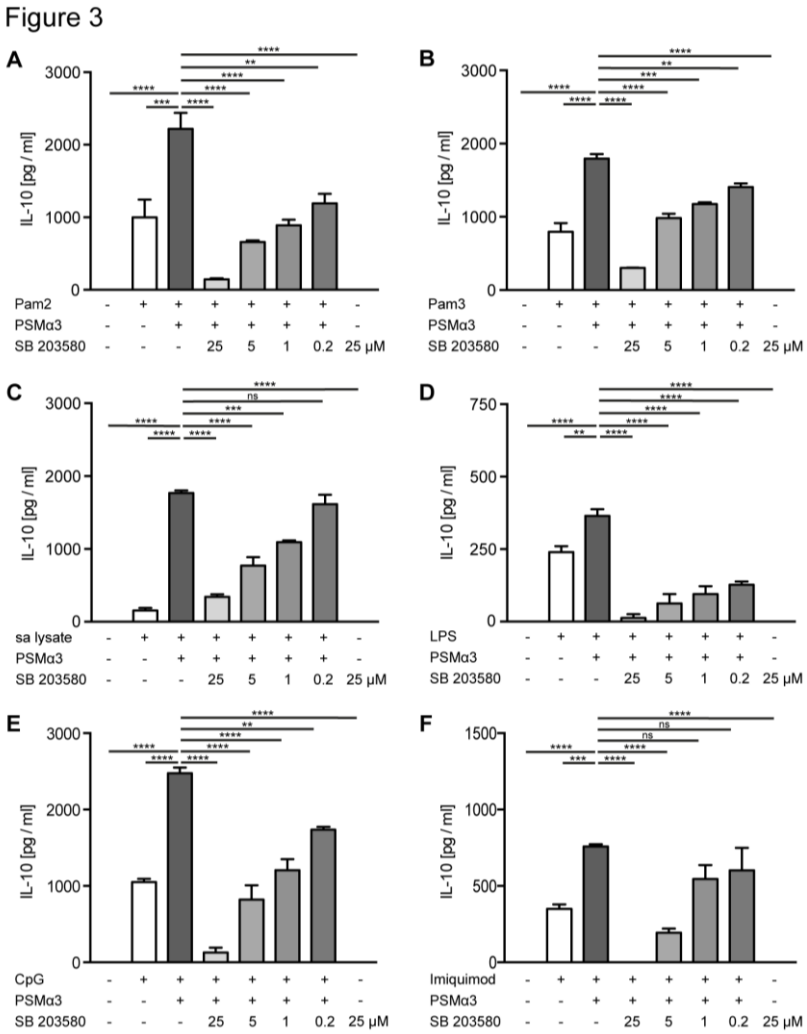

FIGURE 3. PSM-mediated modulation of TLR-induced DC maturation and cytokine secretion is p-p38-dependent. BMDCs were treated for 1h with the indicated concentrations of the p-p38 inhibitor SB 203580 prior to treatment with PSMα3 and Pam2 (A), Pam3 (B), *S. aureus* cell lysate (sa lysate) (C), LPS (D), CpG (E) and Imiquimod (F). 24 h later, cell culture supernatants were collected and analyzed for IL-10 by ELISA. The graphs show one representative out of three independent experiments performed in triplicates (mean ± SEM). **p < 0.005, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Bonferroni posttest.

These data point towards a direct modulation of the MAPK p38-signaling pathway by PSMα3 upon TLR activation with various TLR ligands thereby enhancing MHC class II expression and IL-10 secretion by DCs.

It is known that the MAPK p38 plays a critical role in DC maturation. Hemagglutinin B, a virulence factor of *Porphyromonas gingivalis* that binds to TLR4 activates the MAPK p38 thereby inducing DC maturation, indicated by up-regulation of CD86 (Gaddis et al., 2009). Pre-treatment with the p38 MAPK inhibitor SB 203580 prevents p38 phosphorylation induced by TLR ligands in human DCs and thereby blocks the up-regulation of HLA-DR, CD80 and CD86 (Arrighi et al., 2001). This is consistent with our hypothesis that PSM-mediated p38 phosphorylation accounts for enhanced up-regulation of MHC class II, CD80, and CD86 molecules in DCs. In this study we show that p38, modulated by PSMs, is one of the signaling molecules responsible for inducing maturation (MHC class II upregulation) and thus possibly affect co-stimulatory and adhesion molecule expression on DCs treated with TLR ligands.

TLR activation in general leads to IL-10 expression in DCs via p38 MAPK signaling (Saraiva and O'Garra, 2010). PSM peptides of *S. aureus* (Armbruster et al., 2016), as well as the pathogenicity factor β hemolysin/cytolysin of *Group B Streptococcus* activate the p38 MAPK pathway thereby increasing the secretion of IL-10 (Bebien et al., 2012).

PSMs attenuate antigen uptake of TLR-activated DCs

Several molecules induce the differentiation of immature antigen-capturing DCs to mature antigen-presenting DCs (Merad et al., 2013; Steinman et al., 2003). To investigate whether PSMs affect antigen uptake upon TLR-activation, DCs were treated with various TLR ligands for 24 h, along with PSMα3 or the control peptide. Antigen uptake was analyzed by flow cytometry 30 min after incubation with the fluorescently labeled model antigen Ovalbumin (OVA). DCs treated with TLR ligands with or without the control peptide showed a decrease in OVA uptake compared to untreated immature DCs (medium) (Fig. 4), except for

Imiquimod treatment. TLR ligand treatment together with PSMα3 significantly further reduced OVA uptake by DCs compared to the ligands alone (Fig. 4).

Figure 4

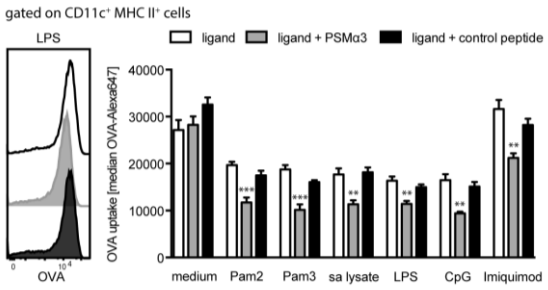

FIGURE 4. PSMs attenuate antigen uptake of TLR-activated DCs. BMDCs from wildtype mice were treated for 24 h with the indicated TLR ligands, TLR ligands in combination with PSMα3 peptides or control peptide, incubated with OVA-Alexa647 for 30 min and analyzed by flow cytometry. Representative histogram overlay of OVA-Alexa647 median in DCs treated with LPS, LPS together with PSMα3 peptide and in combination with control peptide (gated on $CD11c^+$ MHC II^+ cells) (left) and statistical analysis of the OVA-Alexa647 median from TLR ligand-treated DCs (right). Data show one representative out of three independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. **$p < 0.005$, ***$p < 0.001$, one-way ANOVA with Bonferroni posttest.

These data demonstrate that PSMα3 generally impairs OVA uptake by TLR ligand-treated DCs.

The main function of immature DCs is to internalize antigens by endocytosis. In response to pro-inflammatory cytokines and microbial or viral products, which directly or indirectly interact with TLRs, DCs change their phenotype to mature DCs characterized by decreased endocytic but increased antigen presenting capacity (Cella et al., 1997; Garrett et al., 2000; Hackstein et al., 2002). LPS-treated BMDCs demonstrated long-term down-regulation of macropinocytosis via actin remodeling and MAPK signaling (West, 2004). In contrast, toxin B from *Clostridium difficile* completely blocked macropinocytosis in immature BMDCs but did not affect the receptor-mediated endocytosis of FITC-transferrin (Garrett et al., 2000; West, 2004). We used OVA, which is mainly taken up by clathrin-mediated endocytosis via the macrophage mannose receptor but also by macropinocytosis (Autenrieth et al., 2007; Burgdorf et al., 2006). The reduced

OVA uptake 24 h after TLR ligand treatment reflects the down-regulation of macropinocytosis upon DC maturation. As described previously, PSMs additionally impair clathrin-mediated endocytosis of OVA ((Schreiner et al., 2013) and Fig. 4). In general, endocytosis is regulated by p38 MAPK signaling triggered by TLR stimulation (Zaru et al., 2007). Accordingly, OVA uptake by DCs is impaired by YopP, a virulence factor of *Yersinia enterocolitica*, via MAPK inhibition (Autenrieth et al., 2007). These results coincide with the observation that OVA uptake by DCs treated with different TLR ligands is impaired by PSMs via modulation of p38 signaling.

PSMs induce TLR-activated DCs to prime T_{regs}

We recently reported that PSMs lead to enhanced priming of T_{regs} in TLR2-treated DCs by increased activation of the p38-CREB-IL-10 axis (Armbruster et al., 2016). To determine whether this is a TLR2-specific mechanism or also applies to other TLRs, DCs were treated with different TLR ligands in combination with PSMα3 or the control peptide for 24 h. Afterwards DCs were co-cultured with naïve $CD4^+$ T cells for 96 h and T-cell priming was evaluated by flow cytometry. The frequency of $CD4^+CD25^+Foxp3^+$ T cells within the fraction of living cells was significantly increased when DCs were treated with TLR ligands along with PSMα3 compared to DCs treated with TLR ligands alone (Fig. 5A-B). Moreover, we analyzed IFN-γ and IL-17-A in the supernatant of the co-cultures, to investigate T-cell priming towards Th1 and Th17 cells, respectively. $CD4^+$ T cells co-cultured with DCs treated with TLR ligand alone or in combination with the control peptide produced significant amounts of IFN-γ and IL-17A (Fig. 5C-D). However, DCs treated with TLR ligands along with PSMα3 failed to prime Th1 and Th17 cells, as only low amounts of these cytokines were detected in the supernatants (Fig. 5 C-D).

Figure 5

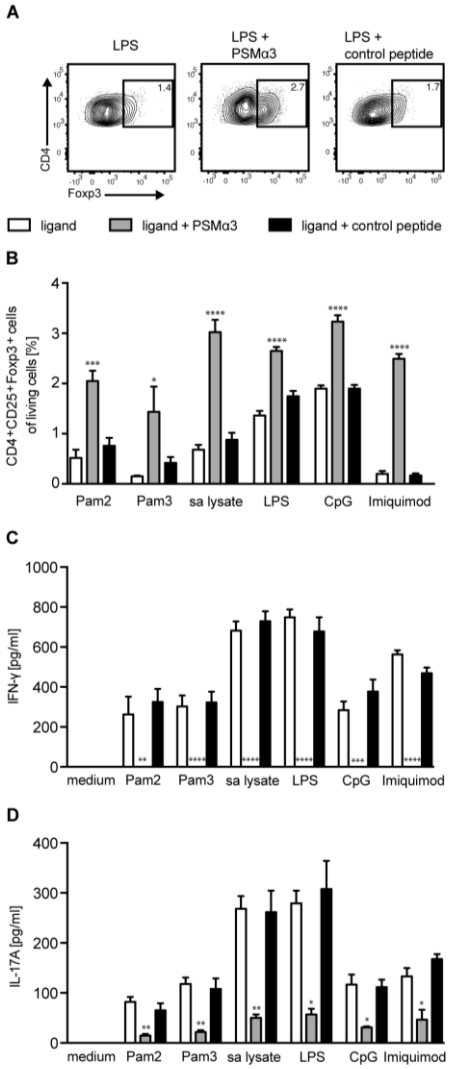

FIGURE 5. PSMs induce TLR-activated DCs to prime T_{regs}. BMDCs from wildtype mice were incubated for 24 h with the indicated TLR ligands, TLR ligands in combination with PSMα3 peptides or control peptide. Then splenic $CD4^+$ T cells isolated from Balb/c mice were added to the culture for 96 h. (A) Representative dot plots show flow cytometry analysis of $CD4^+CD25^+Foxp3^+$ T cells from the co-culture of DCs treated with LPS (gating see Fig S7). Numbers adjacent to outlined areas indicate frequency of $CD4^+CD25^+Foxp3^+$ T cells within the fraction of living cells. (B) Statistical analysis of $CD4^+CD25^+Foxp3^+$ T cells after co-culture with DCs. (C-D) Cell culture supernatants were collected and analyzed for IFN-γ (C) and IL-17A (D) by ELISA. Graph shows one representative out of two (C-D) or three (A-B) independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

Together these data show that priming of T_{regs} by PSMα3-treated DCs, instead of Th1 or Th17, is a common mechanism for the various TLRs.

The main function of mature DCs is to prime naïve T cells and induce their differentiation into various T-cell subsets. Tolerogenic DCs induce T_{regs}, which are beneficial for the host e.g. by mediating mucosal homeostasis against commensal bacteria as well as tolerance against self-antigens. On the other hand, increased numbers of T_{regs} mediating suppressive activity may contribute to the immune escape of pathogens or tumors (Maldonado and Andrian, 2010; Steinman et al., 2003; Yamazaki and Steinman, 2009). Indeed, depletion of T_{regs} in mice with malignancies or chronic *S. aureus* infection improve anti-tumor or anti-infection immune responses, respectively (Tebartz et al., 2015; Zou, 2006). Therefore, understanding how DCs activate T_{regs} is critical for the development of therapeutic strategies in autoimmune dieseases, allograft rejection, allergies, asthma and various forms of hypersensitivity.

Certain pathogens have evolved immune escape mechanisms via the induction of T_{regs} (Maldonado and Andrian, 2010). Still, the contribution of tolerogenic DCs in these settings is unclear. Some pathogens, like *F. hepatica, C. albicans, S. japonicum, S. mansoni, B. pertussis* and *V. cholerae* have been shown to promote DC tolerogenicity and induce T_{reg} differentiation. However, the molecular basis for their recognition and signaling remain largely unknown (Maldonado and Andrian, 2010). Likewise, adoptive transfer of BM-DCs incubated with Cholera toxin primed IL-10 secreting T cells (Lavelle et al., 2003).

The data from this study are a proof of concept of the potential usefulness of *S. aureus'* PSMα3 to induce tolerogenic DCs when different TLRs are activated. One could speculate to use *in vitro* generated PSM-treated tolerogenic DCs for cellular therapy (Lutz, 2012; Maldonado and Andrian, 2010). A major problem of systemic immunosuppressors is that they exert extensive side effects on immune cells. On the contrary, tolerogenic DCs may induce tolerance to the pathologic immune responses in a patient without affecting the immune defense against pathogens or tumors. However, the ability of PSMs to generate

tolerogenic DCs in the human system remains to be confirmed before applying them as cellular therapy.

Acknowledgments

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Supporting Information

Staphylococcus aureus PSM peptides induce tolerogenic dendritic cells upon treatment with ligands of extracellular and intracellular TLRs

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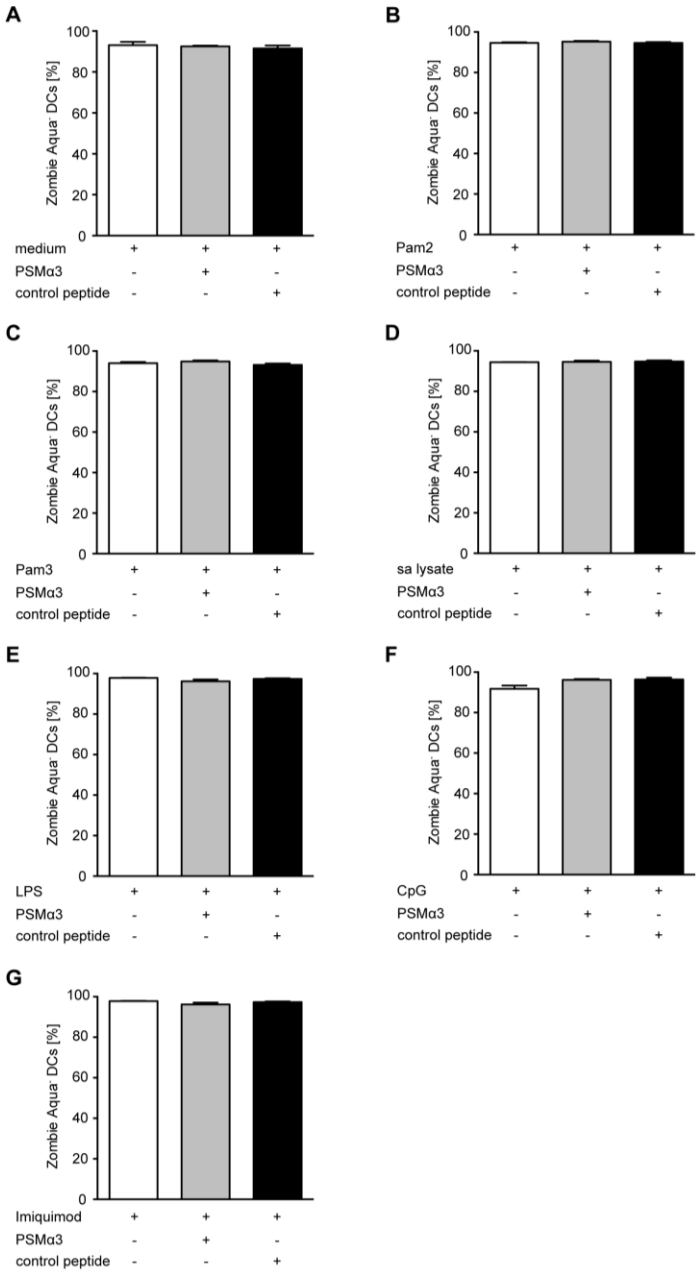

Figure S1: Cell viability assay

BMDCs from wildtype mice were treated for 24 h with the indicated TLR ligands alone, TLR ligands in combination with PSMα3 peptide or TLR ligands in combination with control peptide. Cells were stained with Zombie Aqua, and analyzed by flow cytometry for the frequency of living (Zombie Aqua$^-$) DCs. Graphs show one representative out of more than three independent experiments performed in triplicates (mean ± SEM).

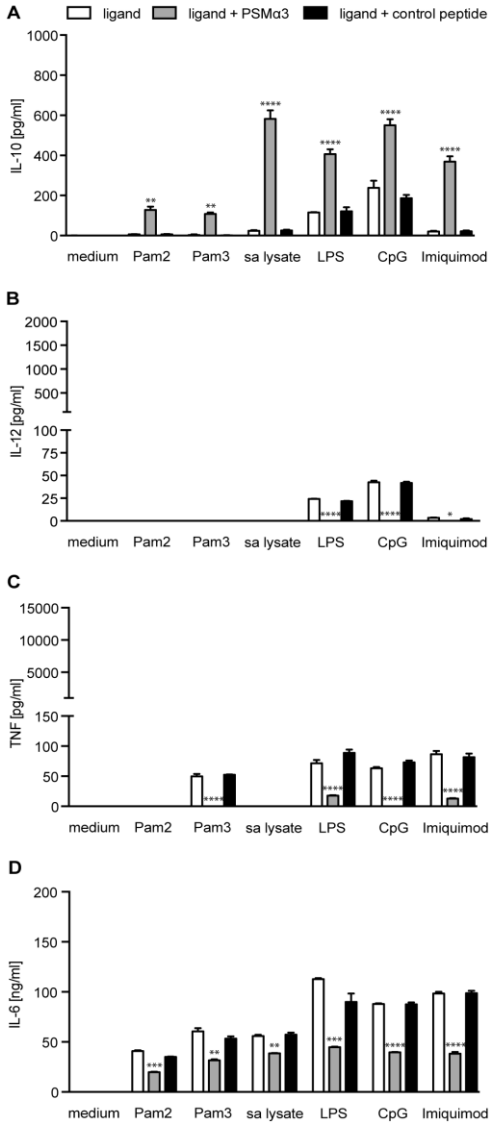

Figure S2: PSMs modulate the TLR-induced cytokine release of wildtype DCs

BMDCs from wildtype mice were incubated for 6 h (A-B) or 24 h (C-D) with the indicated TLR ligands alone, TLR ligands in combination with PSMα3 peptide or TLR ligands in combination with control peptide. Untreated DCs (medium) were used as negative control. Cell culture supernatants were collected and analyzed for IL-10 (A), IL-12 (B), TNF (C) and IL-6 (D) by ELISA. Data show one representative out of two independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

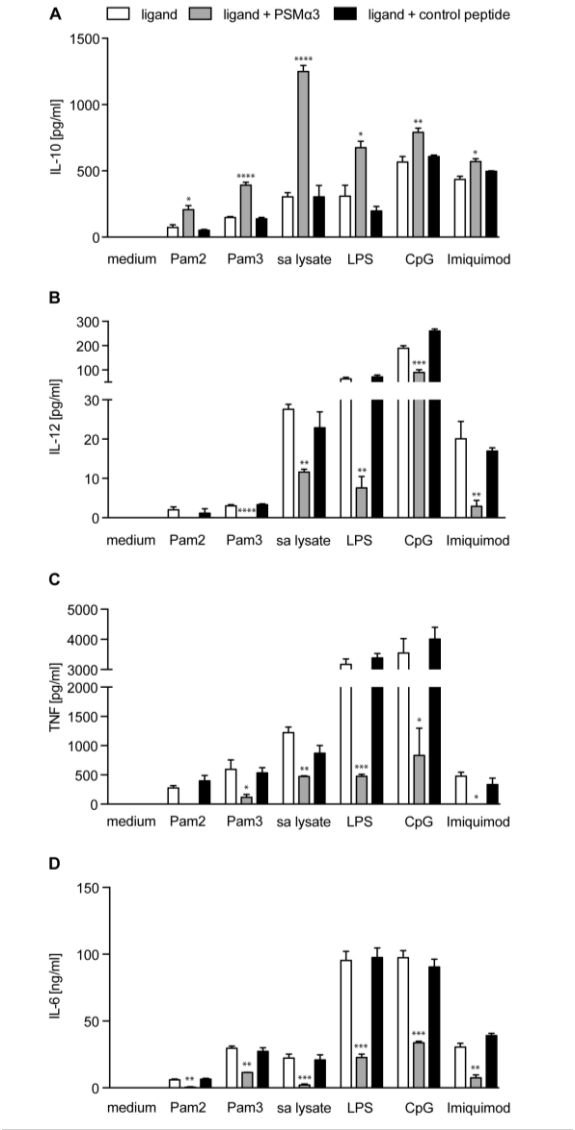

Figure S3: PSMs modulate the TLR-induced cytokine release of mFPR2$^{-/-}$ DCs

BMDCs from FPR2$^{-/-}$ mice were incubated for 24 h (A-B) or 6 h (C-D) with the indicated TLR ligands alone, TLR ligands in combination with PSMα3 peptide or TLR ligands in combination with control peptide. Untreated DCs (medium) were used as negative control. Cell culture supernatants were collected and analyzed for IL-10 (A), IL-12 (B), TNF (C) and IL-6 (D) by ELISA. Data show one representative out of three independent experiments performed in triplicates (mean ± SEM). * Statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

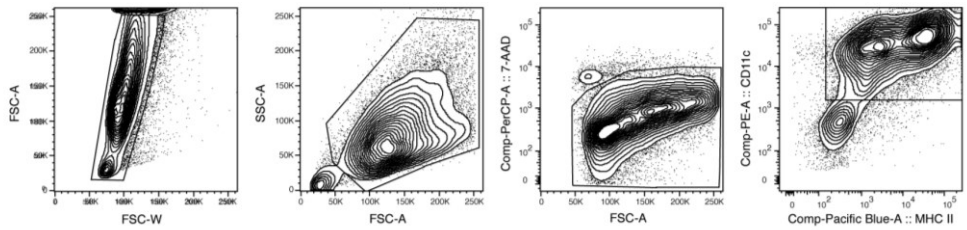

Figure S4: Gating strategy DCs

Dot plots show the gating strategy used to define $CD11c^{+}MHC\ II^{+}$ DCs by flow cytometry. Cells were gated as follows: single cells/leucocytes/living cells/DCs.

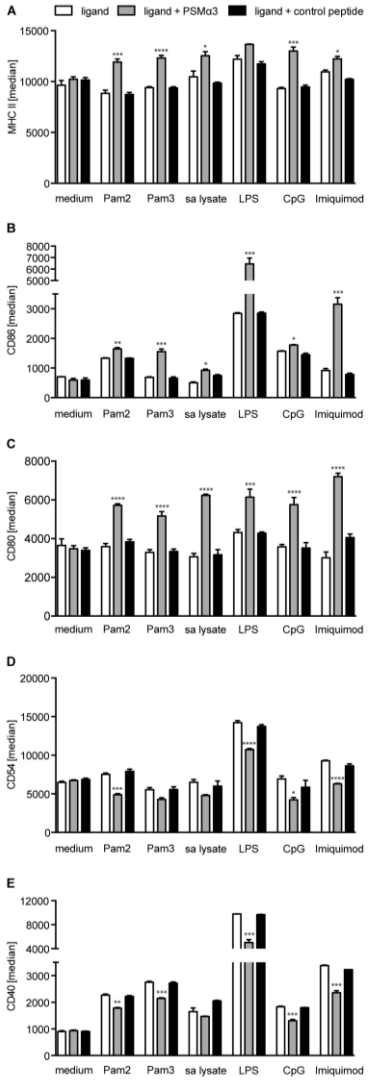

Figure S5: PSMs affect the TLR-induced maturation of DCs after 24h

BMDCs from wildtype mice were incubated for 24 h with the indicated TLR ligands, TLR ligands along with PSMα3 peptides or TLR ligands in combination with control peptide. The cells were stained with CD11c, MHC class II, CD86, CD80, CD54 and CD40 antibodies and analyzed by flow cytometry. Statistical analysis of the median of MHC class II (A), CD86 (B), CD80 (C), CD54 (D) and CD40 (E) expression from TLR ligand-treated DCs (gated on $CD11c^{+}MHC\ II^{+}$ cells). Graphs show one representative out of two independent experiments performed in triplicates (mean ± SEM). * indicate statistically significant differences compared with TLR ligand treated DCs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA with Bonferroni posttest.

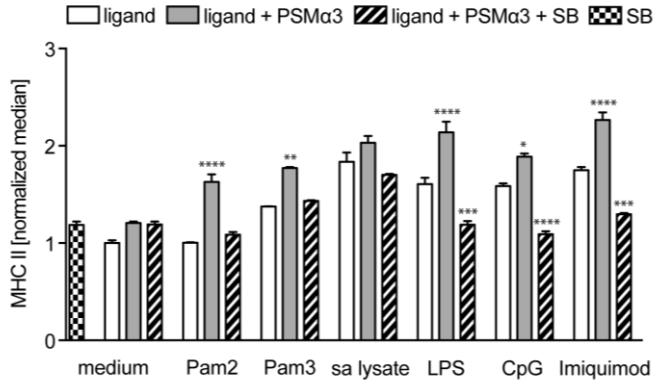

Figure S6: PSMs modulate the maturation of BMDCs in a p38-dependent manner

BMDCs from wildtype mice were pretreated for 1 h with the p-p38 inhibitor SB 203580 and then incubated for 3 h with the indicated TLR ligands and TLR ligands along with PSMα3 peptide. The cells were stained with CD11c and MHC class II antibodies and analyzed by flow cytometry. Bar graphs represent the median MHC class II expression normalized to untreated cells (medium). Data show one representative out of three independent experiments performed in triplicates (mean ± SEM). * Statistically significant differences compared with TLR ligand treated DCs. *p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Bonferroni posttest.

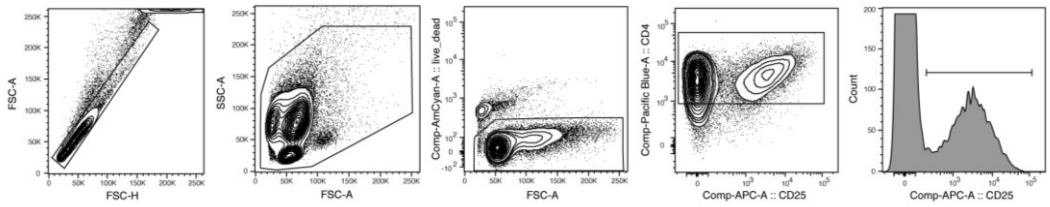

Figure S7: Gating strategy T_{regs}

Dot plots show the gating strategy used to define $CD4^+CD25^+$ T cells by flow cytometry 4 days after co-culture of TLR- and PSM-treated DCs with naïve $CD4^+$ T cells. Cells were gated as follows: single cells/leukocytes/living cells/$CD4^+$/$CD25^+$

4 General discussion

4.1 p38 MAPK signaling in immune cells modulated by virulence factors

The major human pathogen *S. aureus* produces a broad repertoire of secreted and cell surface-associated factors to evade the host immune system. Multi-resistant CA-MRSA strains, such as USA300, are specialized in high-level production and secretion of virulence factors, leading to different pathologies ranging from local SSTIs to severe sepsis (R. Wang et al., 2007). Various immune cells, especially those of the innate immune system, interact with these molecules produced by the invading pathogen. PSMs, a significant group of virulence factors secreted by CA-MRSA fold into a structure that enables these peptides to lyse neutrophils, which are the first line of defense against bacterial infections (R. Wang et al., 2007). DCs, specialized APCs that link the innate and adaptive immunity, are affected by PSMs in their response to TLR2 ligands and are subsequently affected in T cell priming. This effect is characterized by inhibited production of the pro-inflammatory cytokines IL-12, TNF and IL-6 but increased secretion of the anti-inflammatory cytokine IL-10; reduced endocytosis of antigens; and inhibited T_h1 differentiation but increased priming of T_{regs} (Schreiner et al., 2013). These mechanisms allow the pathogen to evade the host immune response. However, it is not known which signaling pathway is modulated by PSMs leading to the increased IL-10 production upon TLR2 ligand treatment in DCs.

Within this work, we show that BM-DCs treated with *S. aureus* cell lysate (a TLR2 ligand) and PSMα3 display increased phosphorylation of the p38-CREB signaling pathway. Blocking this pathway leads to the inhibition of IL-10 secretion, as well as inhibition of the induced T_{reg} priming in the presence of PSMs. However, immunomodulatory properties, including activating the MAPK p38 pathway, are not unusual for various pathogens.

A compromised immune response is an often caused problem by secreting factors of pathogens that mediate host immune system evasion and therefore

are associated with more serious diseases. Other studies have revealed that α-Toxin, a virulence factor of *S. aureus*, induces the phosphorylation of p38 MAPK in the non-virally transformed HaCaT keratinocyte cell line, thereby activating cellular recovery mechanisms, as p38 is considered a survival protein in these cells. (Husmann et al., 2006). PVL, another virulence factor of CA-MRSA, also increases p38 MAPK phosphorylation in human and mouse neutrophils in a concentration-dependent manner to modulate the cytokine immune response, as we observed for PSMs in murine BM-DCs (Yoong and Pier, 2012). Moreover, in line with our findings, one group showed that the β hemolysin/cytolysin toxin from Group B *Streptococcus* leads to the production of IL-10 in macrophages in a p38-dependent manner (Bebien et al., 2012). IL-10 production in response to a microbial stimulus was also observed in a viral context. The A52R protein of Vaccinia virus activates MAPKs and is able to induce the IL-10 promoter. A52R also serves as a co-inducer of TLR4-triggered IL-10 production by LPS (Maloney et al., 2005). Furthermore, the hepatitis B virus modulates the ability of natural killer cells to control infection by suppressing p38 with the two viral antigens HBsAg and HBeAg (Yang et al., 2016). In addition, parasites modulate p38 signaling as a mechanism of immune evasion. For example, *Neospora caninum* increases p38 phosphorylation in macrophages (Mota et al., 2016); in contrast, *Leishmania donovani promastigotes* prevent MAPK activation in naïve macrophages (Privé and Descoteaux, 2000). Moreover, BM-DCs treated with tumor culture conditioning medium have induced p38 MAPK activation but inhibit DC differentiation. This effect allows the tumor to evade detection by immune cells (S. Wang et al., 2006).

Furthermore, we show in BM-DCs both that p38 is modulated and that there is a direct co-localization between PSMα2 and p38 or phosphorylated p38 MAPK. However, PSMs are not the only virulence factors known to directly interact with MAPKs. For example, YopJ of *Yersinia* binds directly to MKKs; however, in contrast to PSMs, YopJ blocks the phosphorylation and activation of MKKs. This interaction results in the inhibition of a pro-inflammatory cytokine response (Orth et al., 1999).

In summary, the capacity of various pathogens or their released factors to modulate the phosphorylation state of the p38 MAPK (or other MAPK family members) highlights the value of future research on this signaling molecule as a potential target for treatment of infections. The signaling molecule p38 plays a variety of key roles in many cellular mechanisms; therefore, specificity is an important factor when targeting MAPKs to avoid side effects, such as the inhibition of other protein kinases. Specific kinase inhibitors often cannot be used because of toxicity or solubility concerns. The development of molecules that interfere with this pathway through inhibition of protein kinase activity can support the discovery of new therapeutic targets against bacteria that are highly resistant to antibiotic treatment, such as CA-MRSA.

4.2 **Mechanism of virulence factor interaction with innate immune cells**

S. aureus utilizes secretion of various types of virulence factors to promote severe infections. Among these are different secreted toxins, which play a key role in the evasion of the host immune response as well as in pathogenesis directly through the killing of several diverse cell types or the interruption of different barriers in the host. *S. aureus* virulence factors are classified into different groups depending on their ability to damage the membrane in a receptor-dependent or independent manner or depending on their enzymatic activity (Otto, 2014). The virulence factors α-Toxin and PVL belong to the receptor-dependent group due to the interaction with their specific receptors ADAM10 and the complement receptor C5aR, respectively, to initiate cytolytic activity (Otto, 2014; Spaan et al., 2013; Wilke and Bubeck Wardenburg, 2010). Although PSMs are able to bind to the FPR2 receptor expressed on different immune cells, they also attach to the membrane in a receptor-independent non-specific mechanism, leading to membrane disruption. The α-helical structure and detergent-like properties of PSMs may contribute to their cell-damaging capabilities. The factors that play important roles in the sensitivity of cells to PSMs are phospholipid constitution and charge of the cell membrane (Otto, 2014).

Our data show that PSMα2 and PSMα3 are internalized by DCs independently of binding to their receptor FPR2 and independently of macropinocytosis or receptor-mediated endocytosis. Instead, PSMα3 peptides penetrate DCs by transient pore formation. For δ-toxin, one member of the PSM group, it is already known that short-lived pores are formed, and these are dependent on peptide density. High concentrations induce polymer structures that disturb the membrane and ultimately lead to membrane fragmentation (Talbot et al., 2001). These findings support our results, as δ-toxin showed the same lactate dehydrogenase (LDH) release levels from DCs as PSMα3, and δ-toxin is classified as an α-type PSM based on its structure and functions. In neutrophils, high concentrations of PSMα3 cause lysis of the cells independent of the FPR2 receptor, which is also likely due to membrane damage (Kretschmer et al., 2010; R. Wang et al., 2007). Thus, PSMs can be classified into the group of pore-forming toxins (PFTs), which are known for their virulence properties in a large number of pathogens. Furthermore, PFTs can induce the production of various cytokines, including TNF-α, IL-1β and IL-6, which are mainly produced by phagocytes (Chopra et al., 2000). PFTs are also beneficial for the bacteria by subverting the host immune response via their cytotoxic activity against immune cells or by supporting intracellular survival of the pathogens. Other studies have already shown that PSMα peptides are essential for the phagosomal escape of *S. aureus* and their intracellular replication, which further supports the theory that PSMs belong to the PFT family (Grosz et al., 2014).

In summary, PFTs are essential for *S. aureus* to ensure survival against the host innate immune response, where α-type PSMs play a crucial role in pathogenesis. In addition, β-type PSMs are critical for the processes of surface colonization and/or biofilm formation. This group of virulence factors has the potential to be used as a target for new therapeutic strategies, as PFTs are widespread among bacterial pathogens. In the search for new potential abtibacterial drugs or new targets for vaccination, targeting virulence factors like PFTs may be a promising possibility in fighting multi-resistant *S. aureus* strains. Furthermore, PFTs can be used as a component of cocktails to attack infected cells.

4.3 Tolerogenic DCs induced by various virulence factors

DCs, which are essential APCs, play an important role in linking innate and adaptive immunity and are also in the position to suppress or resolve ongoing immune responses. These cells are key players in the induction and maintenance of central and peripheral tolerance (Steinman et al., 2003). The so-called tolerogenic DCs induce tolerance through several different mechanisms, including the priming of regulatory T cells, the suppression of T effector cells, the initiation of apoptosis of autoreactive T cells and the production of immunomodulatory and immunosuppressive molecules (Bonasio et al., 2006; Ilarregui et al., 2009; Maldonado and Andrian, 2010). Different frameworks exist to distinguish between a more tolerogenic or more inflammatory DC. The activation status of the DC, as well as the microenvironment of the progenitor cells, plays a key role in this distinction (Tisch, 2016; Xia et al., 2016). Tolerogenic DCs contain different DC subgroups comprising immature DCs as well as DCs with a more mature activation status. At first, immature DCs were described as having a low-level MHC class II and co-stimulatory molecule expression with the ability to prime naïve $CD4^+$ T cells into T_{regs}; however, more mature DCs share this capacity (Spörri and Reis e Sousa, 2005). Immature DCs can be manipulated by exposure to different cytokines or pathogenic factors, leading to a mature DC phenotype characterized by an increased expression of MHC class II and co-stimulatory molecules, but their functions in priming T_{regs} are not altered (Tisch, 2016).

Here, we demonstrate that BM-DCs stimulated with various TLR ligands and PSMα3 of *S. aureus* show a tolerogenic phenotype. The stimulation leads to increased expression of MHC class II, CD80 and CD86 as well as increased priming of T_{regs}, but it also results in reduced production of the T_h17 cytokine IL-17A and complete inhibition of the T_h1 cytokine IFN-γ. *S. aureus* is not the only pathogen that uses the production of toxins to induce tolerogenic DCs, which then support the pathogen by subverting the host immune system. Adenylate cyclase toxin CyaA from *Bordetella pertussis* in combination with a TLR signal reprograms DCs towards a tolerogenic phenotype that induces a T_h2/T_{reg} response. CyaA promotes a similar cytokine profile to PSMα3 in tolerogenic

DCs, including suppression of IL-12 but enhanced IL-10 production combined with an increase in MHC class II, CD80 and CD86 (Ross et al., 2004). Furthermore, VacA, a pore-forming toxin of *Helicobacter pylori*, displays a similar phenotype to PSMα peptides in inducing tolerogenic DCs. VacA-stimulated BM-DCs in combination with the TLR4 ligand LPS prevent IL-12 production and induce priming of T regulatory cells. In contrast, VacA mutants demonstrat an enhanced T_h1 and T_h17 response, further supporting our results (Oertli et al., 2013). Priming of T_{regs} by PSMα3 and TLR-stimulated BM-DCs through increased production of IL-10 by these cells is dependent on the MAPK signaling molecule p38. CyaA and VacA are also well known for their ability to modulate MAPK p38 activation (Hickey et al., 2008; Isomoto et al., 2010).

In general, tolerogenic DCs appear to be an interesting target as they are triggered by various different pathogens and have a wide influence on host cell immune responses in both healthy and disease states. Further understanding the mechanisms that induce tolerogenic DCs will be beneficial in the future to generate tolerogenic DCs *ex vivo* as potential new strategies for different therapeutic approaches. With their increased IL-10 secretion, tolerogenic DCs play key roles in bacterial infections and inflammatory diseases, including multiple sclerosis, and in promoting the long-term survival of transplant patients and people with autoimmune diseases.

4.4 **References**

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List of Publications

1. **Nicole S. Armbruster**, Jennifer R. Richardson, Jens Schreiner, Juliane Klenk, Manina Günter, Dorothee Kretschmer, Simone Pöschel, Katja Schenke-Layland, Hubert Kalbacher, Kristopher Clark, Stella E. Autenrieth.
   PSM Peptides of Staphylococcus aureus Activate the p38-CREB Pathway in Dendritic Cells, Thereby Modulating Cytokine Production and T Cell Priming.
   J Immunol 02/2016

2. **Nicole S. Armbruster**, Jennifer R. Richardson, Jens Schreiner, Juliane Klenk, Manina Günter, Stella E. Autenrieth.
   Staphylococcus aureus PSM peptides induce tolerogenic dendritic cells upon treatment with ligands of extracellular and intracellular TLRs.
   Int J Med Microbiol. 09/2016

Contribution to the publications in the thesis

Paper 1:

PSM Peptides of Staphylococcus aureus Activate the p38-CREB Pathway in Dendritic Cells, Thereby Modulating Cytokine Production and T Cell Priming.

All experiments were performed by me except of figure 8, the colocalization of PSMs with p38 and phosphorylated p38 measured with the Image-Stream^X mk II performed by Jennifer R. Richardson in assistance with the technician Simone Pöschel and the experiments in the supplement figure 1C for the time points 5 min, 15 min and 60 min, the p38 phosphorylation kinetic carried out by Jens Schreiner. The technicians Juliane Klenk and Manina Günter assisted me in the generation of BM-DCs and in the co-culture experiments. I analyzed the data, prepared the figures and wrote the manuscript together with Stella E. Autenrieth. All the other authors contributed materials and ideas to the study as well as the manuscript.

Paper 2:

Staphylococcus aureus PSM Peptides induce tolerogenic dendritic cells upon treatment with ligands of extracellular and intracellular TLRs.

I performed all the experiments except of one of the three independent experiments in figure 1 carried out by Jens Schreiner and one of the three independent experiments in figure 4 performed by Jennifer R. Richardson. The technicians Juliane Klenk and Manina Günter assisted me in the generation of BM-DCs and in the co-culture experiments. Furthermore I analyzed the data, prepared the figures and wrote the manuscript together with Stella E. Autenrieth.

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