Staphylococcus aureus phenol-soluble modulin peptides impair human monocyte-derived dendritic cell functions and thereby affect the adaptive immune response

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Summary

Staphylococcus aureus (Sa) is a major human pathogen that secrets a great variability of virulence factors, which are very effective at subverting the human immune system. Phenol-soluble modulin (PSM) peptide toxins are one group of these secreted virulence factors, which highly contribute to Sa pathogenicity and are not only able to modulate immune cells, but also to lyse blood cells. Dendritic cells (DCs) are professional antigen presenting cells that link the innate and the adaptive immune response. Previously, the analysis of mouse bone marrow-derived DCs showed that PSMs have the ability to impair their protective functions in the immune system. However, the impact of Sa PSM peptides on human DCs was hitherto unknown. We therefore analyzed in this study the effect of PSMα3 on human monocyte-derived DC (moDC) maturation and functions, like cytokine production, antigen uptake, and T cell stimulatory capacity upon simultaneous treatment with either LPS (Toll-like receptor (TLR)4 ligand) or Sa cell lysate (TLR2 ligand).

PSMs were able to penetrate the moDC cell membrane and enter the cytosol upon transient pore formation without cell lysis as shown by imaging flow cytometry. Upon simultaneous treatment with TLR ligands PSMs impaired DC functions, like reducing antigen uptake via clathrin-mediated endocytosis, modulating DC maturation, and preventing cytokine production. As a consequence, the adaptive immune response was affected by impaired T helper (Th) 1 differentiation. Instead the frequency and proliferation of regulatory T cells (T_{reg}) with suppressive capacity was increased. Moreover, this T_{reg} induction was also observed in an allogenic and autologous autoimmune disease setting, when $CD4^+$ T cells from patients with Th1/Th17-associated spondyloarthritis were co-cultured with PSMα3-treated mature DCs.

Thus, PSMs from highly virulent Sa strains affect DC functions not only in the mouse, but also in the human system, by priming tolerogenic DCs (tDCs), which modulate the adaptive immune response and thereby probably increase the tolerance towards the bacteria. The ability of PSMα3 as a novel peptide to prime tDCs could be beneficial for clinical applications, like vaccination strategies. If this approach is feasible for treating autoimmune diseases *in vivo*, by either generating tDCs *ex vivo* or by the administration of PSMα3 has to be further investigated.

Zusammenfassung

Staphylococcus aureus (Sa) ist ein bedeutender humaner Krankheitserreger, welcher eine große Vielfalt an Virulenzfaktoren sekretiert, die sehr effektiv zur Umgehung des humanen Immunsystems beitragen. Phenol-lösliche modulin (PSM) Peptidtoxine sind eine Gruppe dieser sekretierten Virulenzfaktoren, welche stark zur Pathogenität von Sa beitragen, da sie nicht nur imstande sind Immunzellen zu beeinflussen, sondern auch Blutzellen zu lysieren. Dendritische Zellen (DCs) sind professionelle Antigen-präsentierende Zellen, welche die angeborene mit der erworbenen Immunantwort verknüpfen. Bislang konnte anhand der Analyse von aus Maus Knochenmark *in vitro* generierten DCs gezeigt werden, dass PSMs dazu fähig sind deren protektiven immunologischen Funktionen zu beeinträchtigen. Der Einfluss von Sa PSM Peptiden auf humane DCs war bisher unbekannt. Daher wurden in dieser Studie die Auswirkungen von PSMα3 nach gleichzeitiger Behandlung von humanen von Monozyten-abstammenden DC (moDC) mit LPS (Toll-ähnlicher Rezeptor (TLR)4 Ligand) oder Sa Zelllysat (TLR2 Ligand) auf die Reifung, die Zytokinproduktion, die Antigenaufnahme und die Fähigkeit T Zellen zu stimulieren, untersucht.

Durch bildgebende Durchflusszytometrie wurde hier gezeigt, dass PSMs imstande waren die Zellmembran von moDCs durch transiente Porenbildung zu penetrieren, um ins Zytosol zu gelangen, ohne dabei Zelltod zu induzieren. Durch die gleichzeitige Behandlung mit TLR Liganden und PSMα3 wurden die Funktionen von moDCs, wie folgt beeinträchtigt: verringerte Antigenaufnahme via Clathrin-vermittelter Endozytose, veränderte DC Reifung und verminderte Zytokinproduktion. Dies führte, durch eine beinträchtige Differenzierung in T-Helfer (Th) 1 Zellen zur Beeinflussung der adaptiven Immunantwort. Stattdessen war die Anzahl und Proliferation von funktionell suppressiven regulatorischen T Zellen (T_{reg}) erhöht. Darüber hinaus konnte diese Induktion an T_{regs} in allogenen und autologen Autoimmunkrankheitssettings beobachtet werden. Hierfür wurden $CD4^+$ T Zellen von Patienten mit Th1/Th17-assoziierter Spondyloarthritis mit PSMα3-behandelten reifen DCs ko-kultiviert.

Folglich beeinflussen PSMs von hochvirulenten Sa Stämmen nicht nur DCs in der Maus, sondern auch im humanen System. Dies erfolgt durch die Entstehung von tolerogenen DCs (tDCs), welche die adaptive Immunantwort verändern, was womöglich zur erhöhten Toleranz gegenüber dem Bakterium führt. Somit könnte

PSMα3 als ein neuartiges Peptid zur tDC Induktion verwendet werden, was für klinische Applikationen, wie Vakzinierungen, von Nutzen sein könnte. Ob dies durch eine *ex vivo* Generierung von tDCs oder durch Gabe von PSMα3 für die Therapie von Autoimmunkrankheiten *in vivo* möglich wäre, muss noch weiter untersucht werden.

1. Introduction

1.1 Dendritic cells

In the 1970s Steinman and Cohn discovered the dendritic cell (DC) and described its unique role in the immune system (Steinman, 1974). DCs are rare (≈1 % of total hematopoietic cells) bone marrow-derived leukocytes that are crucial for the initiation and control of immune responses; they are found in blood, lymphoid and non-lymphoid tissues (O'Keeffe et al., 2015). DCs are sentinels that continuously sample the environment for antigens (O'Keeffe et al., 2015). As cells of the innate immune system, DCs are able to recognize, take up and process extra- as well as intracellular antigens, thereby functioning as a bridge between the innate and adaptive immunity not only to initiate an acute inflammatory response but also to maintain self-tolerance (Collin & Bigley, 2018). The encounter with antigens leads to the maturation of DCs via the activation of pattern recognition receptors (PRRs) necessary for lymph node homing and T-cell activation (Hackstein & Thomson, 2004). The control of the immune response, directing tolerance or immunity, not only depends on the activation state of DCs but also on the existence of different types of DCs, each of which are specialized to respond to particular pathogens and to interact with specific subsets of T cells (Collin & Bigley, 2018; Collin, McGovern, & Haniffa, 2013; O'Keeffe et al., 2015).

1.1.1 Human dendritic cell subsets

DCs are a heterogeneous group of immune cells and the wide range of immune responses is orchestrated by three major DC subsets: plasmacytoid DCs (pDC) and the two conventional DCs (cDC), cDC1 and cDC2 (Breton et al., 2015; Collin & Bigley, 2018; Lewis & Reizis, 2012; Merad et al., 2013). The development into the different subsets is controlled by specific transcription and growth factors and is characterized by hierarchal differentiation from DC-specific progenitor cells (Collin & Bigley, 2018). The functionally distinct subsets differ in their surface molecule expression. Plasmacytoid DCs produce type I interferons (IFN) in response to viral infections and are identified by the expression of blood DC antigen (BDCA)-2/cluster of differentiation (CD)303 (Colonna, Trinchieri, & Liu, 2004) and their development is driven by the transcription factor E2-2 (Collin & Bigley, 2018). The two subsets of cDCs are distinguished by the expression of BDCA-1/CD1c and BDCA-3/CD141.

The $CD1c^+$ DCs (cDC2) are the major human cDC subset, with a frequency of about 1% of all mononuclear cells in the circulation and are dependent upon the transcription factors IRF4 and Notch2 (Collin & Bigley, 2018). They are pivotal for $CD4^+$ T-cell priming (Collin et al., 2013; Jin et al., 2014; Leal Rojas et al., 2017) promoting T helper (Th)17- and Th2-biased immune responses to extracellular pathogens (Persson et al., 2013; Piccioli et al., 2007; Schlitzer et al., 2013). The $CD141^+$ DCs (cDC1) only make up around 0.1% of mononuclear cells in the circulation and are therefore a fairly rare subset depending on IRF8 and BATF3 (Collin & Bigley, 2018; Jongbloed et al., 2010; Piccioli et al., 2007) specialized in cross-presenting extra cellular antigens to activate $CD8^+$ T cells and promote Th1 responses (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010; Robbins et al., 2008; Villadangos & Shortman, 2010). Despite the fact that DCs are present in almost all tissues in the whole body, only a small percentage of tissue cells are DCs. Hence it is hard to analyze small DC populations, especially in humans (Merad & Manz, 2009). The discovery of differentiating human blood monocytes into DCs (monocyte-derived DCs; moDCs) upon cytokine treatment made the DC research much easier. The key cytokine for this differentiation is granulocyte-macrophage colony-stimulating factor (GM-CSF), but also other cytokines like Interleukin (IL)-4 and tumor necrosis factor (TNF) are needed (Merad & Manz, 2009). These moDCs have been widely used to study human DC biology and as a tool for vaccination strategies in various disease settings, e.g. as cancer therapy (P. Chen et al., 2015).

1.1.2 Antigen uptake, processing and presenting by dendritic cells

DCs are remarkable potent initiators of the adaptive immune response and have the unique ability to activate naïve T cells thereby shaping the immune response by driving differentiation into the distinct effector T-cell subsets (Mellman & Steinman, 2001). DCs sample their surroundings and internalize, process, and present captured antigens on major histocompatibility complex (MHC) class I (MHC I) and MHC class II (MHC II) molecules. In order to initiate a sufficient T-cell response, DCs undergo functional and morphological changes, which is termed DC maturation.

Immature DCs (iDCs) capture and take up antigens via three different mechanisms: macropinocytosis, receptor-mediated endocytosis and phagocytosis (Lanzavecchia, 1996; Sallusto et al., 1995). Macropinocytosis, also referred to as fluid-phase

endocytosis, allows DCs to continuously take up very high levels of fluid phase volume containing possible antigens. This process is dependent on membrane ruffling by cytoskeletal rearrangement in order to form macropinosomes with a size of 0.5 – 3 µm (Sallusto et al., 1995; Villadangos & Schnorrer, 2007). The other two routes of antigen capture and internalization are mediated by receptors found on the DC surface. iDCs have a high endocytic activity mediated by various receptors, like the macrophage mannose receptor (MMR/CD206), Fc receptors, the transferrin receptor and C-type lectin receptors (Cella et al., 1997; Lanzavecchia, 1996; Sallusto et al., 1995). Once an antigen has been bound to one of the receptors, the antigen-receptor complex is internalized into clathrin-coated pits. Further, the cargo is either released at endosomal pH and the receptor recycles, e.g. the mannose-receptor. In a case involving Fc receptors, they are degraded in the endosomes as well (Cella et al., 1997). Lastly, DCs are able to internalize particles by phagocytosis. Phagocytosis is triggered by the encounter of extracellular particles with surface receptors, like Fc receptors and complement receptors (Mellman & Steinman, 2001). This process is used to take up whole microorganisms or apoptotic cells, which are labeled by antibodies or complement proteins (Cella et al., 1997; Lanzavecchia, 1996; Stuart & Ezekowitz, 2008).

Antigens must be processed into peptides in order to be presented on MHC I molecules to activate $CD8^+$ T cells or on MHC II to interact with $CD4^+$ T cells (Sprent, 1995). Endogenous antigens are presented on MHC I molecules after being degraded by the immunoproteasome in the cytosol, transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) and loaded onto MHC I molecules (Muchamuel et al., 2009; Sprent, 1995; Villadangos & Schnorrer, 2007). In contrast, exogenous antigens following internalization are processed by proteases in the endosomal compartments and presented on MHC II molecules. Additionally, cDC1 are able to present exogenous antigens via MHC I molecules to $CD8^+$ T cells, this is known as cross-presentation (Sprent, 1995; Villadangos & Schnorrer, 2007). It has been shown that TAP is involved in this process and that the phagosomes fuse with the ER providing the machinery for peptide loading. An indirect phagosome to cytosol pathway was also proposed, including processing of the peptides via the immunoproteasome (Palmowski et al., 2006).

1.1.3 Maturation of dendritic cells

Antigen presentation to T cells is highly controlled and is dependent upon a mechanism called DC maturation, which is initiated in the periphery. Immature DCs, the sentinels of the peripheral tissues are highly active antigen-capturing cells, with relatively low levels of MHC class II and costimulatory molecules on their cell surface. Therefore, iDCs have low stimulatory activity to activate naïve T cells, whereas mature DCs (mDCs) downregulate endocytic activity and are efficient T-cell activators (Mellman & Steinman, 2001; Tan & O'Neill, 2005). During infection or inflammation, DCs induce immunity after antigens are captured in the presence of activating stimuli like microbial products, inflammatory cytokines, or encounters with TNF receptors (Banchereau et al., 2000; Hackstein, Morelli, & Thomson, 2001; Tan & O'Neill, 2005). The absence of these signals leads to induction of tolerance. Activated and matured DCs undergo morphological changes, downregulate their endocytic capacity, and on the contrary upregulate costimulatory molecules like CD40, CD80 and CD86 and MHC II (Banchereau et al., 2000; Hackstein et al., 2001). Furthermore, chemokine receptors are up-regulated, an important process for the migration to T-cell zones in secondary lymphoid organs (Ardeshna et al., 2000). The recognition of these activating stimuli, danger- or pathogen-associated molecular patterns (DAMPs, PAMPs), is mediated by various germline encoded PRRs triggering maturation. Immature DCs express a wide variety of PRRs, such as like toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, RIG-I-like receptors and C-type lectin receptors (Kanneganti et al., 2007; Kawai & Akira, 2007; Matzinger, 2002; Medzhitov, 2001; Shaw et al., 2010; Tang et al., 2012; Villadangos & Schnorrer, 2007). The TLR family is one of the best-characterized PRRs and is important for the control of DC maturation and cytokine production. TLRs are constitutively expressed and highly conserved throughout different species; so far 12 TLRs have been identified for the mammalian system. While recognizing a great variety of pathogens, TLRs contribute to the immune systems discrimination between self and foreign and thus make the innate immunity specific for microbial products (Kawai & Akira, 2011). TLRs are type I transmembrane receptors found at the cell surface (TLRs 1, 2, 4, 5, 6, 10, and 11) or intra-cellularly in endosomes (TLRs 3, 7, 8, and 9), possessing C-terminal leucine-rich repeats important for recognition, a trans-membrane domain and a Toll/Interleukin-1R (TIR) signaling domain (Kawai & Akira, 2011; Manicassamy &

Pulendran, 2009). Compliant with their role in the immune system and in immunosurveillance, TLRs are highly expressed in tissues involved in immune function (e.g. spleen, peripheral blood), as well as in tissues exposed to the external environment (e.g. lung, gastrointestinal tract). Furthermore, these tissues also display the most diverse repertoire of TLRs (Zarember & Godowski, 2002). The distinct cell types in various tissues express varying levels of TLRs. Analysis of peripheral blood leukocytes showed that the greatest variety of TLRs are expressed by $CD14^+$ mononuclear cells (Zarember & Godowski, 2002). However, the different TLR expression by the various cell types implies that specific roles exist in each population relating to the immunological function (Schreibelt et al., 2010; Zarember & Godowski, 2002). The distinct DC subsets also express different TLRs, which causes diverse effects when activated, reflecting their different functions in the immune response (Schreibelt et al., 2010). PDCs are important in viral infections and express TLR7 and TLR9, which are endosomal pattern recognition receptors, capable of sensing single-stranded RNA and double-stranded DNA, and TLR1 to some extent (Collin & Bigley, 2018; Schreibelt et al., 2010). Required for the efficient control of viral and intracellular antigens are cDC1s that express TLR3, 9 and 10. TLR3 and TLR10 are selectively expressed by this DC subset (Collin & Bigley, 2018). In common with monocytes, cDC2s express TLR2, 4, 5, 6, and 8; they highly respond to extracellular antigens (Collin & Bigley, 2018). Lastly, *in vitro* differentiated moDCs express all TLRs except for TLR9 and TLR10, however, not all to the same extent (Schreibelt et al., 2010). Upon the recognition of their ligands, TLRs dimerize thereby recruiting adaptor proteins such as myeloid differentiation primary response 88 (MyD88), to TIR domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) to the TLR TIR domains. Whereas most of the TLRs are MyD88-dependent, TLR3 exclusively and TLR4 in part signal via TRIF. The interaction of the adaptor protein TIR domains with the TLR TIR domains leads to the activation of the Interleukin-1 receptor-associated kinase (IRAK) family and TNF receptor associated factor 6 (TRAF6), which in turn activates mitogen-activated protein kinases (MAPK) and transcription factors nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κb) and activator protein 1 (AP-1). These are important for inflammatory cytokine induction, type I interferons (IFN), and upregulation of costimulatory molecules and C-C chemokine receptor type 7 (Figure 1-1; (Manicassamy & Pulendran, 2009)).

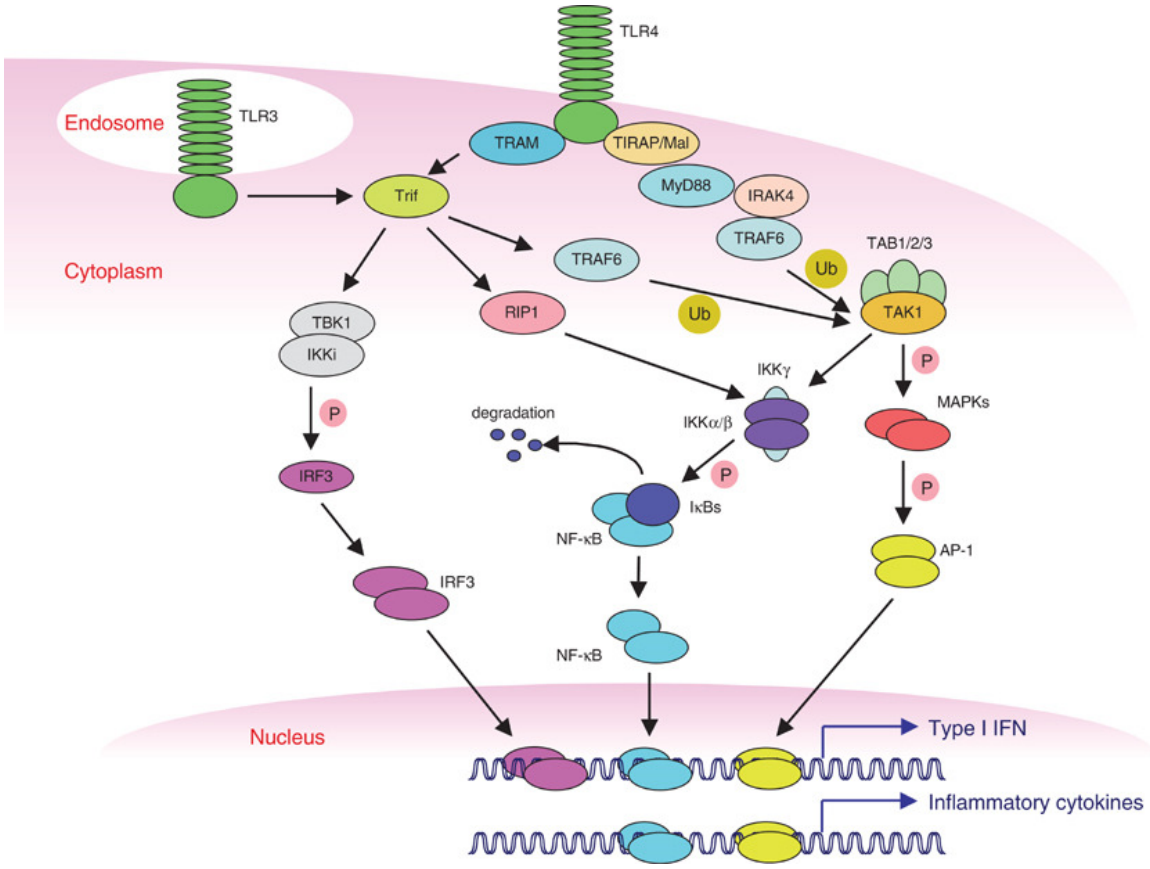

Figure 1-1: TLR signaling pathways (Kawai & Akira, 2006)

TLR are present on cell surfaces (e.g. TLR4) or localized in endosomes (e.g. TLR3). Both cell surface and endosomal TLRs induce downstream signaling, this leads to the induction of inflammatory cytokine and Type I IFNs expression. TLR3 exclusively signals via the MyD88- independent pathway, whereas TLR4 uses both pathways.

1.1.4 Tolerogenic DCs

Apart from inducing an efficient immune response, DCs are also crucial for maintaining immune tolerance against self-antigens in the steady-state. These tolerogenic DCs (tDCs) mediate tolerance by inducing T-cell anergy and deletion by insufficient stimulatory signals or by inducing the differentiation of regulatory T cells (T_{regs}) (Raker et al., 2015). Although the specific phenotype of tDCs and the molecular mechanism involved in tolerance induction by these cells are not entirely defined, especially as the group of tDCs is heterogeneous comprising naturally occurring and induced tDCs, it is thought that the maturation status plays a critical role (Gordon, 2014; Kushwah & Hu, 2011; Raker et al., 2015; Yoo & Ha, 2016). Whereas mDCs mediate immunity, iDCs, with a low MHC II and low costimulatory molecule phenotype and high production of anti-inflammatory cytokines, e.g., IL-10 and TGF-ß, which possess critical immunoregulatory functions like

controlling/regulating the production of pro-inflammatory cytokines were suggested to maintain tolerance. These iDCs are poorly immunogenic and are therefore important in inducing tolerance under steady state conditions, after e.g. taking up apoptotic cells (Kushwah et al., 2009; Ouyang et al., 2011; Raker et al., 2015; Villadangos & Schnorrer, 2007). However, the maturation state alone does not define tDCs. Mature or semi-mature DCs were also found to induce tolerance (Raker et al., 2015; Villadangos & Schnorrer, 2007). Additionally, costimulatory as well as coinhibitory molecule expression, production of anti-inflammatory instead of pro-inflammatory cytokines and indoleamine-2,3-dioxygenase (IDO) expression characterizes the tDC phenotype (Raker et al., 2015). With the production of these molecules, tDCs have the potential to induce T_{reg} expansion thereby impairing effector T-cell responses (Armbruster et al., 2016b; Ouyang et al., 2011; Raker et al., 2015; Schreiner et al., 2013). Various pathogens and tumors are also able to induce tDCs and subsequent T_{reg} differentiation thus escaping the immune response. Various pathogenic products from e.g. *Candida albicans, Schistosoma mansoni* and *Vibrio cholerae* are able to mediate this process and are therefore partially used for the production of immunosuppressive drugs, making tDCs a sufficient tool for therapeutic approaches (Maldonado & Andrian, 2010; Ouyang et al., 2011).

1.2 T-cell activation and polarization

Once DCs are activated they migrate to the draining lymph nodes to activate naïve T cells. This event requires multiple signals; signal 1 is the interaction of the antigen-loaded MHC complex with the T-cell receptor. Signal 2 is the engagement of costimulatory or coinhibitory receptors and their ligands. Signal 3 is mediated by cytokines that are released by the mature DCs (Kambayashi & Laufer, 2014; Tai et al., 2018). $CD4^+$ T cells differentiate into different T effector cell subsets, comprising of T helper (Th) 1, Th2, Th17, T follicular helper cells, and induced regulatory T cells (iT_{reg}) all with distinct functions important for the regulation of immune responses. This differentiation, also known as polarization, is initiated upon the interaction of naïve CD4 T cells and antigen-bearing DCs expressing inflammatory cytokines. This process is further dependent upon, and mediated by, the cytokine milieu and the respective key transcription factor of the distinct subsets (Figure 1-2; (Park et al., 2005; Zhou et al., 2009)). Each of these subsets produces effector cytokines that are important for its respective function. IL-12 induces the polarization of Th1 cells driven by the Th1 master transcription factor T-bet in concert with the transcription factor

signal transducer and activator of transcription 4 (STAT4). Th1 cells are characterized as IFN-γ producing Th cells, which mediate cellular immunity against intracellular microorganisms (Park et al., 2005; Szabo et al., 2003; Zhou et al., 2009). Th2 cells are induced by IL-4. They are further regulated and defined by STAT6 and GATA-binding protein 3 (GATA3) expression and are important for the production of IL-4, IL-5 and IL-13 that induces humoral immunity against extracellular pathogens (Park et al., 2005; Szabo et al., 2003; Zhou et al., 2009). Th17 differentiation is induced by TGF-β, IL-6 and IL-23 and is characterized by the expression of retinoic acid receptor-related orphan receptor-γt (RORγt) and STAT3. Th17 effector cells produce IL-17 and IL-22 and are also necessary for the clearance of extracellular pathogens (Z. Chen et al., 2007; Harrington et al., 2005; Zhou et al., 2009; Zou & Restifo, 2010). As well as inducing immunity, the differentiation of $CD4^+$ T cells into T_{regs} regulates Th cell responses and is therefore crucial for maintaining immune homeostasis (Shevach, 2009; Vignali et al., 2008; Zhou et al., 2009). T_{regs} are induced by TGF-β, retinoic acid (RA), and IL-2 and characterized by the expression of the transcription factor forkhead box P3 (FoxP3). T_{regs} are further divided into natural T_{regs} (nT_{regs}), which develop in the thymus and iT_{regs} arising in the periphery, however, both subsets are important for immune tolerance (Schmitt & Williams, 2013). This differentiation of naïve $CD4^+$ T cells into the different effector T-cell subsets is not terminal. Plasticity allows the switch into one of the other subsets, with Th1 and Th2 being the most stable phenotypes (Zhou et al., 2009).

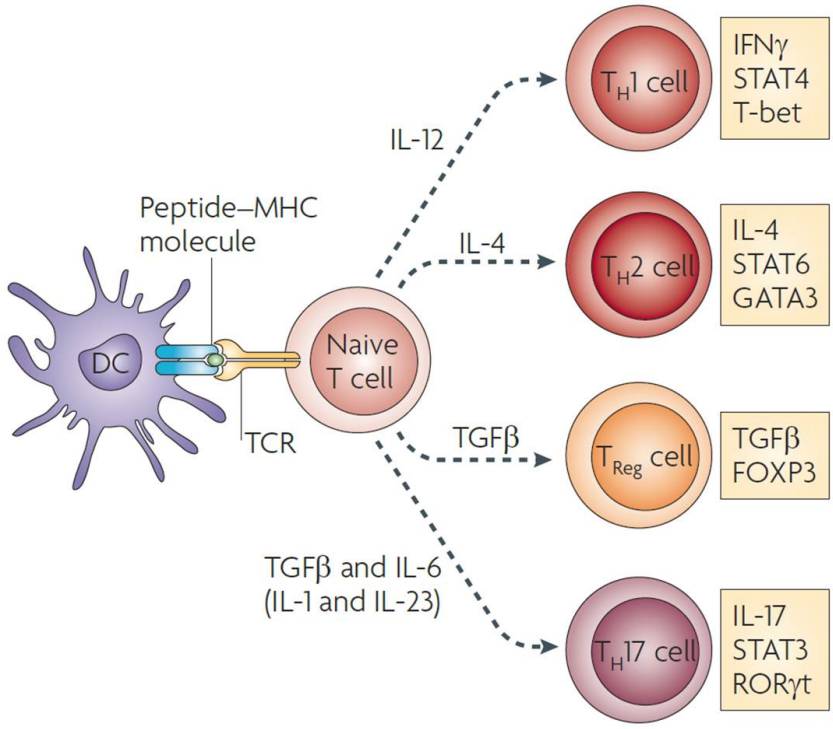

Figure 1-2: $CD4^+$ T-cell polarization into the different Th subsets (Zou & Restifo, 2010)

DCs activate naïve $CD4^+$ T cells by engagement of the antigen-loaded MHC with the TCR. These naïve T cells differentiate into the different Th subsets (Th1, Th2, Th17 or T_{reg}), which is dependent on the local cytokine milieu and driven by the key master transcription factors of each Th cell subset.

1.3 *Staphylococcus aureus* phenol-soluble modulin peptides

Staphylococcus aureus (Sa), a commensal Gram-positive bacterium, is a major human pathogen and a leading cause of morbidity and mortality. Multi-drug resistance and enhanced virulence of Sa constitutes a health threat, especially the community-associated methillin-resistant Sa (CA-MRSA) strains, such as USA300. The wide variability of secreted or cell surface-associated virulence factors and the resulting different strategies to evade the host's immune system are responsible for the high pathogenicity of Sa (Coates et al., 2014; DuMont & Torres, 2014; M. Miller et al., 2009; Peschel & Otto, 2013). Phenol-soluble modulins (PSMs), originally discovered in *Staphylococcus epidermidis* (Mehlin et al., 1999) are a family of secreted short cytolytic peptide toxins produced by highly virulent Staphylococci that greatly contribute to the pathogenesis of Sa (Chatterjee et al., 2013; Cheung et al., 2014; R. Wang et al., 2007). Two types of PSMs with a total of seven members are distinguished according to their length, which all share an amphipathic α-helical structure, a formylated N-terminus, but have limited sequence similarity (Figure 1-3):

the α-type PSMs (PSMα1-4 and δ-toxin; ~20–25 AA) and the β-type PSMs (PSMβ1-2; ~44 AA) (Peschel & Otto, 2013; R. Wang et al., 2007).

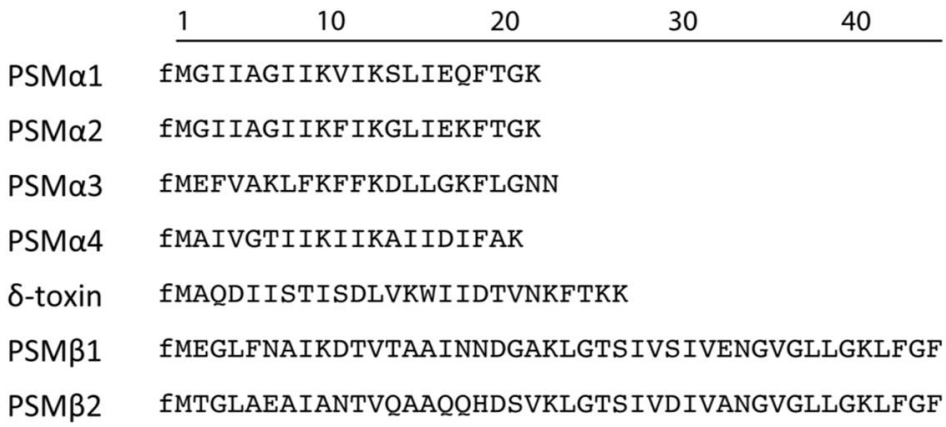

Figure 1-3: Amino acid sequences of the seven PSM family members (R. Wang et al., 2007)

PSMs are capable of lysing many eukaryotic cell types and stimulating inflammatory responses. Due to their surfactant-like properties, they are important in biofilm formation, and are thus key mediators in Sa pathogenesis (Chatterjee et al., 2013; DuMont & Torres, 2014; Grumann et al., 2014; M. Miller et al., 2009; Peschel & Otto, 2013). CA-MRSA strains secrete by far the highest amounts of PSM peptides. However, as the peptides also trigger inflammatory responses, the production is under tight control of the quorum-sensing accessory gene regulator system. This ensures expression only at times of high bacteria density and results in efficient evasion of the immune system (Cheung et al., 2014). Notably, not all PSMs equally contribute to Sa pathogenesis. The PSMα peptides harbor essential functions and are the most potent PSMs with regard to cytolysis. PSMα peptides highly contribute to the virulence and disease manifestation of CA-MRSA (Cheung et al., 2014; Peschel & Otto, 2013; R. Wang et al., 2007).

1.3.1 The effect of phenol-soluble modulins on the immune system

PSM peptide toxins have multiple effects on immune cells, thereby manipulating the immune response. Especially the PSMα peptides, with their high cytolytic activity efficiently contribute to immune evasion. At nanomolar concentrations, PSMs recruit and activate human neutrophils via binding to the N-formyl peptide receptor 2. At higher concentrations, several PSMαs show cytolytic activity with the ability to lyse erythrocytes, monocytes, and human neutrophils. This takes place after phagocytosis by membrane disruption and perturbation in a receptor-independent fashion and

eliminates the cellular innate host defense (Cheung et al., 2011; Forsman et al., 2012; Kretschmer et al., 2010; R. Wang et al., 2007). However, not only the innate immune response but also the adaptive immune system is affected by modulation of DC functions. PSMs affect mouse bone marrow-derived DCs (BM-DCs) by acting as chemoattractants, inhibiting clathrin-mediated endocytosis, which is essential for antigen uptake. Further the secretion of pro-inflammatory cytokines is suppressed. In contrast, IL-10 production is upregulated by increased phosphorylation of the MAPK p38. This results in a reduced Th1 immune response and the frequency of T_{reg} cells is increased, further contributing to immune evasion of Sa (Armbruster et al., 2016b).

1.4 Research objectives

Sa by means of the secretion of PSM peptide toxins is able to evade the innate host defence by lysing neutrophils and monocytes after phagocytosis. Sa is a major human pathogen and as DCs act as link between the innate and adaptive immune response, DC modulation might play an important role in Sa pathogenesis. Indeed, PSMs also affect the adaptive immune response in the mouse by modulating dendritic cell maturation and functions, like antigen uptake and cytokine secretion, leading to a reduced Th1 immune response and to an increased frequency of T_{regs}. Herein, we addressed the question if Sa-derived PSM peptides also affect human dendritic cells and their ability to activate T cells. Human monocyte-derived DCs were simultaneously treated with TLR ligands and PSMα3. The expression of costimulatory, as well as coinhibitory molecules, antigen uptake and T cell stimulatory capacity was analyzed by flow cytometry. Cytokine secretion by moDCs or in co-culture with and therefore upon interaction with T cells was assessed by performing enzyme-linked immunosorbent assays or bead-based immunoassays. Antigen uptake and PSM uptake was further evaluated by imaging flow cytometry. If Sa PSM peptides also show the ability to prime tolerogenic human DCs, it could be beneficial for clinical use of the peptide. Therefore, the ability of the treated moDCs to activate and prime T cells in an autoimmune disease setting was additionally assessed in a mixed lymphocyte reaction and analyzed by flow cytometry.

2. Material and Methods

2.1 Research Subjects

Buffy coats from healthy volunteers were obtained from the ZKT Tübingen GmbH. Fresh blood was obtained from healthy volunteers with informed consent. This was approved by the ethical review committee of the medical faculty of the Eberhard-Karls-University of Tübingen with the project number 633/2012BO2. Blood from patients with Th17-associated autoimmune diseases were obtained from the division of Rheumatology, Department of Internal Medicine II, University Hospital Tübingen. This was approved by the ethical review committee of the medical faculty of the Eberhard-Karls-University of Tübingen with the project number 046/2015BO2.

2.2 Reagents

Formylated PSM peptides (PSMα2, PSMα3, δ-Toxin) were synthesized at the Interfaculty Institute of Cell Biology, Department of Immunology, University of Tübingen. FITC-labeled PSMα2 was synthesized at the Group of Hubert Kalbacher, Interfaculty Institute of Biochemistry, University of Tübingen. Sa cell lysate (Sa lysate) containing lipopeptides and specifically activating TLR2 was prepared from a protein A-deficient Sa mutant strain (SA113) and provided by Andreas Peschel, Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen. The AlexaFluor647-conjugated model antigen Ovalbumin (OVA) was purchased from Invitrogen.

2.3 Isolation of peripheral blood mononuclear cells

Buffy coats or fresh blood was diluted with Dulbecco's PBS (Life Technologies) (Buffy Coats 1:7 blood: PBS; Fresh blood 1:1 blood: PBS). Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation at 2000 rpm for 20 min at room temperature with 35 mL cell suspension stacked on 15 mL Biocoll separation solution (Biochrom). The interphase containing the PBMCs was abstracted and washed twice with PBS. PBMCs were further used to generate human moDCs and for the isolation of naïve $CD4^+$ T cells and $CD4^+$ T cells.

2.4 Generation of human monocyte-derived DCs

PBMCs were plated in a tissue-treated 6-well plate ($6x10^6$ cells per well) in DC medium (RPMI1640 (Merck), 10% FBS (Sigma), 2 mM L-Glutamine (Life

Technologies), 100 U/mL Penicillin-Streptomycin (Life Technologies), 1x non-essential amino acids (Merck), 1 mM sodium pyruvate (Merck) and 50 µM 2-mercaptoethanol (Roth)) and incubated for 1 h at 37°C, 5% CO_2. After that, wells were washed with medium and PBS discarding the non-adherent cells. 3 mL/well DC medium containing 50 ng IL-4 and 100 ng GM-CSF (both from Miltenyi) was added to the remaining cells. Cells were incubated for 6 d at 37°C, 5% CO_2. Cytokines were again added on day 2 and day 4. At day 6 the cells were used for the following experiments. The purity of the moDC culture was always >90% of lymphocytes.

2.5 Stimulation of human monocyte-derived DCs

For stimulation moDCs were seeded in non-tissue culture treated 96-well or 48-well plates (cell number see the particular experiment) and treated with 3 µg/mL Sa lysate or 100 ng/mL LPS in combination with or without PSMα3 (10 µM) and incubated for 6 h, 24 h or 48 h, if not indicated differently for the individual experiments.

2.6 Cytokine/ indolamin-2,3-Dioxygenase production by moDCs

MoDCs ($2{,}5x10^5$) were seeded in a 96-well plate and treated with Sa lysate or LPS in combination with or without PSMα3. Supernatants were collected after 6 h, 24 h or 48 h and analyzed for TNF, IL-10, IL-12 and IDO production, respectively. Cytokines and IDO in the supernatants were determined by sandwich ELISA (eBioscience (TNF, IL-10, IL-12), R&D Systems (IDO)) according to the manufacturer's instructions.

2.7 Dendritic cell surface molecule expression analysis by flow cytometry

For moDC ($CD11c^+HLA\text{-}DR^+$) surface marker analysis of costimulatory and coinhibitory molecules, moDCs ($2x10^5$) were seeded in a 96-well plate and treated with Sa lysate and LPS with or without PSMα3 or with only PSMα3 for 6 h (costimulatory) or 24 h (costimulatory and coinhibitory). Cells were removed from the plate using Accutase (Sigma-Aldrich) and treated with IgG from human serum (1µg of human IgG per 100,000 cells; Sigma-Aldrich) for 20 min at room temperature to avoid unspecific binding via Fc receptors.

Table 2-1: Panel for the analysis of DC surface markers

Panel	Antigen	Fluorochrome	Clone	Company
DC costimulatory Molecules				
	HLA-DR	BV650	L243	BioLegend
	CD11c	APC	MJ4-27G121	Miltenyi
	CD40	FITC	5C3	BioLegend
	CD83	PE/Dazzle594	HB15e	BioLegend
	CD80	PE/Cy7	2D10	BioLegend
	CD86	BV605	IT2.2	BioLegend
		Zombie Aqua		
DC coinhibitory Molecules				
	HLA-DR	BV650	L243	BioLegend
	CD11c	PE/Cy7	Bu15	BioLegend
	PD-L1	PE	29E.2A3	BioLegend
	PD-L2	PE	MIH18	BioLegend
	ILT3	PE	ZM4.1	BioLegend
		Zombie Aqua		BioLegend

Cells were stained according to Table 1 for 20 min at 4°C. Zombie Aqua was used to exclude dead cells. FACS buffer (PBS containing 1% FBS, 2 mM EDTA (Merck) and 0.09% NaN_3 (Sigma-Aldrich)) was used for all incubations and washing steps. At least 50,000 cells were acquired using a LSR Fortessa flow cytometer (BD Biosciences) with the DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.4.2 software (Tree Star).

2.8 Phospho flow cytometry

For the experiments analyzing phosphorylation of p38 and NF-κB, moDCs ($2x10^5$) were seeded in a 96-well plate and treated with LPS with or without PSMα3 or with only PSMα3 for 30 min or 1 h. Cells were removed from the plate using Accutase and incubated with IgG from human serum (1µg of human IgG per 100,000 cells) for 20

min at room temperature to avoid unspecific binding via Fc receptors. Cells were stained with the antibodies listed in Table 2, where Zombie Aqua was used to exclude dead cells and the fluorochrome conjugated HLA-DR and CD11c were used for extracellular staining, which were incubated for 20 min at 4°C.

Table 2-2: Antibody panel for phospho flow cytometry

Panel	Antigen	Fluorochrome	Clone	Company
Phospho flow				
	HLA-DR	PE	L243	BD Biosciences
	CD11c	APC/Cy7	Bu15	BioLegend
	p-NF-κB p65 (Ser536)	-	93H1	Cell Signaling
	p-p38 MAPK (Thr180/Tyr182)	-	12F8	Cell Signaling
	Rabbit IgG	DyLight649		Jackson ImmunoResearch
		Zombie Aqua		BioLegend

To detect intracellular phospho-p38 (p-p38) and phospho-NF-κB (p-NF-κB) cells were fixed with 2% paraformaldehyde (VWR) in PBS, permeabilized with 90% freezing methanol (Applichem) overnight and stained with the primary Abs to p-p38 MAPK or phospho-NF-κB p65 for 1 h in the dark at room temperature followed by staining with secondary DyLight649-conjugated AffiniPure Goat At-Rabbit IgG antibody for 15 min at 4°C. PBS with 0.5% BSA (Biomol) was used for incubation and washing steps of intracellular antibody staining. At least 50,000 cells were acquired using a Canto-II (BD Biosciences) with DIVA software (BD Biosciences) and were further analyzed using the FlowJo 10.4.2 software (Tree Star).

2.9 Measurement of antigen uptake by flow cytometry or multispectral imaging flow cytometry

MoDCs ($5x10^5$) were seeded in a 48-well plate and treated for 24 h with Sa lysate or LPS with or without PSMα3 or with only PSMα3 prior to the incubation with OVA-AlexaFluor647 (5 µg/mL, Invitrogen) together with PSMα2 FITC (0.5 µM) for 30 min at 37°C, 5% CO_2. Unspecific binding of OVA/PSMα2 was assessed by incubating the cells on ice. Cells were washed twice with ice-cold PBS containing 2% FBS.

Subsequently, cells were blocked with IgG from human serum for 20 min at room temperature and stained according to Table 3 for flow cytometry analysis and Table 4 for imaging flow cytometry analysis for 20 min at 4°C.

Table 2-3: Antibody panels for antigen uptake analysis at the flow cytometry and the Image-Stream mkII

Panel	Antigen	Fluorochrome	Clone	Company
Antigen uptake FACS				
	HLA-DR	BV650	L243	BioLegend
	CD11c	PE/Cy7	Bu15	BioLegend
		Zombie Aqua		BioLegend
Antigen uptake ISX				
	HLA-DR	PE	L243	BD Biosciences
	CD11c	PE/Cy7	Bu15	BioLegend
		DAPI		Sigma

At least 50,000 cells were acquired using a LSR Fortessa flow cytometer (BD Biosciences) with the DIVA software (BD Biosciences) and further analyzed using the FlowJo 10.4.2 software (Tree Star). Images of 10,000 living moDCs were acquired using the Image-Stream mkII (ISX; Amnis) with the INSPIRE instrument controller software. The data were analyzed using the IDEAS analysis software (Merck Millipore).

2.10 Lactate dehydrogenase release

MoDCs ($2x10^5$) were seeded in a 96-well plate and treated with Triton X100 (1%; Sigma-Aldrich), DMSO (2%, Fluka), PSMα2 (10 µM), PSMα3 (2.5 µM, 5 µM, 7.5 µM, 10 µM), δ-Toxin (10 µM) or OVA (10 µg, Sigma-Aldrich) for 10 min at 37°C, 5% CO_2. Supernatants were used for the analysis of lactate dehydrogenase (LDH) release using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. Absorbance was measured at 492 nm and 620 nm over a period of 1 h with an interval of 5 min using the Spark 10M microplate reader (Tecan). Cell death was determined by staining the cells with 7-aminoactinomycin D (7-AAD, Biomol) and acquiring on a Canto II flow cytometer with the DIVA software.

2.11 T-cell assay/ allogenic T-cell assay

MoDCs ($5x10^4$) were seeded in a 96-well plate and treated with Sa lysate or LPS with or without PSMα3 or with only PSMα3 for 24 h. For some experiments moDCs were pre-treated with the IDO Inhibitor 1-Methyl-D-tryptophan (1-DMT; 200 µM) or with a human IL-4 Rα blocking antibody (R&D Systems) for 1 h prior to the stimulation. The human IL-4 Rα antibody was again added on day 2 of the co-culture. Human naïve $CD4^+$ T cells were isolated from PBMCs using the MojoSort™ Human CD4 Naïve T Cell Isolation Kit (BioLegend) according to the manufacturer's instructions. For the allogenic T cell assay $CD4^+$ T cells from patients were isolated using CD4 Microbeads (Miltenyi Biotech) according to the manufacturer's instructions. For the magnetic cell separation, a LS column (Miltenyi Biotech) was placed into the QuadroMACS Separator (Miltenyi Biotech) and rinsed with MACS buffer (PBS containing 0,5% BSA (Biomol) and 2 mM EDTA). The cell suspension was applied to the column, and the column was washed three times with 3 mL MACS buffer. The untouched naïve $CD4^+$ T cells were collected in the flow through. The purity of isolated (naïve) $CD4^+$ T cells was always ≈85%. The naïve $CD4^+$ T cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE; 5 µM, BioLegend) according to the manufacturer's instructions. $2x10^5$ T cells diluted in 100 µL T cell medium (RPMI1640 (Merck), 10% FBS (Sigma), 2 mM L-Glutamine (Life Technologies), 100 U/mL penicillin-streptomycin (Life Technologies), 1x non-essential amino acids (Merck), 1 mM sodium pyruvate (Merck), 10 mM HEPES (Biochrom) and 50 µM 2-mercaptoethanol (Roth)) were added to the moDCs. To investigate whether secreted factors from DCs upon PSM-treatment mediate T_{reg} priming, T cells were co-cultured with untreated moDCs adding conditioned medium from LPS or LPS + PSMα3 stimulated DCs. In a third assay moDCs treated as described above were splitted using Accutase (Sigma-Aldrich) for 5 min at room temperature and again sowed with either fresh or conditioned DC medium (TLRL or TLRL + PSMα3). In some conditions, DC were fixed with 1% paraformaldehyde for 10 min at 4°C to address the impact of newly secreted factors on T_{reg} priming by DCs. 3-4 d after co-culture T cells were blocked with IgG from human serum for 15 min at room temperature and subsequently stained extracellularly with the antibodies listed in Table 5 for 20 min at 4°C. For intracellular staining, cells were fixed and permeabilized with the Foxp3/ Transcription Factor Staining Buffer Set

(eBiosciences), blocked and stained with the intracellular antibodies listed in Table 5 for 45 min at 4°C.

Table 2-4: Antibody panel for T-cell subset analysis

Panel	Antigen	Location	Fluorochrome	Clone	Company
T cells					
	CD3	Extracellular	Pacific Blue	SK7	BioLegend
	CD4	Extracellular	APC-Vio770	REA623	Miltenyi
	CD25	Extracellular	PE-Cy7	BC96	eBioscience
	CD45RA	Extracellular	BV605	HI100	BioLegend
	CD127	Extracellular	PE	eBioRDR5	eBioscience
	FoxP3	Intracellular	AlexaFluor647	259D	BioLegend
	T-bet	Intracellular	PE/Dazzle594	4B10	BioLegend
	GATA3	Intracellular	PerCP/Cy5.5	16E10A23	BioLegend
	RORγt	Intracellular	BV650	Q21-559	BD Biosciences
			CFSE		BioLegend
			Zombie Aqua		BioLegend

FACS buffer was used for all incubations and washing steps for the extracellular staining, and 1x permeabilization buffer (Foxp3/ Transcription Factor Staining Buffer Set (eBiosciences)) was used for all incubations and washing steps for the intracellular staining. At least 70,000 cells were acquired using an LSR Fortessa flow cytometer (BD Biosciences) with the DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.4.2 software (Tree Star).

2.12 Autologous T- cell assay

$CD14^+$ cells from PBMCs of patients with Th17-associated autoimmune diseases were isolated by MACS using CD14 MicroBeads (Miltenyi Biotech) and plated in a tissue-treated 6-well plate ($1.3x10^6$ cells per well) in DC medium containing 50 ng IL-4 and 100 ng GM-CSF for 6 d to generate moDCs. The remaining $CD14^-$ cells were frozen at -80°C in RPMI1640 supplemented with 20 % FBS and 10% DMSO. After 6 d moDCs ($5x10^4$) were seeded in a 96-well plate and treated with LPS with or without PSMα3 for 24 h. The $CD14^-$ cells were thawed and used to isolate $CD4^+$ T cells by MACS with CD4 MicroBeads (Miltenyi Biotech). The $CD4^+$ T cells were labeled with CFSE (5 µM) according to the manufacturer's instructions and $2x10^5$ T cells diluted in

100 µL T-cell medium were added to the moDCs. 3-4 d after co-culture T cells were stained as above (see Table 5) and iT_{regs} ($CD3^+CD4^+CD127^-CD25^{hi}CD45RA^-$ $FoxP3^{hi}$) were analyzed by flow cytometry using an LSR Fortessa flow cytometer (BD Biosciences) with the DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.4.2 software (Tree Star).

2.13 Cytokine production in the moDC-T-cell co-culture

50 µL cell culture supernatants from the T cell assay were collected on day 1, 2 and 3 and cytokine production from 15 µL was analyzed by performing bead-based immunoassays in a 96-well plate (LEGENDplex human B cell Panel (13-Plex) and LEGENDplex Free Active/Total TGF-β1 (BioLegend)) according to the manufacturer's instructions, using the Lyric flow cytometer with autosampler (BD Bioscience).

2.14 T-cell suppression assay

MoDCs ($2x10^5$) were seeded in a 48-well plate and stimulated with LPS and PSMα3 for 24 h. Human $CD4^+$ T cells were isolated from PBMCs using the human CD4 MicroBeads Kit (Miltenyi) according to the manufacturer's instructions using LS columns. $8x10^5$ T cells were added to the stimulated moDCs and cultured for 4 d at 37°C, 5% CO_2. T cells were stained according to Table 6, where DAPI (16,7 ng/mL) was used to exclude dead cells. T_{regs} ($CD4^+CD127^-CD25^{hi}CD45RA^-$) were purified by FACS sorting using an ARIA IIu cell sorter (BD Bioscience).

Table 2-5: Panel for sorting regulatory T cells

Panel	Antigen	Fluorochrome	Clone	Company
T_{reg} Sort				
	CD4	APC-Vio770	REA623	Miltenyi
	CD25	PE-Cy7	BC96	eBioscience
	CD45RA	PerCP	HI100	BioLegend
	CD127	PE	eBioRDR5	eBioscience
		DAPI		Sigma

$CD4^+$ T cells, isolated from PBMCs from a different donor using the CD4 MicroBeads Kit (Miltenyi) were used as T effector (T_{eff}) cells. The T_{effs} were labeled with CFSE (5 µM), and $8x10^4$ cells were seeded in a 96- well plate in T-cell medium together with the indicated numbers of sorted T_{regs}. For T-cell activation of the T_{effs} Dynabeads

(Human T-Activator CD3/CD28 Proliferation; Gibco) were added according to the manufacturer's instructions. The proliferation of T_{effs} was assessed after 3 d by analyzing the CFSE signal by flow cytometry. Dead cells were excluded by staining the cells with Zombie Aqua. 20,000 cells were acquired at the Canto II with the DIVA software (BD Biosciences) and were further analyzed using the proliferation tool in FlowJo 10.4.2 software (Tree Star).

2.15 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 7.0a software (GraphPad, San Diego, CA). Statistical differences were determined using one-way ANOVA with Turkey's posttest or an unpaired student's T-test in case data were normally distributed (Shapiro-Wilk normality test). Otherwise, data were analyzed using the Kruskal-Wallis nonparametric test. The differences were considered as statistically significant if $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****).

3. Results

3.1 PSMs modulate DC maturation

DCs are key regulators of the adaptive immune response. However, in order to successfully activate T cells, DCs have to undergo phenotypical and morphological changes, known as maturation (Banchereau et al., 2000). This includes the upregulation of maturation markers (e.g., CD83 and the human MHC II molecule Human Leukocyte Antigen – DR isotype (HLA-DR)), costimulatory (e.g., CD80, CD86, CD40), as well as coinhibitory molecules (e.g., Programmed death-ligand (PD-L)1, PD-L2 or Immunoglobulin-like transcript (ILT)3). To address whether the Sa-secreted peptide toxin PSMα3 has an effect on DC maturation, moDCs were treated with either the TLR2 ligand Sa lysate (Schreiner et al., 2013) or the TLR4 ligand LPS in combination with, and without, PSMα3. MoDCs were characterized as living $CD11c^{+}HLA\text{-}DR^{+}$ leukocytes (**Figure 3-1 A**) and surface marker expression was assessed by flow cytometry after 6 h or 24 h. Except for CD80 after 6 h, the expression of all surface markers increased after 6 h or 24 h upon TLR ligand (TLRL) treatment compared to untreated cells (**Figure 3-1 B-D**); this indicated DC maturation. Co-treatment with PSMα3 for 6 h showed a slight but not significant increase of CD83 and CD86 and a significant higher expression of HLA-DR compared to the treatment with TLRLs alone (**Figure 3-1 B**). On the contrary, CD40 was significantly less expressed on TLR4 treated mDCs when co-treated with PSMα3 for 6 h or 24 h (**Figure 3-1 B, C**). Likewise, CD80 expression was hampered on TLR4-stimulated mDCs when treated in combination with PSMα3 for 24 h (**Figure 3-1 C**). No significant results were obtained from the analysis of the coinhibitory molecules PD-L1, PD-L2 and ILT3 after 24 h; however, PSMα3 seems to inhibit the up-regulation of PD-L1 expression on TLR4-treated mDCs (**Figure 3-1 D**). In conclusion, PSMα3 affects surface molecule expression of TLRL-treated moDCs by enhancing early costimulatory molecule and HLA-DR expression while simultaneously inhibiting CD80 and CD40 upregulation upon TLR4 stimulation.

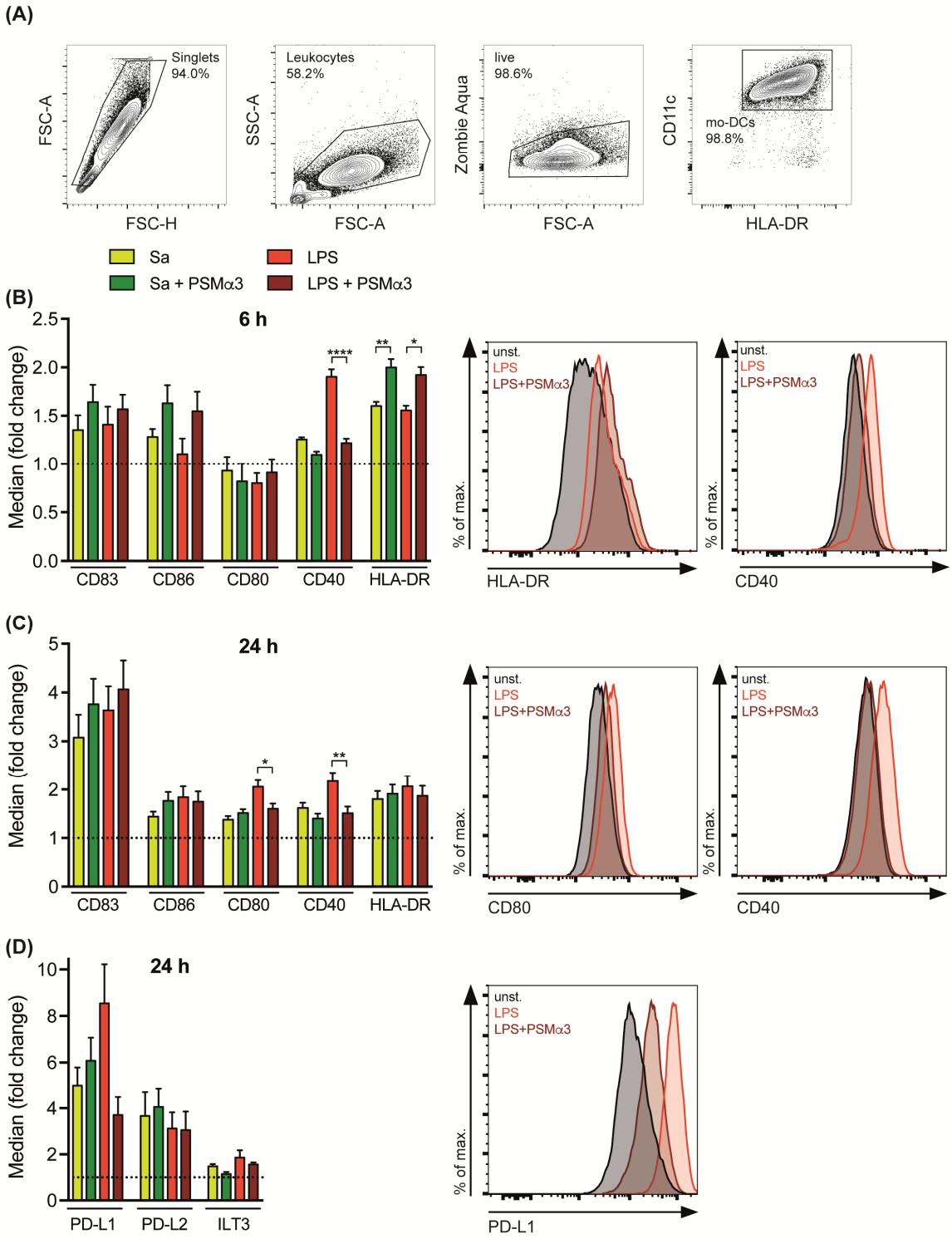

Figure 3-1: PSMs modulate DC maturation by affecting surface molecule expression of TLRL-treated moDCs (from Richardson et al., 2018)

(A) Gating strategy: moDCs were characterized as living $CD11c^{+}HLA\text{-}DR^{+}$ leukocytes. (B-D) MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3 for 6 h (B) or 24 h (C, D) and analyzed by flow cytometry. The expression of the indicated costimulatory (B, C) and inhibitory molecules (D) was determined as mean fluorescence intensity (MFI). The MFI of the respective marker expression is shown in the bar graphs as fold change of untreated cells (left). The graphs represent n≥3 independent experiments (mean ± SEM) performed in triplicates. Representative histogram overlays (right) of HLA-DR and CD40 after 6 h (B), CD80, CD40 (C), and

PD-L1 (D) after 24 h. *p < 0.05, **p < 0.005 or ****p < 0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

3.2 Cytokine secretion of TLR4-treated moDCs is impaired by PSMs

Upon activation and maturation DCs express cytokines, important for T-cell activation and priming (Blanco et al., 2008a). Previously, it was shown that PSMα3 inhibits pro-inflammatory cytokine secretion of mouse bone-marrow derived DCs (BM-DCs) after stimulation with various TLRLs and conversely induced the expression of IL-10, thereby manipulating the immune response (Armbruster et al., 2016b). To investigate if PSMα3 has similar effects on human DCs, cell culture supernatants from TLRL-treated moDCs in combination with, and without, PSMα3 were analyzed after 6 h for the pro-inflammatory cytokine TNF (**Figure 3-2 A**) and after 24 h for the pro-inflammatory cytokine IL-12 (**Figure 3-2 B**) or the anti-inflammatory cytokine IL-10 production (**Figure 3-2 C**).

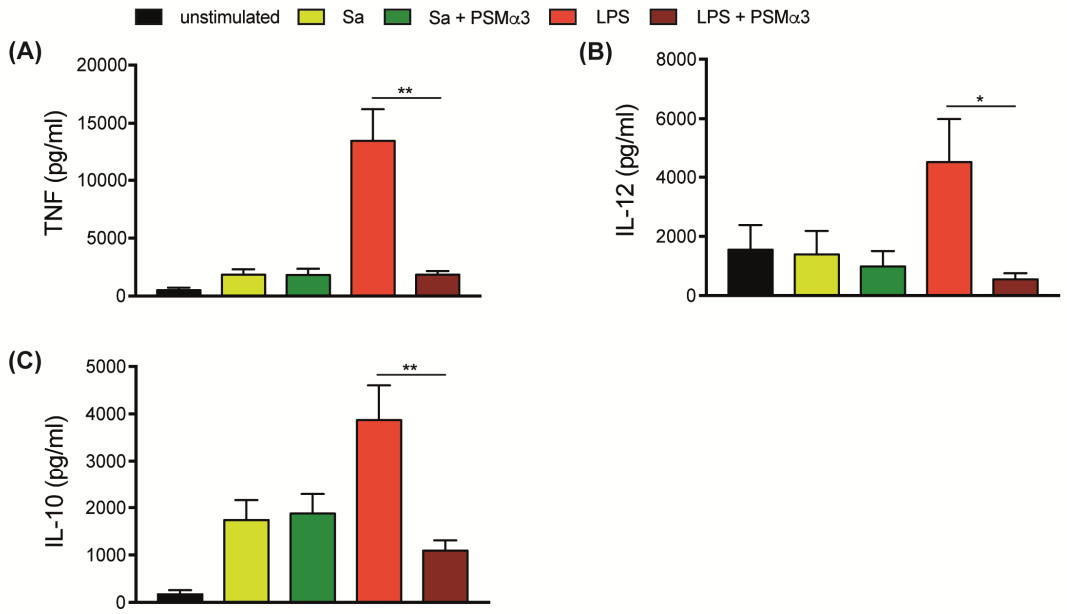

Figure 3-2: PSMs impair cytokine secretion by TLR4-treated moDCs (adapted from Richardson et al., 2018)

MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3 for 6 h (A) or 24 h (B, C). Cytokine production of TNF (A), IL-12 (B) and IL-10 (C) was analyzed in the cell culture supernatants by sandwich ELISA. The graphs represent n ≥10 independent experiments (mean ±SEM) performed in triplicates. *p < 0.05 or **p < 0.005, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

Treatment with TLRLs induced overall secretion of pro- and anti-inflammatory cytokines compared to untreated moDCs (**Figure 3-2 A-C**), except for IL-12 in TLR2-

treated cells (**Figure 3-2 C**). PSMα3 had no effect on pro- or anti-inflammatory cytokine secretion of moDCs treated with the TLR2 ligand Sa lysate (**Figure 3-2**). However, the LPS-induced secretion of TNF, IL-12 and also IL-10 was significantly inhibited when cells were co-treated with PSMα3 (**Figure 3-2**). In summation, PSMα3 impairs pro- as well as anti-inflammatory cytokine secretion by TLR4-treated mDCs.

3.3 PSMs dampen phosphorylation of NF-κB and p38 in TLR4-treated moDCs

The maturation of DCs is induced by engagement of stimuli with various PRRs, like TLRs, which activate signaling pathways regulating e.g. cytokine production. The activation of these signaling pathways is often mediated by the phosphorylation of certain proteins, including NF-κB and p38 MAPK. The p38 MAPK signaling pathway is connected to DC maturation and to cytokine production and NF-κB is a transcription factor involved in regulating inflammatory responses (Nakahara et al., 2006). To investigate whether these signaling pathways are affected and responsible for the impaired cytokine production in TLR4-treated cells in the presence of PSMα3, DCs were treated with LPS in the presence and in the absence of PSMα3 for 30 min or 60 min and phosphorylation of NF-κB (p-NF-κB; **Figure 3-3 A, B**) and p38 (p-p38; **Figure 3-3 C, D**) was determined via flow cytometry analysis. The treatment with PSMα3 alone showed no changes in p-NF-κB (**Figure 3-3 A, B heatmap**) or p-p38 (**Figure 3-3 C, D heatmap**) after 30 min or 60 min compared to untreated moDCs. LPS treatment, however, showed an increase in phosphorylation in both NF-κB and p38. The phosphorylation of NF-κB was increased 3.4 fold after 30 min of LPS treatment and 2.5 fold increased after 60 min of stimulation compared to untreated cells (**Figure 3-3 A, B heatmap**). The phosphorylation of p38 did not increase as much, with only a 2.95 fold increase after 30 min (**Figure 3-3 C heatmap)** of LPS treatment and 1.9 fold after 60 min (**Figure 3-3 D heatmap**). The presence of PSMα3 in TLR4-treated moDCs showed a reduced phosphorylation of both NF-κB and p38. Simultaneous stimulation of LPS with PSMα3 had no effect on NF-κB phosphorylation after 30 min compared to LPS-treated moDCs (3.37±1.1 vs. 3.1±1.18) which was analyzed in 4 individual donors (**Figure 3-3 A**).

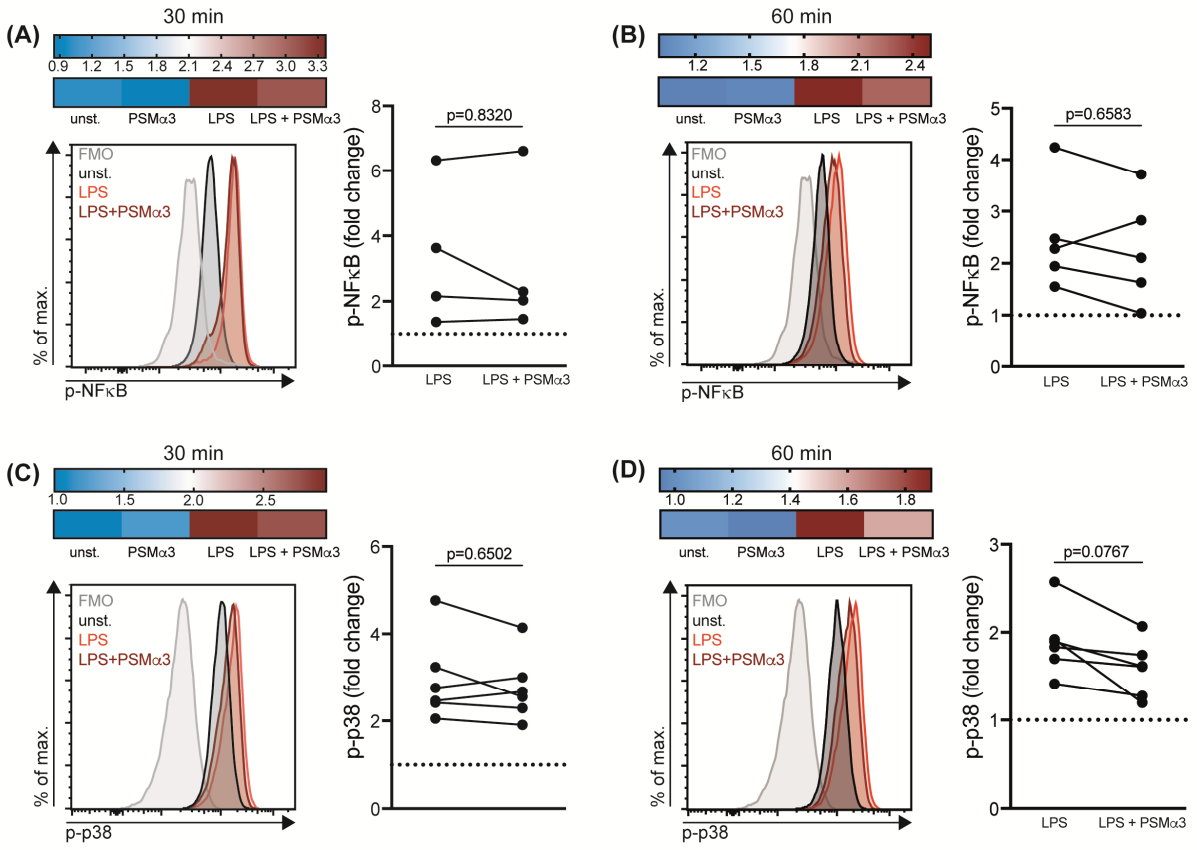

Figure 3-3: PSMs dampen the phosphorylation of NF-κB and p38 LPS-Treated moDCs (adapted from Richardson et al., 2018)

MoDCs were treated with LPS with, and without, PSMα3 or with only PSMα3 for 30 min (A, C) or 60min (B, D). Phosphorylation of NF-κB (p65 Ser536) and p38 (Thr180/Tyr182) was analyzed in $CD11c^{+}HLA\text{-}DR^{+}$ cells. Representative histogram overlays of p-NF-κB after 30 min (A) or 60 min (B) and of p-p38 after 30 min (C) or 60 min (D). The heat map shows the fold-change of p-NF-κB (A, B) or p-p38 (C, D) normalized to untreated DCs (unst.). The graphs show the fold change of p-NF-κB (A n=4, B n=5) and p-p38 (C, D n=6) normalized to untreated DCs (dotted line) from n ≥ 4 different donors. One-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

However, treatment of LPS with PSMα3 for 60 min showed a reduced phosphorylation of NF-κB (2.5±0.46 vs 2.26±0.46), which was not significant but observed for most donors (**Figure 3-3 B**). The analysis of p-p38 in TLR4-stimulated moDCs upon PSMα3 treatment showed a slight reduction of phosphorylation after 30 min (2.95±0.4 vs 2.76±0.31) and 60 min (1.9±0.16 vs 1.58±0.13), this was observed for most of the analyzed donors after 30 min and all the analyzed donors after 60 min (**Figure 3-3 C, D**). The analysis of these signaling pathways indicates an effect of PSMα3 on the phosphorylation of both NF-κB and p38 in TLR4-treated moDCs, which may be responsible for the observed changes in DC maturation and cytokine production.

3.4 PSMs reduce antigen uptake by moDCs

The main function of DCs in the periphery is to take up antigen for presentation and activation of T cells (Trombetta & Mellman, 2005). The preferred routes of antigen uptake by iDCs are via clathrin-mediated endocytosis and macropinocytosis, whereas mDCs lose most of their endocytic capacity and retain antigen uptake only by clathrin-mediated endocytosis (Burgdorf et al., 2007; Platt et al., 2010). To elucidate whether PSMs have an effect on this pivotal task, moDCs were treated with Sa lysate or LPS in the presence and in the absence of PSMα3 or with only PSMα3 for 3 h (**Figure 3-4 A**) or 24 h (**Figure 3-4 B**) and AlexaFluor647-labeled OVA uptake was assessed by flow cytometry analysis.

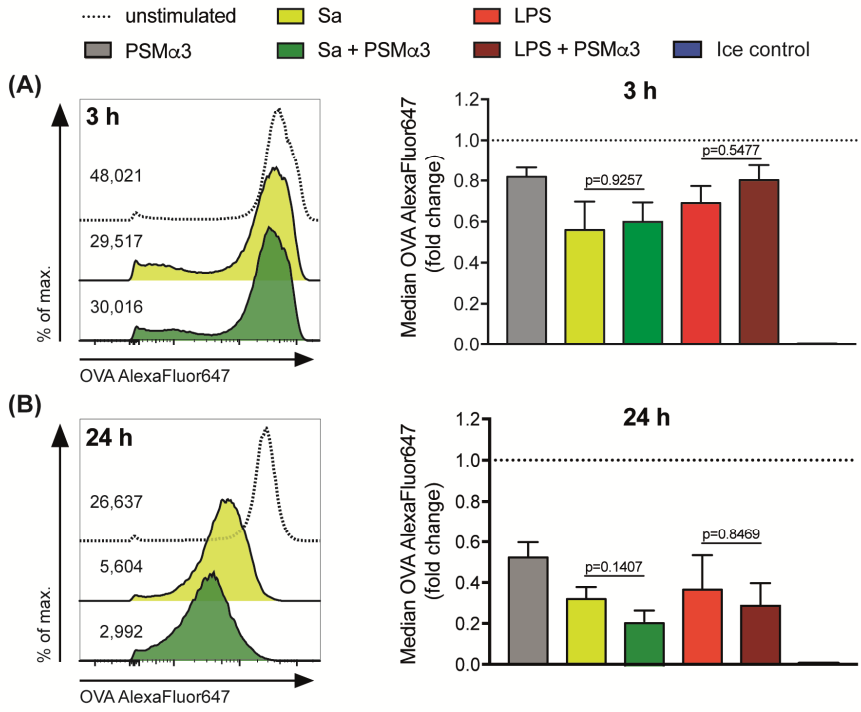

Figure 3-4: PSMs areduce antigen uptake by moDCs (adapted from Richardson et al., 2018)

MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3, or with only PSMα3 for 3 h (A) or 24 h (B). Subsequently, moDCs were incubated with AlexaFluor647-labeled OVA at 37°C or on ice for 30 min. OVA uptake was assessed in $CD11c^{+}HLA\text{-}DR^{+}$ moDCs by flow cytometry. The histogram overlays show the MFI of original data from one representative for 3 h (A) or 24 h (B). The bar graphs show the normalized MFI as fold change to untreated cells (n ≥ 3, performed in triplicates, mean ± SEM). One-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

To exclude unspecific binding, the incubation of moDCs with OVA was performed on ice. This prevents the mobilization and remodeling of the actin cytoskeleton, which is required for antigen capture via clathrin-mediated endocytosis and micropinocytosis (West et al., 2004). As expected, no OVA signal was detected for moDCs incubated

on ice (**Figure 3-4 A, B**). Maturation of moDCs via stimulating TLR2 with Sa lysate or TLR4 with LPS leads to a reduced uptake of OVA already after 3 h by ~44% or ~31% (**Figure 3-4 A**), but also after 24 h by ~68 or ~63%, respectively (**Figure 3-4 B**), confirming the loss of endocytic capacity of mDCs. Treatment of iDCs with PSMα3 alone led to a reduction of OVA uptake by ~18% after 3 h ($p<0.319$) (**Figure 3-4 A**) and by ~48% after 24 h (***$p<0.0003$) (**Figure 3-4 B**) indicating that PSMα3 affects antigen uptake of iDCs. The analysis of PSMα3 treatment in mDCs revealed no difference in TLR2-treated cells after 3 h and only a slight increase in TLR4-treated cells (**Figure 3-4 A**). However, after 24 h the co-treatment of TLRLs and PSMα3 showed a further reduction of OVA uptake by ~11% and ~8% respectively compared to only TLRL-treated moDCs (Sa $p=0.1407$, LPS $p=0.8459$), which is shown for Sa cell lysate in the histogram overlay of the original data (**Figure 3-4 B**). In summary, PSMs affect antigen uptake by reducing OVA uptake especially by iDCs after 3 h and 24 h, but also slightly decreasing that of mDCs after 24 h.

3.5 PSMs form transient pores into the moDCs membrane thereby entering the cell

PSMs have cytolytic activity with the ability to form transient pores into the cell membrane. This feature not only allows PSMs to lyse human immune cells but also to enter the cytosol of cells (Armbruster et al., 2016b). To investigate whether PSMs can form transient pores in the membrane of human moDCs, or if these peptides are internalized via an active process of antigen uptake, cells were treated with either Sa lysate or LPS in the presence and in the absence of PSMα3 for 24 h. Thereafter, moDCs were incubated with fluorescently-labeled PSMα2 and AlexaFluor647-labeled OVA peptides and cells were analyzed by flow cytometry or multispectral imaging flow cytometry (**Figure 3-5 A, B**). Despite the maturation state of moDCs, PSMα2-FITC was detected in all uniquely treated and analyzed moDCs. Whereas a slightly higher MFI was detected in TLR2-treated cells and a slightly minor MFI in TLR4-treated moDCs compared to iDCs, there was no difference between TLRL compared to TLRL and PSMα3 treatment (**Figure 3-5 A**). PSMα2-FITC was also detected, although reduced by ~54% ($p=0.0003$) in cells incubated on ice, indicating that uptake of PSMα2-FITC is not entirely dependent on actin rearrangement (**Figure 3-5 A**). When analyzing PSMα2-FITC internalization and localization via multispectral imaging flow cytometry, the observed quantitative data from the flow cytometry

analysis were confirmed. PSMα2-FITC was detected in moDCs regardless of the treatment (**Figure 3-5 B**).

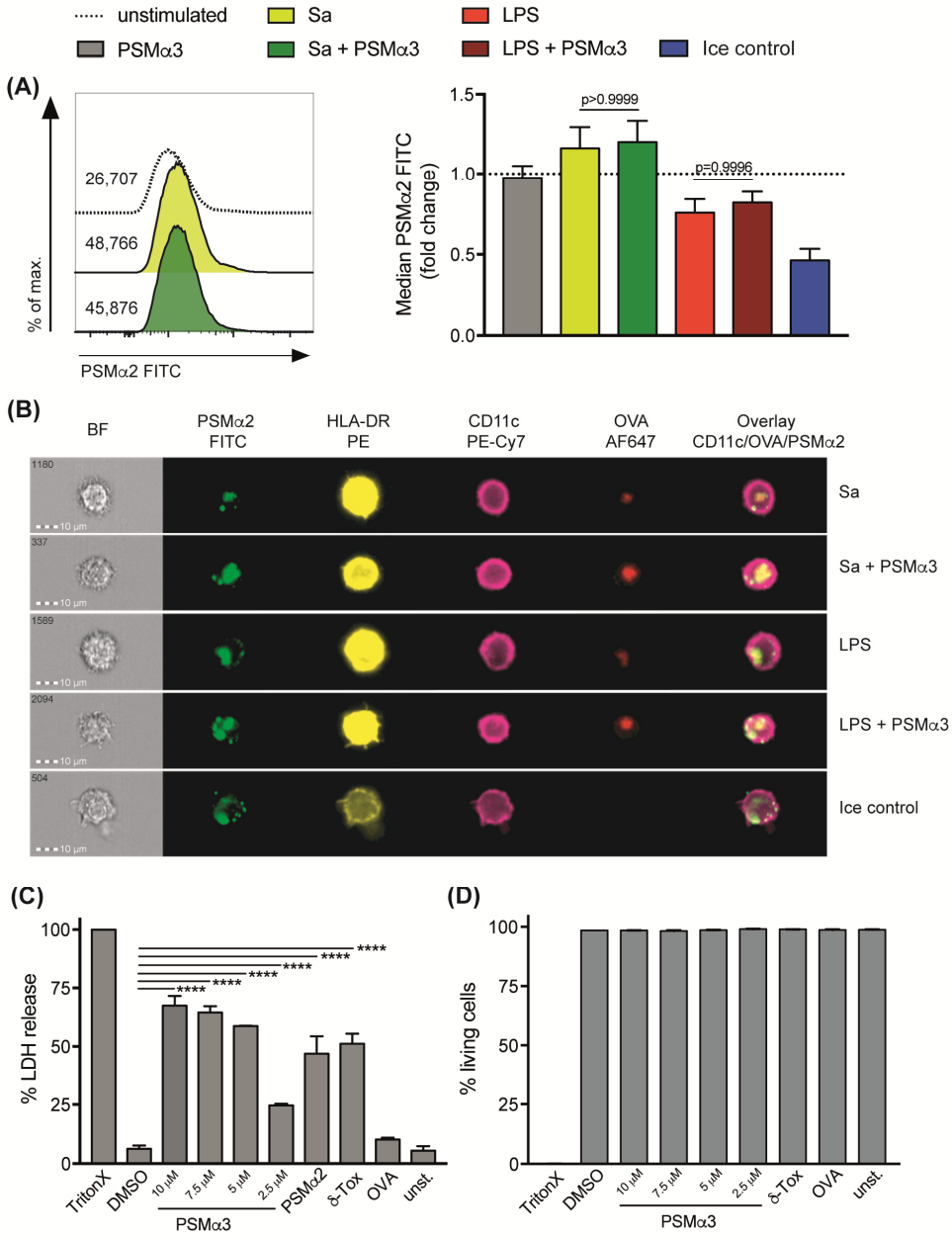

Figure 3-5: PSMs penetrate the moDC membrane via transient pore formation (adapted from Richardson et al., 2018)

(A, B) MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3 for 24 h. Subsequently, moDCs were incubated with AlexaFluor647-labeled OVA and FITC-labeled PSMα2 at 37°C or on ice for 30 min. The signal of PSMα2 FITC was assessed in $CD11c^{+}HLA\text{-}DR^{+}$ moDCs as MFI by flow cytometry (A) or by imaging flow cytometry (B). (A) The histogram overlay shows the MFI of original data from one representative. The histograms show the normalized MFI as fold change to untreated cells (n ≥ 5, performed in triplicates, mean ± SEM). (B) The localization of PSMα2-FITC in $CD11c^{+}HLA\text{-}DR^{+}$ moDCs was analyzed by multispectral imaging flow cytometry (one representative experiment of n = 3 independent experiments; mean ± SEM). Representative bright field (BF) and fluorescence images of moDCs are shown for the different treatments. (C, D) MoDCs were either not treated (unst.) or incubated with 1% Triton X-100, 2% DMSO, PSMα3 (2.5 µM, 5 µM, 7.5 µM or 10 µM), 10 µM PSMα2,

10 µM δ-Toxin or 5 µg/mL OVA for 10 min. (C) LDH release was determined in the cell culture supernatants (one representative experiment of n = 3 independent experiments; mean ± SEM). (D) The treated cells were used to assess cell death of moDCs by flow cytometry using 7-AAD. ****p < 0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

The fluorescently labeled PSMα2 peptides were detected as spots close to the membrane when incubated at 4°C or 37°C, and colocalized with AlexaFluor647-labeled OVA when incubated at 37°C. As PSMα2 was detected inside the cells when incubated at 4°C, its internalization is independent of actin rearrangement (**Figure 3-5 B**). To further analyze how PSMs enter moDCs, pore formation was assessed by measuring the release of L-lactate dehydrogenase (LDH) in the supernatants of PSM treated moDCs. LDH is a cytosolic enzyme and only present in the supernatant when released via pores in the cell membrane. This is often used to measure cellular cytotoxicity. Triton X-100, a detergent capable of lysing cells was used as positive control, as it disrupts the cell membrane. δ-Toxin, a peptide belonging to the family of PSM peptides was also used as positive control, as it was shown to form transient pores (Pokorny et al., 2006). Neither untreated cells nor cells treated with OVA nor DMSO, which was used to dissolve PSM peptides, released LDH into the supernatants (**Figure 3-5 C**). However, treatment with PSMα2, δ-Toxin or PSMα3 significantly induced the release of LDH, for PSMα3 in a concentration-dependent manner (**Figure 3-5 C**). Measuring cell viability by flow cytometry showed that aside from Triton X-100, the various treatments had no effect on the viability of moDCs (**Figure 3-5 D**). This indicates that PSMs are able to enter moDCs, most likely by transient pore formation and are located close to the membrane or colocalized with OVA inside the cell without having cytolytic effects.

3.6 Th1 cell differentiation induced by TLRL-treated moDCs is suppressed by PSMs

DCs play a crucial role in the activation of naïve T cells thereby shaping the immune response by driving differentiation into the distinct effector T-cell subsets (Mellman & Steinman, 2001). Specific transcription factors, called master regulators of the T helper (Th) lineages, control the differentiation of these T-cell subsets, with T-bet important for Th1 cell, GATA-3 for Th2 cell, RORγt for Th17 cell, and FoxP3 for T_{reg} differentiation (Li et al., 2014).

To address whether PSMs also have effects on the adaptive immune response by modulating DC functions, moDCs were treated with Sa lysate or LPS in the presence

and in the absence of PSMα3 for 24 h. After, treated moDCs were co-cultured with naïve $CD4^+$ T cells for 3 d and Th subsets were analyzed by flow cytometry (**Figure 3-6 A**). Treatment of moDCs with PSMα3 alone had no effect on Th1 polarization and showed only a slight increase in Th2 differentiation compared to untreated iDCs (**Figure 3-6 B**). TLRL-treated moDCs increased the frequency of both Th subsets; however, co-treatment with PSMα3 significantly reduced the priming of Th1 cells in TLR4, as well as TLR2-stimulated mDCs, characterized by less expression of the transcription factor T-bet (**Figure 3-6 A, B**). PSMα3 showed the same tendency for priming the Th2 cells by TLRL-treated moDCs with a slightly less GATA3 expression (Sa 1.32 vs. 1.1%, p = 0.27; LPS 1.25 vs. 1.1%, p = 0.8) (**Figure 3-6 A, B**). In contrast, no difference in the frequency of RORγt$^+$ Th17 cells was observed when T cells were co-cultured with mDCs or PSMα3-treated mDCs (**Figure 3-6 A, B**).

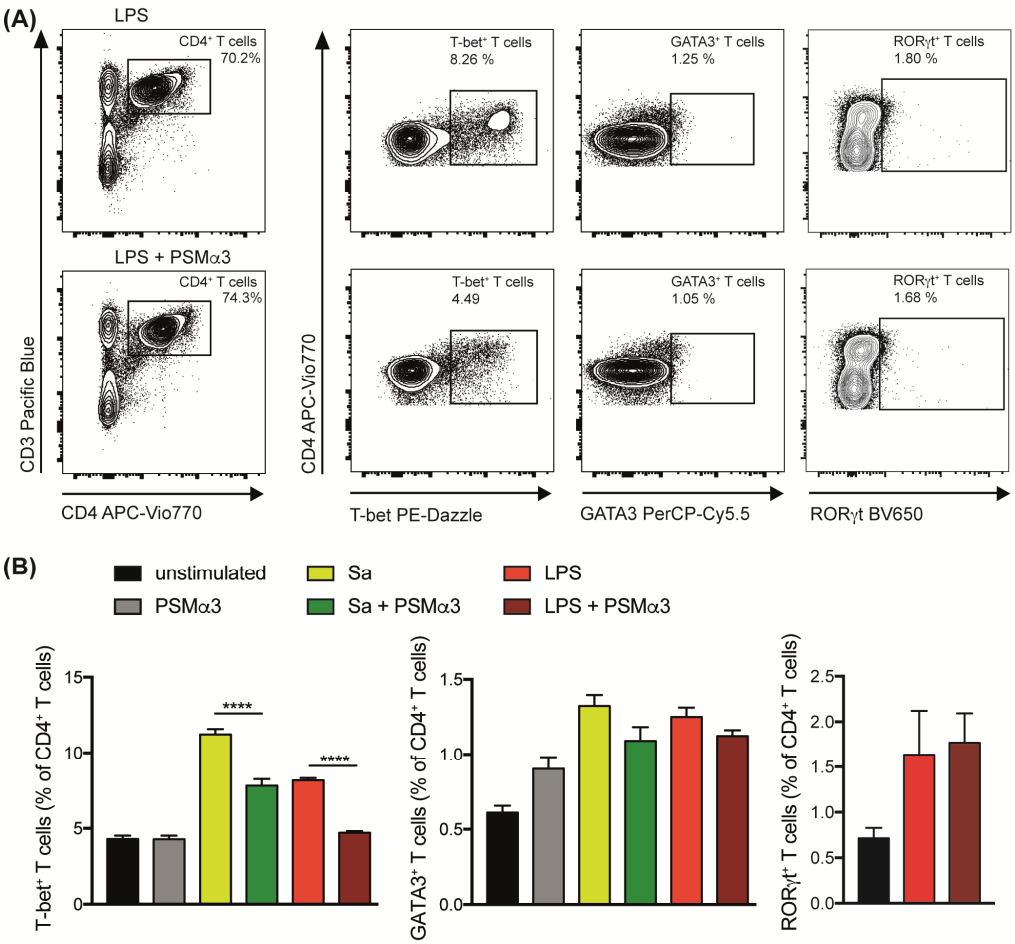

Figure 3-6: Th1 cell differentiation induced by TLRL-treated moDCs is suppressed by PSMs (adapted from Richardson et al., 2018)

MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3, or with only PSMα3 for 24 h. After, cells were co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 d. Th subsets were analyzed by flow cytometry; Th1 cells were characterized as $CD3^+CD4^+$T-bet$^+$ T cells, Th2 cells were characterized as $CD3^+CD4^+GATA3^+$ T cells, and Th17 cells were characterized as $CD3^+CD4^+$RORγt$^+$ T cells. (A) Gating strategy with frequencies of the

three Th subsets in LPS or LPS with PSMα3 treated cells. (B) The bar graphs show the frequency of T-bet$^+$ Th1, GATA3$^+$ Th2, and RORγt$^+$ Th17 cells of CD4$^+$ T cells (one representative of n = 4 independent experiments performed in triplicates, mean ± SEM). ****p < 0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

Not only the interaction between DCs and T cells, but also the local cytokine environment control T-cell polarization. Th1 cells require IL-12 for their differentiation, which then predominantly produce IFN-γ, whereas Th2 cells depend on IL-4 and also mainly produce IL-4 and IL-13. Th17 cells are characterized as IL-17A producing cells and are induced among others by IL-6 (Li et al., 2014). To address whether PSMs have an effect on the cytokine environment in the DC-T-cell co-culture, supernatants were collected each day and analyzed by a bead-based cytokine array. TLRL-treatment induced the secretion of all analyzed cytokines after 1 d or 2 d (**Figure 3-7**). The analysis of the impact of PSMs on the effector cytokines of the different Th subsets showed a drastically decreased secretion of IFN-γ in TLR4-stimulated cells when PSMα3 was present after 2-3 d; indications of this drastic decrease were observed after only 1 d (**Figure 3-7 A**). This was also observed in TLR2-stimulated cells after 2-3 d; although here the effect was not significant (**Figure 3-7 A**). PSMα3 had no effect on the TLRL-induced secretion of IL-17A or IL-4 (**Figure 3-7 B, C**).

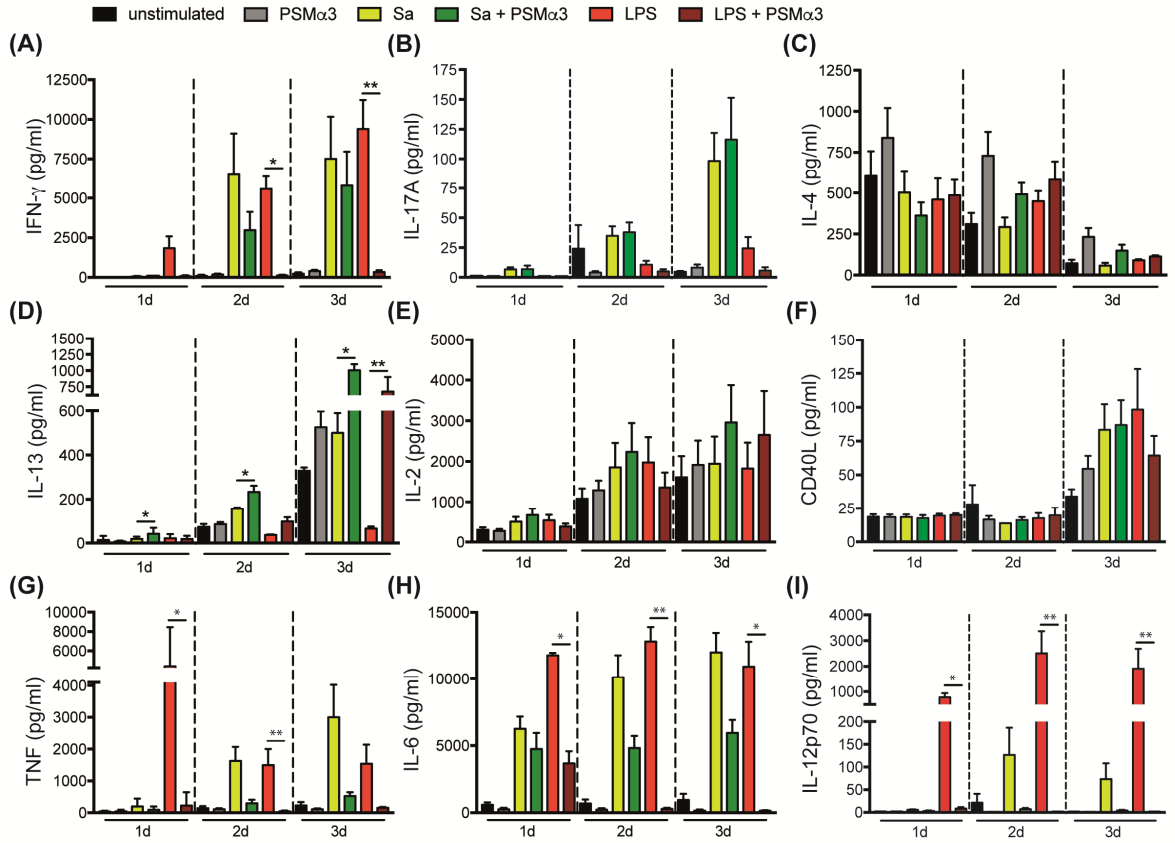

Figure 3-7: PSMs modulate the cytokine milieu of the moDC-T-cell co-culture (adapted from Richardson et al., 2018)

(A-I) MoDCs were treated with Sa lysate (Sa) or LPS with or without PSMα3 or with only PSMα3 for 24 h and co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 d. Every day supernatants of the co-culture were collected and analyzed via a bead-based cytokine array to determine IFN-g (A), IL-17A (B), IL-4 (C), IL-13 (D), IL-2 (E), CD40L (F), TNF (G), IL-6 (H) and IL-12p70 (I) levels in the moDC-T-cell co-culture. (n ≥ 3 independent experiments performed in triplicates, mean ± SEM). *p < 0.05, or **p < 0.005, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

Co-treatment of moDCs with TLRLs and PSMα3, however, significantly increased the production of IL-13 in the co-culture, for TLR2 stimulated cells as early as after 1 d and in combination with LPS after 3 d (**Figure 3-7 D**). IL-2 and CD40L are key stimulators for the proliferation of immune cells. PSMα3 showed only a trend to increase IL-2 after 3 d compared to TLRL-stimulated cells, but had no effect on CD40L secretion (**Figure 3-7 E, F**). Further, PSMα3 completely inhibited the TLR4-induced secretion of the pro-inflammatory effector cytokines TNF, IL-6 and IL12p70, which was observed at all the indicated times (**Figure 3-7 G-I**). TLR2-treated cells showed lower levels of TNF and IL-12p70, but here PSMα3 also completely inhibited the production at all times (**Figure 3-7 G, I**). TLR2-induced IL-6 production was also inhibited by PSMα3, but this effect was not significant and not as pronounced as in TLR4 stimulated cells (**Figure 3-7 H**). In summary, PSMα3 suppresses Th1

polarization by inhibiting the expression of T-bet and by completely blocking cytokine secretion of IL-12p70 and IFN-γ in LPS-treated cells; this is not only important for Th1 development but also for Th1 function. PSMα3 additionally inhibited the secretion of pro-inflammatory cytokines in the co-culture, which was also observed for moDC cytokine production and on the contrary increases the secretion of IL-13.

3.7 PSMs increase the frequency of induced regulatory T cells with suppressive capacity

It was previously shown that PSMs induce T_{reg} proliferation and frequency upon priming mouse tDCs (Armbruster et al., 2016b; Schreiner et al., 2013). As PSMα3-treated mDCs attenuated the priming of Th1 cells, the effect of PSMα3 on T_{reg} priming was assessed. Therefore, moDCs were treated with Sa lysate or LPS in the presence or absence of PSMα3 for 24 h. These treated moDCs were then co-cultured with naïve $CD4^+$ T cells for 3 d or 4 d and iT_{regs} were analyzed by flow cytometry, characterized as $CD3^+CD4^+CD127^-CD25^{hi}CD45RA^-FoxP3^{hi}$ cell subset ((Santegoets et al., 2015); **Figure 3-8A**). Whereas TLRL- or PSMα3-treatment of moDCs alone had no effect on iT_{reg} priming compared to untreated cells after 3 d, combined treatment of DCs with Sa lysate with PSMα3 or LPS with PSMα3 showed a significantly higher frequency of iT_{regs} (**Figure 3-8 B**). After 4 d TLRL- or PSMα3-treatment of moDCs alone already showed an increased frequency of iT_{regs} compared to untreated cells, however, combined treatment again showed an even higher significant increase of iT_{regs} (**Figure 3-8 A, B**). Analysis of the proliferation capacity of iT_{regs} induced by TLRL-treated or in combination with PSMα3-treated moDCs by CFSE staining showed that iT_{reg} proliferation is increased in the presence of PSMα3 (**Figure 3-8 C**).

Close analysis shows that PSMα3 modulates human mDCs to induce iT_{reg} polarization and proliferation.

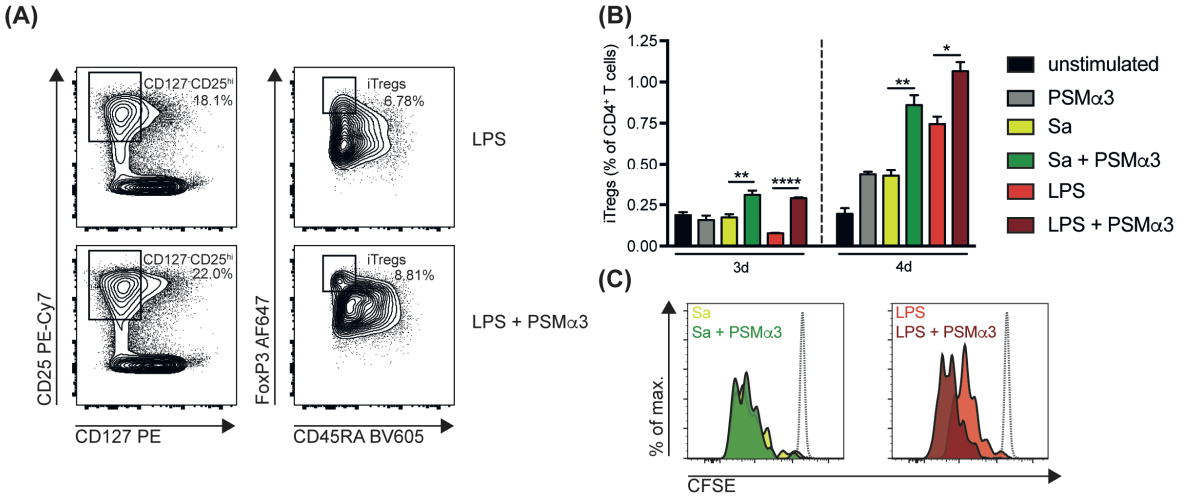

Figure 3-8: PSM peptides induce iT_{reg} frequency and proliferation (adapted from Richardson et al., 2018)

MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3, or with only PSMα3 for 24 h. After, moDCs were co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 d or 4 d. iT_{regs} were analyzed by flow cytometry and characterized as $CD3^+CD4^+CD127^-CD25^{hi}CD45RA^-FoxP3^{hi}$ cells (A). The frequency of iT_{regs} from $CD4^+$ T cells (B) and the proliferation of iT_{regs} (C) were determined by flow cytometry (n ≥ 5 performed in triplicates, mean ± SEM). *p < 0.05, **p < 0.005, or ****p <0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

To analyze whether these iT_{regs} are functional and have suppressive capacity, iT_{regs} were sorted after being primed by LPS and PSMα3-treated moDCs for 4 d. T_{regs} were sorted as live $CD4^+CD127^-CD25^{hi}CD45^-$ cells and showed a purity of >99% after sorting (**Figure 3-9 A**). The purified iT_{regs} were then co-cultured in different concentrations with CFSE-labeled $CD4^+$ T cells (effector T cells; T_{eff}), which were activated with anti-CD3/CD28 Dynabeads and proliferation of T_{effs} was analyzed after 3 d. T_{effs} only, either activated with beads or not activated, served as control. Activated T_{eff} cells only showed the maximal proliferation capacity in the experiment, whereas not activated T_{eff} showed no proliferation at all (**Figure 3-9 B**). The proliferation of the T_{eff} cells was significantly inhibited in a concentration-dependent manner, with the highest inhibition of ~38% at a ratio of 1:1 (T_{eff}:iT_{regs}), ~25% at a 2:1 ratio (T_{eff}:iT_{regs}) and no significant but still a ~12% inhibition of the proliferation with a ratio of 4:1 (T_{eff}:iT_{regs}) (**Figure 3-9 B**). This shows that PSMα3-primed mDCs not only induce the frequency and proliferation of iT_{regs}, but that these iT_{regs} are functionally capable of inhibiting T_{eff} cell proliferation.

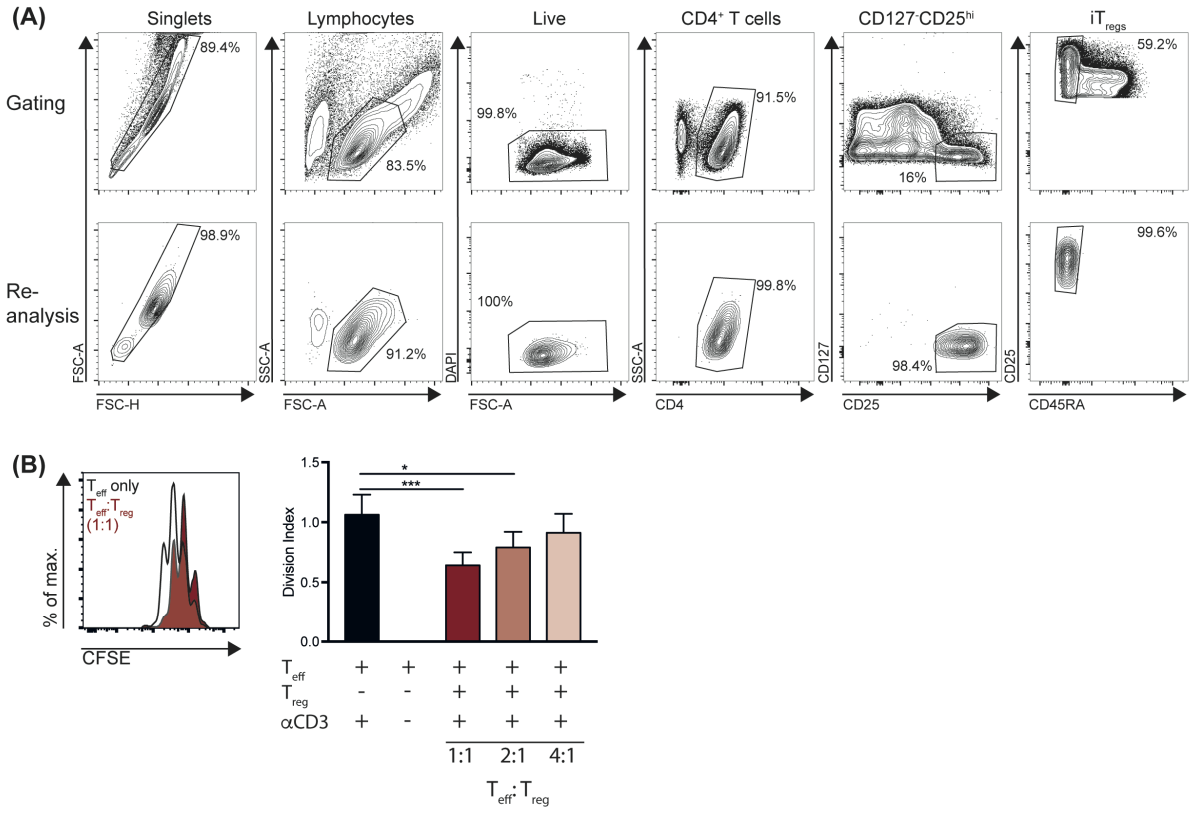

Figure 3-9: PSMα3-treated mDCs induce iT_{regs} with suppressive capacity (adapted from Richardson et al., 2018)

MoDCs were treated with LPS and PSMα3 for 24 h and co-cultured with naïve $CD4^+$ T cells for 4 d. (A) iT_{regs} were purified using an ARIA IIu cell sorter from BD. The upper panel shows the gating of the iT_{regs}, which were sorted as $CD4^+CD127^-CD25^{hi}CD45RA^-$ cells and the lower panel shows the re-analysis of the purified iT_{regs} after sorting. (B) The purified iT_{regs} were co-cultured with CFSE-labeled and CD3/CD28 Dynabeads activated $CD4^+$ T effector cells (T_{eff}) and proliferation of the T_{effs} was assessed after 3 d by flow cytometry. The histogram overlay shows the proliferation peaks of T_{effs} only (black) or T_{effs} co-cultured together with iT_{regs} in a ratio of 1:1 (red). The bar graphs show the division index of activated or not activated T_{effs} without or with the presence of iT_{regs} in various ratios. (n = 3, performed in triplicates, mean ± SEM). *p < 0.05, or ***p <0.001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

3.8 Induced regulatory T-cell polarization is mediated by PSMα3-treated moDCs via direct cell interaction and upon production of soluble factors

To investigate which mechanisms or factors are important for iT_{reg} priming by PSMα3 treated mDCs, naïve $CD4^+$ T cells were co-cultured with differently treated moDCs. As described above, PSMα3-treated mDCs significantly increased the priming of iT_{regs} compared to mDCs alone (**Figure 3-10 A, B condition 1**). First, to investigate whether soluble factors produced by mDCs are important for the priming, naïve T cells were co-cultured with iDCs adding medium from primed moDCs (**conditioned**

medium; Figure 3-10 A condition 2). The medium from LPS and PSMα3 primed moDCs showed a similar increase of iT_{regs} as in condition 1, whereas the LPS conditioned medium showed no effect (**Figure 3-10 B condition 2**). This indicates that the medium from PSMα3-primed mDCs contains soluble factors necessary for iT_{reg} priming.

Next, to exclude soluble factors secreted by mDCs in the first 24 h (stimulation time), moDCs were treated with LPS or LPS and PSMα3 for 24 h, washed after the incubation time, re-seeded and cultured with naïve T cells for 3 – 4 d in fresh medium (**Figure 3-10 A condition 3**). These mDCs induced an even higher frequency of iT_{regs} than mDCs without medium change, also with LPS-treated cells. Similarly, the combination with PSMα3 significantly increased the frequency of iT_{regs}, suggesting that the interaction of mDCs and $CD4^+$ T cells, as well as secreted factors upon the interaction of the two cell types are important for iT_{reg} priming (**Figure 3-10 B condition 3**).

Lastly, to address the impact of direct DC-T-cell interaction on iT_{reg} priming, mDCs were fixed after treatment with LPS or LPS and PSMα3 for 24 h. The fixed moDCs were either cultured with naïve $CD4^+$ T cells in fresh medium or in conditioned medium for 3 – 4 d (**Figure 3-10 A condition 4**). The co-culture of $CD4^+$ T cells with fixed mDCs in fresh medium showed low frequencies of iT_{regs} regardless of whether PSMα3 was present or not (**Figure 3-10 B condition 4**). Similar results were obtained when fixed mDCs were co-cultured with T cells in conditioned medium (**Figure 3-10 B condition 4**), showing that the direct and intact interaction of mDCs with naïve $CD4^+$ T cells is crucial for iT_{reg} polarization.

To summarize these findings, direct interaction of PSMα3-treated mDCs and T cells is necessary for sufficient iT_{reg} induction. Furthermore, soluble factors produced by moDCs upon the treatment with TLRLs and PSMα3 and/or after interaction with T cells, are essential.

Several factors are known to induce iT_{reg} priming, like TGF-β, IL-10, IL-2, and IDO (Raker et al., 2015; Rosenzwajg et al., 2015; Schmitt & Williams, 2013). To investigate whether cytokines produced by PSMα3-treated moDCs mediate iT_{reg} priming, moDCs were treated with Sa lysate or LPS in the presence or absence of PSMα3 for 24 h. These treated moDCs were co-cultured with naïve $CD4^+$ T cells for 3 d and co-culture supernatants were analyzed over time for TGF-β, IL-10, and IL-2. Neither IL-10 nor TGF-β were increased in the co-culture of PSMα3-treated mDCs

with naïve CD4$^+$ T cells, but rather decreased in the case of TLR4-treated cells compared to the co-culture with mDCs (**Figure 3-10 C, D**). Analysis of IL-2 production in the co-culture revealed no difference after 1 d or 2 d, but a slight increase after 3 d (**Figure 3-7 E**). The secretion of cytokines important for Th priming however was almost completely inhibited in LPS and PSMα3 treated mDCs (**Figure 3-7**).

Lastly, examination of IDO secretion showed a noticeably increased production by LPS-treated moDCs in response to PSMα3 for both analyses (**Figure 3-10 E**) compared to LPS-treated cells alone. IDO was shown to be important for T_{reg} differentiation from naïve T cells (Raker et al., 2015) and LPS treatment alone led to IDO secretion, which had been previously described (Salazar et al., 2017). IDO was not detected in Sa lysate-treated cells but slightly increased upon PSMα3 treatment, which however, was insignificant (**Figure 3-10 E**).

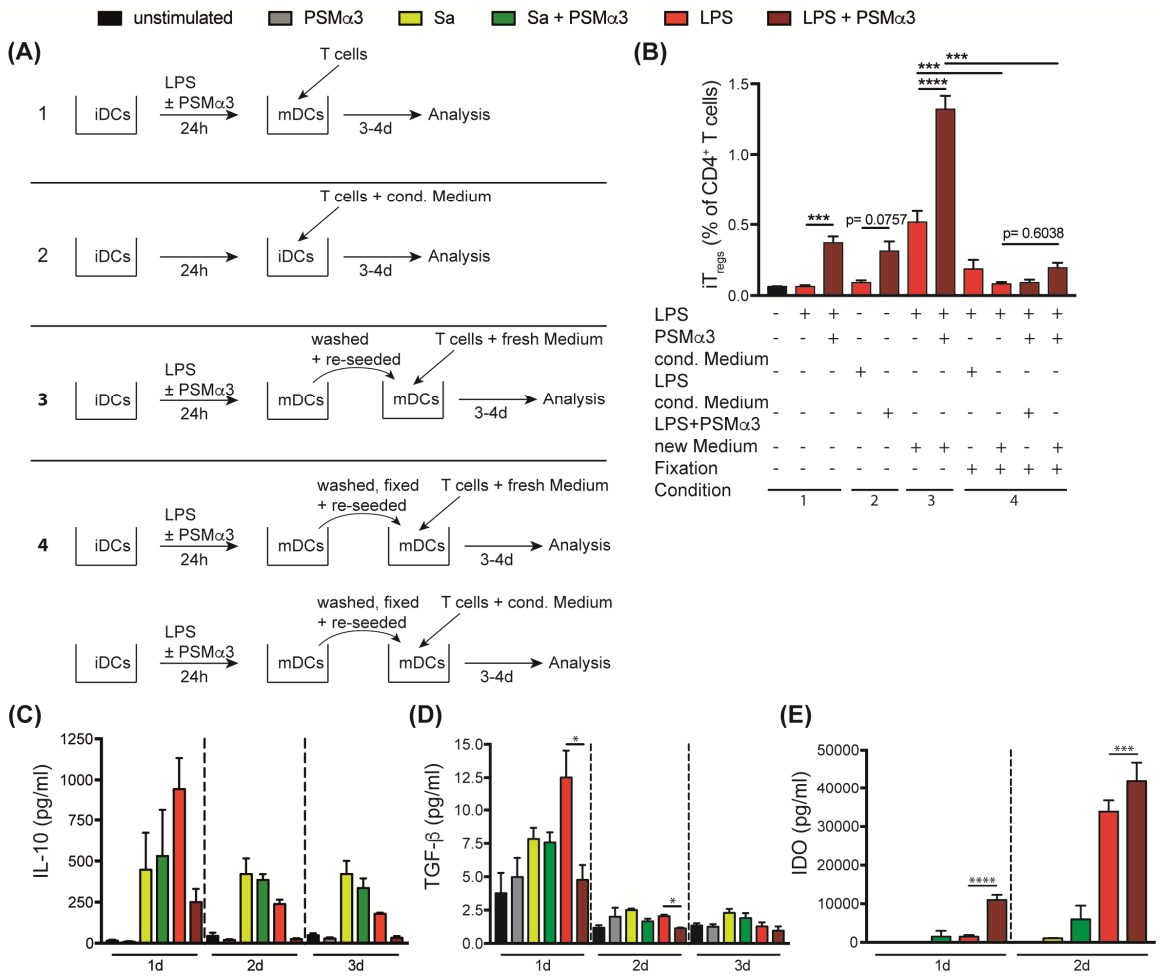

Figure 3-10: PSMα3-treated mDCs induce iT$_{regs}$ by secretion of soluble factors and direct DC-T- cell-interaction (from Richardson et al., 2018)

(A) The experimental setting in a schematic overview: T cells were co-cultured for 3 – 4 d (1) with mDCs (treated with LPS or LPS + PSMα3 for 24 h), with (2) untreated moDCs (iDCs) in the presence of conditioned medium from LPS or LPS + PSMα3 treated DCs, with (3) washed and re-seeded mDCs together with new medium or (4) with fixed moDCs together with either new medium or conditioned medium. (B) The frequencies of iT_{regs} from $CD4^+$ T cells from the experiments described in (A) (one representative of n ≥ 3 independent experiments performed in triplicates; mean ± SEM). (C, D) MoDCs were treated with Sa lysate (Sa) or LPS with, and without, PSMα3 for 24 h and co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 d. Every day supernatants of the co-culture were collected and analyzed via a bead-based cytokine array to determine IL-10 (C) and TGF-β (D) levels in the moDC-T-cell co-culture. (n ≥ 3 independent experiments performed in triplicates, mean ± SEM). (E) MoDCs were treated with Sa or LPS with, and without, PSMα3 for 1 d or 2 d and cell culture supernatants were analyzed for IDO production by sandwich ELISA (n = 2 performed in triplicates; mean ± SEM). *p < 0.05, ***p < 0.001, or ****p <0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest.

The most salient observation is that the induction of iT_{regs} by PSMα3-treated mDCs is dependent on the direct interaction between DCs and T cells and on soluble factors produced by mDCs, most likely the enzyme IDO.

In order to test if the secretion of IDO by PSMα3-treated moDCs really accounts for the increase in iT_{reg} frequency, moDCs were pre-treated with the specific IDO inhibitor 1-Methyl-D-Tryptophan (1-DMT) prior to LPS or LPS and PSMα3 treatment. Again, mDCs were co-cultured with naïve $CD4^+$ T cells and the iT_{reg} frequency was determined. IDO inhibition surprisingly did not lead to diminished iT_{reg} frequencies, but rather revealed even higher frequencies of iT_{regs} independent of whether cells were treated with LPS with or without PSMα3 or not treated at all. Additionally, the difference in inducing iT_{regs} between LPS-treated and LPS and PSMα3-treated moDCs was still significantly higher with PSMα3 (**Figure 3-11 A**). Considering this, the increased IDO production by PSM-treated mDCs seems not to be the cause for the increased iT_{reg} polarization.

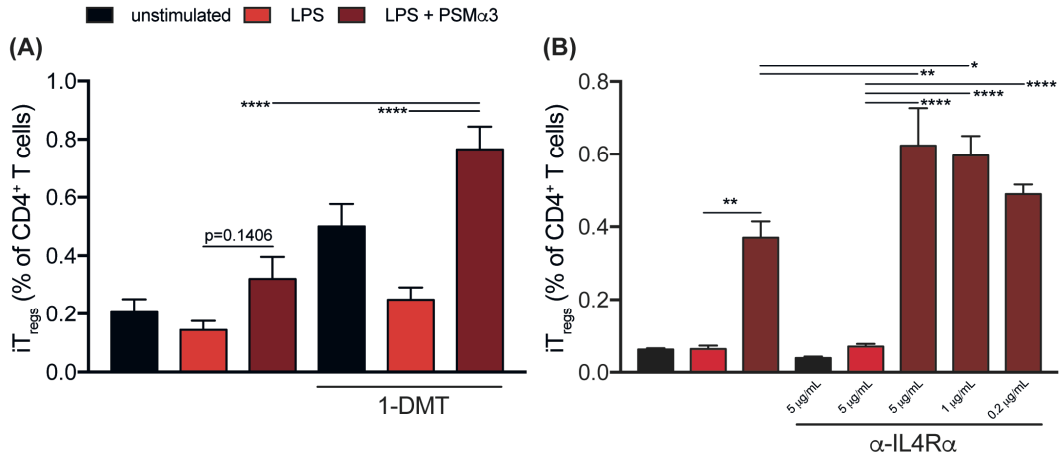

Figure 3-11: Inhibition of IDO and IL4-Rα has no impact on iT_{reg} priming by PSMα3-treated mDCs (adapted from Richardson et al., 2018)

MoDCs were pre-treated with either (A) the IDO inhibitor 1-Methyl-D-Tryptophan (1-DMT) or (B) a blocking antibody against the IL-4Rα subunit for 1 h prior the stimulation with LPS with, and without, PSMα3 for 24 h. MoDCs were then co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 d or 4 d and iT_{reg} frequency was analyzed by flow cytometry. (one representative of n ≥ 3 independent experiments performed in triplicates; mean ± SEM). *p < 0.05, *p < 0.005, or ****p <0.0001, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest

Additionally, it was proposed that IL-13 is able to induce iT_{reg} differentiation (Skapenko et al., 2005). IL-13 was increased in the co-culture of PSM-treated mDCs with T cells (**Figure 3-7 D**). IL-13 and IL-4 exclusively bind to the IL-4Rα subunit (Skapenko et al., 2005); therefore to address whether IL-13 is the essential soluble factor in the co-culture leading to iT_{reg} induction, moDCs were treated with various concentration of an inhibiting IL4Rα antibody prior to treatment with LPS or LPS and PSMα3. MDCs were afterwards co-cultured with naïve $CD4^+$ T cells and the iT_{reg} frequency was determined. Likewise, to the inhibition of IDO, the inhibition of the IL-4Ra had no effect on the induction of iT_{regs} by PSM-treated mDC (**Figure 3-11 B**). The inhibitory antibody had no effect on iT_{regs} primed by untreated or LPS-treated moDCs; the frequencies were comparable to those of cells not treated with the antibody. However, when PSMα3 and LPS treated moDCs were pre-treated with the antibody, they induced a significantly higher iT_{reg} frequency compared to PSM-treated mDCs without antibody incubation. The frequency of iT_{regs} was reduced with decreasing concentrations of the antibody (**Figure 3-11 B**), this suggests that the inhibition of the IL-4Ra leads to an even greater iT_{reg} polarization and means that IL-13 is not essential for iT_{reg} priming by PSM-treated mDCs.

3.9 Induced regulatory T-cell polarization from CD4$^+$ T cells of patients with autoimmune diseases upon interaction with PSMα3-treated mDCs

The peptide toxin PSMα3 is able to modulate moDCs to prime iT$_{regs}$. To address whether this feature can be used for therapeutic approaches in an autoimmune disease setting, moDCs from healthy donors were treated with LPS or LPS and PSMα3 for 24 h and co-cultured with CD4$^+$ T cells from patients with spondyloarthritis. Spondyloarthritis is the name for a family of inflammatory rheumatic diseases with patients suffering from spondylitis and/or enthesitis, conditions which affect the spine and joints. Especially pro-inflammatory Th1 and Th17 cells are increased in these diseases (Smith & Colbert, 2014; C. Wang et al., 2015).

The co-culture of PSMα3-treated mDCs from healthy donors with patient CD4$^+$ T cells showed, as already described above for T cells from healthy donors, that PSMα3 significantly increased the capacity of LPS-treated moDCs to induce iT$_{regs}$ compared to LPS-treated moDCs (**Figure 3-12 A**). However, PSMα3 had no effect on the polarization into the different Th subsets from T cells from spondyloarthritis patients (**Figure 3-12 A**). The analysis of an autologous setting for better therapeutic applicability with moDCs and T cells from the same patient showed similar but not significant results (**Figure 3-12 B**).

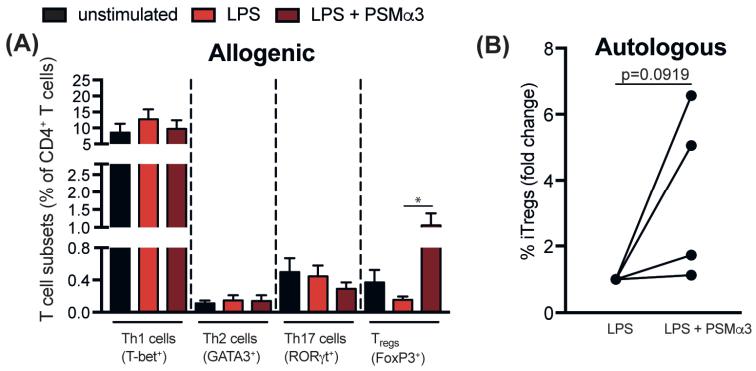

Figure 3-12: PSMα3-treated mDCs increases iT$_{reg}$ frequency from CD4$^+$ T cells of spondyloarthritis patients (from Richardson et al., 2018)

(A) Allogenic T-cell assay: MoDCs from healthy donors were treated with LPS with, and without, PSMα3 for 24 h and co-cultured with CFSE-labeled CD4$^+$ T cells from patients with spondyloarthritis for 3 d or 4 d. The different T cell subsets were analyzed by flow cytometry. The graph shows the frequency of the T-bet$^+$ Th1, GATA3$^+$ Th2, RORγt$^+$ Th17 cells and CD3$^+$CD4$^+$CD127$^-$CD25^{hi}CD45RA$^-$FoxP3^{hi} iT$_{regs}$ from CD4$^+$ T cells (n = 6 patients; mean ± SEM). *p < 0.05, one-way ANOVA with Turkey's posttest or Kruskal-Wallis with Dunn's posttest. (B) Autologous T-cell assay: MoDCs from spondyloarthritis patients were treated as described in (A) and co-cultured with CFSE-labeled CD4$^+$ T cells from the same patients. CD3$^+$CD4$^+$CD127$^-$CD25^{hi}CD45RA$^-$FoxP3^{hi} iT$_{regs}$ were analyzed by

flow cytometry. The graph shows the frequency of iT_{regs} as fold change from LPS-treated cells of 4 different spondyloarthritis patients. Unpaired student's T-test.

In summation, PSMα3-treated mDCs also lead to iT_{reg} polarization from $CD4^+$ T cells in allogenic and autologous inflammatory disease settings. Induction of tolerogenicity by PSMs being feasible in a clinical setting indicates their potential for DC therapy in chronic inflammatory diseases.

4. Discussion

DCs not only initiate acute inflammatory responses but also maintain self-tolerance (Collin & Bigley, 2018). Various pathogens and tumors are able to modulate DC function and thus escape the immune response (Maldonado & Andrian, 2010; Ouyang et al., 2011). Sa is a major human pathogen and a leading cause of morbidity and mortality induced by a wide variability of secreted or cell surface-associated virulence factors (Coates et al., 2014; DuMont & Torres, 2014; M. Miller et al., 2009; Peschel & Otto, 2013). The Sa PSMs are short cytolytic peptide toxins produced by highly virulent Staphylococci that greatly contribute to their pathogenesis and are capable of lysing many eukaryotic cell types. Sa PSMs are also capable of stimulating and modulating inflammatory responses. (Chatterjee et al., 2013; DuMont & Torres, 2014; Grumann et al., 2014; M. Miller et al., 2009; Peschel & Otto, 2013). PSMα peptides particularly harbor essential functions and are the most potent PSMs with regard to cytolysis and have been shown to modulate immune cell functions, e.g. that of DCs (Cheung et al., 2014; Peschel & Otto, 2013; Schreiner et al., 2013; R. Wang et al., 2007). It was previously shown that PSMs modulate mouse DCs and have the ability to impair their protective functions in the immune system. However, the impact of Sa PSM peptides on human DCs was hitherto unknown. In this study, the effect of PSMα3 on human moDC functions and the hence resulting T-cell response was assessed.

4.1 PSMs enter human moDCs via pore formation

Pore-forming toxins (PFTs) represent the largest class of bacterial toxins and are important virulence factors (M. R. Gonzalez et al., 2007). Sa produces many virulence factors including PFTs divided into three classes: α-hemolysin, PSMs, and bicomponent leukocidins (Blake et al., 2017). When analyzing PSMα2 uptake, the peptide was detected in moDCs when incubated at 37°C, as well as on ice. This was further confirmed with imaging flow cytometry that gave additional spacial information regarding the location of PSMs, which were observed as spots either close to the moDC membrane or colocalized with OVA inside the cells. The treatment of moDCs with different PSM peptides, like PSMα2, PSMα3 (in varying concentrations), or δ-Toxin led to a concentration-dependent LDH release into the supernatant, without leading to cell lysis.

The presence of PSMα2 inside moDCs when incubated at 4°C indicates that its uptake does not rely on actin remodeling, which is necessary for active antigen uptake (West et al., 2004). PSMs rather form transient pores into the moDC cell membrane, as LDH, a cytosolic enzyme, which is only present in the supernatant when released via pores (Leroueil et al., 2007) was detected in the supernatants. This correlates with previous findings that showed δ-Toxin forms receptor-independent short-lived transient pores (Otto, 2014; Pokorny et al., 2002). PSMs also form receptor-independent transient pores into membranes, thereby gaining access to the cytosol, which was shown for neutrophils (R. Wang et al., 2007), and DCs (Armbruster et al., 2016b), but in the case of DCs without cell lysis. The cytolytic activity of PFTs is mediated by pore formation in the membrane leading to an efflux of important molecules. PFTs from Sa either mediate lysis in a receptor-dependent manner and subsequent formation of pores, like α-hemolysin and bicomponent leukocidins; or in a receptor-independent fashion by attaching to the membrane, disintegration and short-lived pore formation (Otto, 2014). Therefore, PSMs presumably also form short-lived pores in the membranes of human moDCs and work similarly as Sa δ-toxin. It is, however, not clear why PSM peptides lyse neutrophils but show no cytolytic effects on DCs. One possible explanation could be a different composition and integrity of the cell membranes of neutrophils and DCs. The lipid composition of biomembranes is unique and enables various functionalities of cells and cell organelles depending on their functions (van Meer et al., 2008), whereas the integrity determines the fate of cells (Y. Zhang et al., 2018). The ability of PSM peptides to lyse neutrophils was inhibited in the presence of human serum and its comprised lipoproteins, which directly interacted with PSMs (Surewaard et al., 2012). All mammalian sera contain lipoproteins (Chapman, 1986). Fetal bovine serum was used as medium supplement in this study, as well as when mouse DCs were analyzed (Armbruster et al., 2016b; Schreiner et al., 2013). This might explain why PSM peptides were not able to lyse DCs, as they were treated in the presence of serum lipoproteins. Whether PSMs are able to lyse human and mouse DCs in the absence of lipoproteins must be conducted in another study by treating the cells without using FBS-supplemented medium.

The fluorescently-labeled PSMα2 peptides were either localized as spots close to the membrane or in the cytosol overlapping with the model antigen OVA when incubated at 37°C. If this colocalization of the two peptides implies that PSMs are inside the

phagosome, it must be further confirmed by investigating endosomal markers, like Lamp1 (Cook et al., 2004). The analysis of PSM localization in mouse DCs also showed spots close to the membrane and revealed that the peptide colocalized with p38, as well as with its phosphorylated form (Armbruster et al., 2016b), suggesting that PSMα2 is present in the cytosol as p38 is located in the cytoplasm and not in subcellular fractions (Ben-Levy et al., 1998; Roux & Blenis, 2004). This supports the observation that PSMs are located in the cytosol of human moDCs. The overlapping of PSMs and p38 further indicated direct interaction of the two. Molecularly, PSMα3 enhanced the activation of the p38-cAMP response element-binding (CREB) protein pathway upon TLR ligation, which consequently diminished pro-inflammatory cytokine production but induced IL-10 secretion by mouse BM-DCs (Armbruster et al., 2016b). In human moDCs, this activation was not detected but rather a reduced phosphorylation of p38 and NF-κB. Instead of an induction of IL-10, a diminished secretion was observed, as well as for IL-12 and TNF, which was similar in the mouse. Whether PSMs interact with p38 in human moDCs remains elusive. The modulation of certain host' pathways by PFTs is common; among them are the p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK)/ mitogen-activated protein kinase (MAPK) pathways, which are the main mediators of defense pathways (Los et al., 2013). Hence, it is not surprising that these signaling pathways are modulated as mechanisms to evade the host immune response: a common function of PFTs (Los et al., 2013; Yoong & Pier, 2012). It was further demonstrated that PSMα peptides are important for phagosomal escape due to their lytic function, another immune evading mechanism for cytoplasmic replication of the pathogen (Grosz et al., 2014). PSMs presumably exert their cytolytic function primarily inside the cell and are capable of lysing neutrophils after phagocytosis (Surewaard et al., 2013). Still, the presence of PSMs inside DCs did not lead to cell death.

The pore-forming capability of PSMα peptides is important for Sa pathogenesis; not only are these peptides able to lyse certain immune cells, like erythrocytes, monocytes, and human neutrophils (Cheung et al., 2011; 2010; Forsman et al., 2012; Kretschmer et al., 2010; R. Wang et al., 2007), but they also penetrate immune cells to enter the cytosol of cells, like DCs, to modulate signaling pathways (Armbruster et al., 2016b; R. Wang et al., 2007) and escape phagosomal degradation (Grosz et al., 2014). PSMs are therefore essential for Sa survival, where α-type PSMs play a

crucial role as these peptides are highly cytolytic, with PSMα3 being by far the most active (Geiger et al., 2012; Otto, 2014) and developing drugs that target these peptides might attenuate Sa virulence.

4.2 PSMs modulate DC functions thereby inducing a tolerogenic phenotype

DCs initiate all antigen-specific immune responses and are therefore master regulators in the immune system by linking the innate with the adaptive immune response. Not only do they activate the immune system in response to foreign antigens, but they also avoid responses to self antigens, thereby maintaining self-tolerance and preventing autoimmunity (Mellman, 2013). In order to successfully activate T cells, DCs must undergo maturation, including the upregulation of maturation markers, costimulatory, as well as coinhibitory molecules (Banchereau et al., 2000). Upon activation and maturation, DCs express cytokines, essential for T-cell activation and priming (Blanco et al., 2008b). Here, almost all investigated functions of moDCs to induce immunity against inflammatory stimuli were counteracted by PSMs.

The treatment of human moDCs with TLRLs showed a decrease of antigen uptake, indicating DC maturation of moDCs after TLR stimulation. The treatment with PSMα3 of either iDCs or mDCs showed reduced antigen uptake, revealing that PSMα3 affects moDCs in their capacity to take up antigens independently of their maturation state. The analysis of DC surface molecule expression showed that not only moDCs treated with TLRLs did undergo maturation but also when co-treated with PSMα3. PSMα3, however, changed the surface molecule expression of TLRL-treated moDCs by enhancing early costimulatory molecule and HLA-DR expression, but inhibiting CD80, CD40, and PD-L1 upregulation upon TLR4 stimulation. The treatment with PSMα3 further impaired pro-inflammatory, as well as anti-inflammatory cytokine secretion by TLR4-treated moDCs. This impairment might be explained by the prevented activation of the NF-κB, as well as the p38 MAPK signaling pathway in TLR4-treated moDCs indicated by diminished phosphorylation of the two molecules. These effects of PSMα3 on moDCs show that the Sa peptide toxin targets DC maturation and thereby dampens the immune response (Figure 4-1).

In the periphery iDCs preferentially take up antigen via clathrin-mediated endocytosis and micropinocytosis, whereas mDCs retain uptake only via clathrin-mediated endocytosis (Burgdorf et al., 2007; Platt et al., 2010; Trombetta & Mellman, 2005).

Stimulation with TLRLs and the thus resulting reduced antigen uptake is due to maturation of moDCs, which loose most of their endocytic capactity, with only clathrin-mediated endocytosis remaining. The diminished antigen uptake of mDCs upon PSMα3 treatment indicated that the peptides target clathrin-mediated endocytosis, thereby further reducing moDCs endocytic capacity. Analysis of PSM-treated mouse BM-DCs also revealed reduced clathrin-mediated endocytosis that resulted in less efficient antigen presentation leading to the observed reduced T-cell activation, whereas there were no effects on macropinocytosis and phagocytosis (Schreiner et al., 2013). This supports the generated results on human moDCs. The reduction of antigen uptake as possible immune evasion strategy is not specific for Sa; Yops, virulence factors of *Yersinia enterocolitica*, also inhibit OVA uptake (Autenrieth et al., 2007) and antigen degradation (Adkins et al., 2008). Another example is *Salmonella typhimurium,* which is further able to impair antigen presentation by DCs (Tobar et al., 2004). This impairment of antigen uptake and presentation by DCs shows that it is commonly advantageous for various bacteria as evasion strategy to ensure survival and dissemination. Antigen processing and presentation of human moDCs affected by PSMα3-treatment was not assessed and must be further investigated. This could be addressed by analyzing antigen processing with OVA-DQ, a self-quenching conjugate of OVA that is detected upon degradation (Adkins et al., 2008) or by using OVA-specific T cells (N. S. Wilson et al., 2004) to compare T-cell activation upon interaction with untreated or PSMα3-treated moDCs.

For a sufficient T-cell activation, the interaction of costimulatory molecules and their ligands is required. PSMα3 affected surface molecule expression of moDCs by inhibiting the costimulatory molecules CD80 and CD40, and also the coinhibitory molecule PD-L1. This was in accordance with the inhibited CD40 expression in mouse BM-DCs upon PSM-treatment, which further impaired the expression of CD54. On the contrary, CD80, MHC II, and CD86 expression was increased upon PSMα3-treatment, another similarity found in human moDCs (Armbruster et al., 2016a). The aforementioned results show that surface molecule expression of both human and mouse DCs was altered by PSMs. Other pathogens and their secreted virulence factors also have effects on the surface molecule expression of DCs. Cholera toxin in combination with LPS is able to induce CD80 and CD86 expression, but decreases that of CD40 and CD54 by DCs, which modulates DC cell activation

and thereby promotes T_{reg} induction (Lavelle et al., 2003). This is in part consistent with the effects on the studied human moDCs and the thereby responding T-cell activation.

Not only surface molecule expression is induced by maturation, but also cytokine production (Hackstein & Thomson, 2004), which is completely prevented by PSMα3 treatment. Mechanistically, the inhibition of TNF production, which among others regulates CD40 expression, upon synergistic treatment with TLRLs and PSMα3 could explain the impaired up-regulation of CD40. This correlates with the previous observed downregulation of the CD40/CD40L pathway upon TNF blockade (Danese et al., 2006). NF-κB, as well as p38 activation is necessary for DC maturation including upregulation of CD80, CD86, and CD40, and is further important for cytokine production mediated by TLR signaling (Ardeshna et al., 2000; Dalod et al., 2014; Kaisho & Akira, 2001). Simultaneous treatment of moDCs with TLRLs and PSMα3 did not show activation by phosphorylation of these molecules. Prevented activation of NF-κB and p38 corresponds with the abrogated CD40 and CD80 expression and also with impaired cytokine secretion, however, does not explain the enhanced early costimulatory molecule expression. Mouse BM-DCs in contrast showed a greater activation of these signaling pathways after PSM-treatment thereby having similar effects on pro-inflammatory cytokine secretion and surface molecule expression (Armbruster et al., 2016a; Armbruster et al., 2016b; Schreiner et al., 2013). This contrast might be due to species-specificity and indicates that different signaling pathways with possible similar outcomes might be regulated differently. TLR4-treated moDC function was more affected by PSMα3 incubation, suggesting that PSMα3 prevents TLR4-activation either extracellularly and/or intracellularly. Direct extracellular interaction of PSMα1–3 with TLR4 was shown to prevent binding of HMGB1 to TLR4 and thus downstream activation of NF-κB (Chu et al., 2018). This might explain the inhibited CD40 and CD80 expression, as well as the impaired cytokine production in TLR4-treated moDCs in the presence PSMα3. The Sa-derived peptide toxins were also detected inside DCs and colocalized with p38 thereby modulating this signaling pathway (Armbruster et al., 2016b). It is therefore not known if the greater effects on TLR4-treated moDCs is mediated by direct interaction of PSMα3 with the signaling molecules or also by blocking binding of LPS to TLR4, thereby preventing activation of the signaling cascade (Chu et al., 2018). Albeit PSMs dramatically changed the cytokine secretion pattern of mouse BM-DCs upon

TLR2 stimulation via the activation of NF-kB, p38, and ERK. This also led to an inhibited pro-inflammatory cytokine production of TNF, IL-6, and IL-12, however, induced the secretion of the anti-inflammatory IL-10 (Armbruster et al., 2016b; Schreiner et al., 2013). This differs to that found in human moDCs, which again might be due to differences in the species. The pro-inflammatory immune response implemented by the secretion of TNF plays a significant role in the clearance of Sa (Hultgren et al., 1998), therefore inhibiting cytokine production additionally is beneficial for pathogen survival. Again, targeting the MAPK signaling pathways is not specific for Sa PSM peptides. Other virulence factors, like YopJ from *Yersinia pseudotuberculosis* inhibits the phosphorylation of p38 and JNK thereby preventing the production of TNF in macrophages (Palmer et al., 1998). In addition, YopP from *Yersinia enterocolitica* not only suppresses TNF and IL-12 secretion, but also IL-10 production by DCs (Erfurth et al., 2004). Many bacteria evade the host immune response by manipulating signaling pathways, like the NF-kB-, MAPK-, phosphatidylinositol 3-kinase-, and p21-activated kinase pathways (Krachler et al., 2011). Targeting DC functions is an effective evasion strategy by pathogens to prevent inflammation and allowing uncontrolled spreading of the pathogen. Several groups described the induction of tDCs by pathogens (Couper et al., 2008; DePaolo et al., 2008; McGuirk et al., 2002). NF-κB inhibition favors an immature or tolerogenic DC phenotype, which stimulates the expansion of Foxp3-expressing T_{regs} (Iruretagoyena et al., 2006; Martin et al., 2007; Raker et al., 2015; Thompson et al., 2004). Further, also other pathways and proteins, like p38 MAPK, JNK, ERK1/2 and IDO are differently regulated in tDCs compared to cDCs, with p38 MAPK and NF-κB inhibition leading to markedly down-regulated IL-12 production in cDCs after LPS treatment (Dáňová et al., 2015). This might molecularly explain the observed tolerogenic phenotype of human moDCs after synergistic treatment with TLRLs and PSMα3. The entire molecular mechanism however is still unclear and has to be further investigated to explain all the observed effects of PSMs on DC functions, which might help to better understand and control Sa pathogenesis.

4.3 The PSM-primed tDCs induce iT_{reg} differentiation and inhibit Th1 polarization

Upon activation by DCs, $CD4^+$ naïve T cells differentiate into different T effector cell subsets with distinct functions important for regulating immune responses (Tai et al., 2018). The differentiation into T_{regs} regulates Th cell responses and is therefore

crucial for maintaining immune homeostasis (Shevach, 2009; Vignali et al., 2008; Zhou et al., 2009). As almost all functions of human moDCs were affected by PSMs, the subsequent T-cell activation and therefore the effect of PSMs on the adaptive immune response was assessed.

PSMα3-treated mDCs suppressed Th1 polarization by inhibiting the expression of T-bet and completely blocking cytokine secretion of IL-12p70 and IFN-γ in LPS-treated cells, not only important for Th1 development but also for their function. On the contrary, PSMα3-treated mDCs induced iT_{reg} polarization and proliferation (Figure 4-1). These iT_{regs} were functionally capable of inhibiting T_{eff} cell proliferation. When addressing the possible mechanism behind the induction of these iT_{reg} the analysis of cytokine secretion in the moDC- T-cell co-culture showed that PSMα3 inhibited secretion of pro-inflammatory cytokines, which was also observed for moDC cytokine production. IL-10 production was also suppressed upon PSM-treatment by both mDCs and in the co-culture, indicating that this cytokine is not accountable for the induced T_{reg} proliferation. Similar, the analysis of TGF-β levels in the co-culture showed that also this cytokine is not responsible for the here observed increased iT_{reg} priming upon PSM-treatment of mDCs. In contrast, the secretion of the enzyme IDO was significantly increased by TLRL-treated moDCs, as well as that of the cytokine IL-13 in the moDC-T-cell co-culture in response to PSMα3. Neither IDO inhibition by 1-DMT (Agaugue et al., 2006), nor blocking of the IL-4Rα subunit, to which IL-13 and IL-4 exclusively bind to (Skapenko et al., 2005), prevented iT_{reg} induction upon PSM-treatment, but rather enhanced it. Both, IDO and IL-13, therefore do not mechanistically contribute to the PSM-mediated T_{reg} induction. One requirement of PSM-mediated T_{reg} induction certainly is the direct DC-T-cell interaction; lacking this direct interaction abrogates iT_{reg} polarization by PSM-treated mDCs.

The here observed impairment of Th1 differentiation is consistent with findings of PSM-treated BM-DCs, which prevented Th1 priming, whereas that of Th17 cells was unaffected (Schreiner et al., 2013). The impaired ability to prime Th1 responses is not Sa-specific but was also shown for other infections, e.g. with Hepatitis C virus, leading to less IL-12p70 production by DCs (Kanto et al., 2004), which was likewise observed for human moDCs. Further, infection with the Hepatitis B virus compromises the antigen-presenting function of moDC with concomitant impairment of Th1 responses (Beckebaum et al., 2003). PSMs affected antigen uptake of human

moDCs, which might also lead to reduced antigen presentation, consequently leading to less Th1 polarization; this however remains to be shown. Not only the suppressed Th1 polarization but also the functional impairment by less effector cytokine secretion is critical in infections. Th1 and Th17 cells mediate protective $CD4^+$ T-cell responses against Sa and *Candida albicans* due to the secretion of IFN-γ and IL-17 (Cho et al., 2010; Lin et al., 2009; McLoughlin et al., 2008). Further, IFN-γ has an important protective role during systemic infection, by activating macrophages and neutrophils and thereby enhancing phagocytosis of the bacteria (Zhao & Tarkowski, 1995). This is in accordance with the impaired IFN-γ and IL-17A secretion in the moDC-T-cell co-culture upon PSMα3 treatment. Impairment of inflammatory Th-cell responses due to the presence of PSM peptides might therefore lead to reduced clearance and is important for Sa immune escape, which was already observed in mice, where the mortality rate was higher when PSMα3 was expressed by Sa (R. Wang et al., 2007). The further observed induction of iT_{reg} polarization and proliferation by PSMα3-treated human mDCs is comparable to previous *in vitro* studies of mouse BM-DCs. PSM-treated BM-DCs had a tolerogenic phenotype and increased the priming of T_{regs} (Armbruster et al., 2016b; Schreiner et al., 2013). The balance between T_{regs} and T_{effs} is highly controlled, which is necessary during infections, on the one side to combat the infection, but on the other side to prevent immune pathologies (Belkaid, 2007). The induction of iT_{regs} by a tolerogenic DC phenotype induced by the pathogen itself thus might lead to an enhanced immune evasion. A hallmark of tDCs is their immature phenotype characterized by low surface levels of MHC class II and costimulatory molecules. Phenotypically mature DCs mediate immunity, whereas it has been suggested that immature DCs, with a low MHC II and low costimulatory molecule and therefore poorly immunogenic phenotype, maintain tolerance (Kushwah et al., 2010; Ouyang et al., 2011; Raker et al., 2015; Villadangos & Schnorrer, 2007). The maturation state alone, however, does not define tDCs, as mature or semi-mature DCs were also found to induce tolerance (Raker et al., 2015; Villadangos & Schnorrer, 2007) and are capable of promoting T_{regs} and being superior in activating their suppressor function (Pletinckx et al., 2011; Spörri & Reis e Sousa, 2005). Although PSMα3 had little influence on most TLR-induced up-regulated surface molecules, up-regulation of CD80, PD-L1, and CD40 was impaired, thereby preventing full DC-maturation. This modulation, however, did not keep the moDCs in an immature state, as surface molecule expression was definitely higher

than in unstimulated cells. The lack of CD40 expression on DCs was shown to be important for the generation of T_{regs} while suppressing primary immune responses (Quezada et al., 2004), which might also be the case here and lead to PSM-induced iT_{reg} differentiation. Further, tDCs produce low amounts of pro-inflammatory cytokines (Manicassamy & Pulendran, 2011), which further supports the hypothesis that PSMs prime tDCs, as the secretion of all measured pro-inflammatory cytokines was prevented. T-cell differentiation is mainly controlled by cytokines that mediate polarizing signals (Campbell & Koch, 2011). Factors or molecules, like IL-10, TGF-β, retinoic acid, and IDO, are associated with T_{reg} polarization (Kushwah & Hu, 2011; Raker et al., 2015). IL-10 production, however, was suppressed by human mDCs upon PSM-treatment, which is in contrast to the previously observed findings that PSMα3-induced mouse tDCs are characterized by the enhanced production of IL-10 (Armbruster et al., 2016b; Schreiner et al., 2013). The suppression capacity of T_{regs} induced by PSM-primed mouse tDCs though was just partly dependent on IL-10 and TGF-β and suggested other mechanisms, like contact-mediated suppression (Schreiner et al., 2013). IL-10 therefore might be only important for the induction of T_{regs} but not for their functional tasks and further not important in PSM-induced iT_{reg} induction by human DCs. Similarly, TGF-β that was reported to be required for T_{reg} induction (Schmitt & Williams, 2013) also did not account for the observed iT_{reg} induction also questioning its pivotal role in this process. It was further proposed that IL-13 is also able to induce iT_{reg} differentiation (Skapenko et al., 2005), whose production was increased in the co-culture of PSM-treated mDCs with T cells. Blocking of its receptor, however, revealed that this was not the case for the here observed iT_{reg} induction, suggesting that IL-13 is not essential for iT_{reg} priming by PSM-treated mDCs. The enhanced T_{reg} differentiation might be due to blocking the polarization of naïve T cells into Th2 cells and the plasticity of T cells allows the switch into one of the other subsets, in this case into T_{regs} (Zhou et al., 2009). The analysis of IDO showed a significantly higher secretion in PSMα3-treated mDCs. The adaptive immune response is modulated by IDO expression by DCs, which promotes immune suppression and tolerance (Bubnoff et al., 2011; Harden & Egilmez, 2012; Munn & Mellor, 2013). LPS treatment alone already induced IDO, which was recently reported (Salazar et al., 2017), however, IDO production by DCs was significantly enhanced upon PSMα3 treatment. Similar to blocking of the receptor for IL-13, IL-4Rα, inhibition of IDO by the specific inhibitor 1-DMT (Agaugue et al., 2006) did not

prevent iT_{reg} induction, arguing against IDO as an important factor for the increased iT_{reg} differentiation by PSM-treated mDCs. The soluble factor or factors necessary for the here observed iT_{reg} differentiation are not known so far, but some secreted factors have to be responsible as molecules secreted by DCs and upon DC-T-cell interaction were important. Apart from soluble factors, PSM-mediated T_{reg} induction certainly is dependent on the direct DC-T-cell interaction, which is in accordance with previously reported findings (Corthay, 2009). Thus, PSMα3-treated mDCs induce T_{reg} differentiation via direct DC-T-cell interaction, with presumably less CD40/CD40L interaction and less antigen presentation upon fewer uptake in combination with the absence of Th-priming cytokines and yet unknown DC-secreted factors. Various pathogens or pathogenic toxins are able to induce tDCs and subsequent T_{reg} differentiation thus escaping the immune response (Maldonado & Andrian, 2010; Manicassamy & Pulendran, 2009; Ouyang et al., 2011). *Candida albicans* induces tDCs by modulating signaling pathways and enhancing IDO activation (Bonifazi et al., 2009; Maldonado & Andrian, 2010). The *Yersinia pestis* virulence factor LcrV highly contributes to immune evasion by priming tDCs and promoting IL-10 production (DePaolo et al., 2008). Further, *Fasciola hepatica*- derived excretory-secretory products modulate BM-DCs by affecting maturation and their capacity to stimulate T-cell responses. These products increased T_{reg} polarization with increased IL-10 and TGF-β production (Falcón et al., 2010). Therefore, the priming of tDCs by PSMs as an effective evasion strategy to block inflammation and allowing uncontrolled spreading of the pathogen is not Sa-specific.

Hence, PSMs are beneficial for Sa by preventing inflammatory responses by inhibiting Th1 polarization and priming iT_{regs}, leading to uncontrolled spreading and immune evasion of Sa. On the contrary, inducing tolerance by priming tDCs thereby inhibiting inflammatory responses might be useful, especially in autoinflammatory diseases.

4.4 PSMs as therapeutic approach in autoimmune diseases

New therapeutic approaches for preventing or inhibiting hyper-reactive immune activations and responses like in autoimmune diseases, allograft rejection, or allergies are needed. Current therapies mainly use nonspecific systemic immunosuppressants, like glucocorticoids or cytostatics, which are associated with severe side effects (Maldonado & Andrian, 2010; Tamesis et al., 1996). DCs are regulators of the adaptive immune response and tolerance; therefore, the specific

generation and adoptive transfer of DCs, which are able to induce tolerance, into patients is attractive for clinical applications to enhance, maintain or restore immunological tolerance (Lutz, 2012). Thus, *ex vivo* generated tDCs are a new therapeutic option. PSMα3-treated mDCs induced iT_{reg} priming, which was also observed in allogenic and autologous inflammatory disease settings, with either DCs generated from healthy donors or from the same patient. This showed that Sa-derived PSMα3 has the potential to induce tolerogenic human DCs with the ability to prime iT_{regs} indicating its potential for DC therapy in inflammatory diseases. Various pathogens escape the immune system by mechanisms exploiting T_{regs} (Belkaid, 2007; Grainger et al., 2010; Mills, 2004). Pathogenic products and toxins, like here the Sa PSM peptides, are able to promote DC tolerogenicity thereby inducing iT_{reg} differentiation, e.g. products from *Fasciola hepatica*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Bordetella pertussis*, and *Vibrio cholerae* (Maldonado & Andrian, 2010; Ouyang et al., 2011). It is therefore not surprising that compounds, like cyclosporin, tacrolimus, voclosporin, and rapamycin are commonly used as immunosuppressive drugs to treat immune disorders and transplant rejection (Korom et al., 2009; Maldonado & Andrian, 2010). These therapeutic approaches, with the intake of such immuno-modulatory drugs are unspecific. They suppress the entire immune system and have serious side effects that lead to immunodeficiency (Maldonado & Andrian, 2010; Ouyang et al., 2011). DC vaccination strategies by applying tDCs are therefore an attractive alternative. Using DCs would lead to a more specific treatment option with less side effects as these are antigen-specific and only affect the auto-reactive inflammatory response (Maldonado & Andrian, 2010; Raker et al., 2015; Van Brussel et al., 2014; Yoo & Ha, 2016). The therapeutic potential of tDCs has been demonstrated in pre-clinical animal models of autoimmune diseases, like arthritis (van Duivenvoorde et al., 2007), EAE (Menges et al., 2002), and type 1 diabetes (Feili-Hariri et al., 2002), as well as with *in vitro* generated human tDCs (Van Brussel et al., 2014). The generation of such tDCs, using different agents, like dexamethasone (Maldonado & Andrian, 2010; Piemonti et al., 1999) or vitamin D3 (Maldonado & Andrian, 2010; Penna & Adorini, 2000), and their *in vivo* stability are critical parameters (Phillips et al., 2017). Previous clinical trials already revealed that DC administration is well tolerated (Dhodapkar & Steinman, 2002; Dhodapkar et al., 2001; Raker et al., 2015; Steinman & Banchereau, 2007), which is pivotal for a sufficient therapy. Several clinical studies were conducted using tDCs for treating e.g.

rheumatoid arthritis (Thompson et al., 2004) or type I diabetes (Giannoukakis et al., 2011), indicating that tDCs are a promising tool for therapy of autoimmune diseases. PSMα3, secreted by highly virulent CA-MRSA strains, might have the potential to induce stable human tDCs for therapeutic use in cellular therapy for autoimmune diseases. The stability of the PSM-induced tDCs, especially in the presence of inflammatory stimuli is crucial and has to be confirmed by all means before using the peptide for DC therapy. Lastly, it has to be investigated whether *ex vivo* generation of tDCs or direct administration of PSMs is more effective and if this approach is tolerable. While using PSM peptides for tDC induction seems to be a promising approach, remaining open questions have to be answered before the potential of PSMs can be exploited for DC therapy.

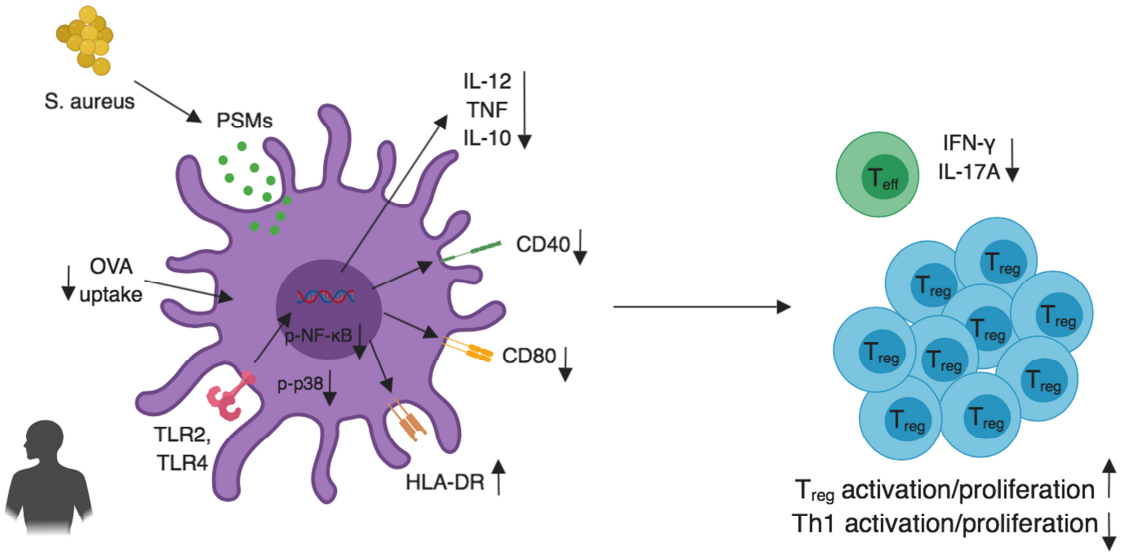

Figure 4-1: Summary of the effects of Sa-produced PSM peptides on human moDCs (designed with BioRender – biorender.com)

Abbreviations

1-DMT	1-Methyl-D-tryptophan
BDCA	Blood dendritic cell antigen
BM-DCs	Bone marrow-derived DCs
CA-MRSA	Community-associated Staphylococcus aureus
CD	Cluster of differentiation
cDC	Conventional DCs
CFSE	Carboxyfluorescein succinimidyl ester
CREB	cAMP response element-binding
DAMPs	Danger-associated molecular pattern
DCs	Dendritic cells
FACS	Fluorescence-activated cell sorting
FoxP3	Forkhead box protein P3
GATA3	GATA binding protein 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-DR	Human Leukocyte Antigen – DR isotype
iDC	Immature DCs
IDO	Indolamin-2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
ILT	Immunoglobulin-like transcript
IRAK	Interleukin-1 receptor-associated kinase-like
ISX	Image-Stream mkII
iT_{regs}	Induced T_{reg}
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
mDC	Mature DCs
MHC	Major histocompatibility complex
MMR	Macrophage mannose receptor
moDC	Monocyte-derived DC
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD	Nucleotide-binding oligomerization domain
nT_{regs}	Natural T_{regs}
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid DC
PD-L	Programmed death-ligand
PFT	Pore-forming toxin
p-NF-κB	Phospho-NF-κB
p-p38	Phospho-p38
PRR	Pattern recognition receptors
PSM	Phenol-soluble modulin
RA	Retinoic acid
RORyt	Retinoic acid receptor-related orphan receptor gamma
Sa	*Staphylococcus aureus*
Sa lysate	*Staphylococcus aureus* cell lysate
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
tDC	Tolerogenic DC
T_{eff}	Effector T cells
TGF-β	Transforming growth factor beta
Th	T helper cell
TIR	Toll/interleukin-1 receptor
TIRAP	Toll/interleukin-1 receptor domain-containing adapter protein
TLR	Toll-like receptor
TLRL	Toll-like receptor ligand
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
T_{reg}	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β

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