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

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ABSTRACT

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

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1. Introduction

Dendritic cells (DCs) are the most essential antigen presenting cells that link the innate and adaptive immunity by activating T and B cells (Merad et al., 2013). Immature DCs are able to actively internalize antigens because of their high endocytic capacity. Simultaneously, inflammatory signals induce the maturation of DCs. As a result, DCs reduce antigen uptake, yet enhance antigen processing and presentation thereby enabling efficient T-cell activation (Garrett et al., 2000). DCs recognize pathogen-associated molecular patterns by their pattern recognition receptors (Saraiva and O'Garra, 2010) e.g. Toll-like receptors (TLRs) (Merad et al., 2013). TLRs respond to exogenous microbial products resulting in the activation of the adaptive immune system by downstream signaling leading to the expression of cytokines, chemokines and interferons (Michelsen, 2001; Re and Strominger, 2004). Additionally, TLR signaling in DCs leads to the downregulation of endocytosis, up-regulation of the co-stimulatory molecules CD80 and CD86, as well as MHC class II molecules and cytokine production (Merad et al., 2013). The pro-inflammatory cytokines e.g. TNF-α, IL-6 and IL-12 recruit other immune cells for pathogen clearance and induce T helper cell differentiation (Dinarello, 2000; Elenkov and Chrousos, 2002). In contrast, the anti-inflammatory cytokine IL-10 elicits important immunoregulatory function by inhibiting IL-12 production which regulates regulatory T-cell (T_{reg}) development (Ouyang et al., 2011).

The Gram-positive bacterium *Staphylococcus aureus* is the leading cause of more than fifty percent of skin and soft-tissue infections worldwide (Brown et al., 2015; Talan et al., 2011). In treatment of this disease, antibiotic abuse has led to the emergence of methicillin-resistant *S. aureus* (MRSA) strains. Both healthy and immunocompromised patients are susceptible to community-associated (CA) MRSA strains e.g. USA300, which is the most prevalent strain in the world (DeLeo et al., 2009; Otto, 2010). Because *S. aureus* possesses many virulence factors it is very effective at evading the host's innate and adaptive immune system (Brown et al., 2015; Otto, 2010). For example, CA-MRSA strains express α-toxin, Panton-Valentine Leukocidin and phenol-soluble modulin peptides (PSMs) (Otto, 2010; Thammavongsa et al., 2015).

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

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Also contributing to CA-MRSA strains pathogenicity is its ability to secrete large amounts of PSMs compared to other MRSA strains (Wang et al., 2007). PSMs contain five α-peptides (δ-toxin and PSMα1-4) and two β-peptides (PSMβ1-2) (Peschel and Otto, 2013; Wang et al., 2007). PSMs attract human neutrophils by binding to the cell surface human formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010; Wang et al., 2007). Furthermore, mouse DCs expressing the mouse FPR2 are also attracted by PSMs (Schreiner et al., 2013). The α-helical and amphipathic PSMs possess a pore forming activity (Wang et al., 2007). Previously, we showed that PSMs, most likely via pore-formation, modulate the cytokine production of DCs independently of the mouse FPR2 (expression) by increasing the TLR2 ligand-induced production of the anti-inflammatory cytokine IL-10 via specific interaction with the MAPK p38 (Armbruster et al., 2016). In contrast, PSMs inhibit the TLR2 ligand-induced pro-inflammatory cytokine secretion of TNF-α, IL-12 and IL-6 (Armbruster et al., 2016; Dinarello, 2000; Elenkov and Chrousos, 2002; Schreiner et al., 2013). As a consequence, the PSM-treated DCs show an impaired T helper 1 cell priming capacity, but an increased induction of Foxp3$^+$ T_{regs} via p38-CREB-IL-10 modulation in DCs (Armbruster et al., 2016; Schreiner et al., 2013). As p38 MAPK signaling is activated upon TLR-stimulation in general, we hypothesize that PSMs affect DC functions including maturation, cytokine production and T-cell priming upon treatment with various TLR ligands. The aim was to test whether PSMs could be useful as general inducers of tolerogenic DCs for therapeutic applications.

The major innate immune-stimulating compounds of *S. aureus* are lipoproteins (Hashimoto et al., 2006; Stoll et al., 2005). They induce a fast and strong cytokine release by mouse peritoneal macrophages via TLR2-MyD88 signaling leading to increased pathogenicity (Schmaler et al., 2009). Lipoproteins of *S.aureus* are either di- or tri-acylated, depending on the growth phase (Kurokawa et al., 2012). Therefore, we tested di- and tri-acylated lipopeptides (Pam2- and Pam3-Cys) in this study. Moreover, we used ligands for TLR4, and the intracellular TLR7 and TLR9. We show that PSMα3 generally modulates antigen uptake, maturation and cytokine production in DCs upon extracellular as well as intracellular TLR-stimulation. In consequence, these tolerogenic DCs increased priming of T_{regs}.

2. Materials and methods

2.1. Mice

Female C57BL/6JolaHsd and BALB/cAnNRj mice were purchased from Janvier (St. Berthevin Cedex, France). FPR2$^{-/-}$ mice (Chen et al., 2010) with a genetic C57BL/6 background were bred in the animal facilities of the University Clinic of Tübingen. All mice were held under specific pathogen-free conditions, were provided with food and water ad libitum and used for experiments between 6 and 12 weeks of age. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Anzeige 09.01.2014).

2.2. Generation of bone marrow-derived DCs (BMDCs)

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

2.3. Reagents for stimulation of BMDCs

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

2.4. Cytokine production by BMDCs

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

2.5. Flow cytometry staining of stimulated BMDCs

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

2.6. OVA uptake by BMDCs after stimulation

To test the antigen uptake capability of BMDCs, 5×10^6 cells were seeded in 48-well plates and treated for 24 h as described above. Thereafter, cells were incubated with AlexaFluor647-labeled Ovalbumin (OVA) (10 μg/ml) for 30 min at 37 °C. Then the cells were washed with ice-cold PBS with 2% FCS followed by extracellular staining with MHC class II-eFluor450 (M5/114.15.2; eBioscience) and CD11c-PE (N418; eBioscience) antibodies and flow cytometry measurement as described above.

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