

Formalin-Fixed *Staphylococcus aureus* Particles Prevent Allergic Sensitization in a Murine Model of Type I Allergy

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Key Words

Mouse model of type I allergy · IgE · Allergic airway inflammation, experimental · *Staphylococcus aureus* · Protein A · Immune deviation · Bone marrow-derived dendritic cells · Hygiene hypothesis

Abstract

Background: Bacterial infections are supposed to act counterregulatory to the development of allergen-specific Th2 immune responses. We analyzed whether administration of extracellular *Staphylococcus aureus* inhibited experimental sensitization against allergens. **Methods:** BALB/c mice were immunized with alum-adsorbed ovalbumin (OVA) together with formalin-fixed *Staphylococcus* particles. OVA-specific antibody production and cytokine synthesis by spleen cells was analyzed. Airway reactivity and cellular infiltration into the airways was assessed after intranasal challenge of mice with OVA. In addition, the capacity of *Staphylococcus* particles to modulate cytokine production by bone marrow-derived dendritic cells was analyzed in vitro. **Results:** Simultaneous application of OVA and *Staphylococcus* particles very efficiently inhibited production of specific IgE and IgG1 as well as secretion of IL-4 and IL-5 by splenocytes, while enhancing IgG2a formation and production of IFN- γ , indicating a shift from a Th2 response towards a Th1-biased response. This effect was not dependent on the expression of protein A by *Staphylococcus*. An enhanced frequency or ac-

tivity of regulatory T cells after administration of *Staphylococcus* particles was not apparent. Treatment of mice with *Staphylococcus* particles during the sensitization phase prevented lung inflammation (airway hyperreactivity, eosinophilia) after local challenge with OVA. Culture of bone marrow-derived dendritic cells with *Staphylococcus* particles induced IL-12p35 and p40 mRNA expression as well as secretion of IL-12p70, and increased production of IL-10 mRNA and protein. **Conclusions:** Administration of formalin-fixed *Staphylococcus* particles induced Th1-biased immune responses and prevented allergic sensitization.

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Introduction

The incidence of atopic disorders including bronchial asthma, hay fever and atopic dermatitis (AD) in developed countries has risen steadily in recent decades [1]. A number of theories have been proposed to explain the increasing occurrence of allergic diseases, all considering the complex interaction between genetic and environmental factors in the elicitation of these hypersensitivities. The controversially discussed hygiene hypothesis describes an inverse correlation between the frequency of recurrent microbial infections in early childhood and the prevalence to develop allergies in later life [2, 3]. This postulate was reasoned by several epidemiological and clinical

cal studies which suggest that an encounter with infectious agents leads to the stimulation of T helper (Th)1-biased immunity and prevents the development of Th2 immune responses following natural exposure to allergens [4, 5]. More recent work has emphasized the reduced activity of regulatory T cells (Treg) in allergic patients which is likely to be due to a diminished microbial burden in early life [6]. Thus, the anti-allergic response triggered by the exposure to pathogens during childhood may include both mechanisms, immune deviation and expansion of Treg activity [7].

The inhibitory capacity of microbial infections on the development of allergic reactions has also been shown in animal studies. Exposure of mice to live or heat-killed *Mycobacterium* species (*M. bovis*-bacillus Calmette-Guérin (BCG), *M. vaccae*) prior to allergen sensitization reduced allergen-specific immunoglobulin (Ig) E and Th2 cytokine production and inhibited lung hyper eosinophilia as well as the development of airway hyper reactivity following bronchial allergen challenge [8–11]. The suppressive effect was also observed when the mycobacteria were administered to presensitized animals [10–12]. In addition, infection with live or heat-killed *Listeria monocytogenes*, another facultative intracellular bacterium, has been reported to interfere with the development of Th2 responses and to prevent allergic airway inflammation [13] as well as to reduce anaphylactic symptoms in a mouse model of peanut allergy [14]. Likewise, *Bordetella pertussis* infection modulated allergen priming and attenuated the severity of airway pathology following subsequent allergen aerosol challenge [15].

A common feature of bacterial infections, which contributes to the inhibition of allergic immune responses, is the strong polarization of CD4+ T cells into Th1 lymphocytes. The major reason for the potent induction of Th1 cells is the expression of conserved microbial molecules referred to as pathogen-associated molecular patterns (PAMPs). Most PAMPs are specifically recognized by Toll-like receptors (TLRs) which are expressed on host immune cells [16]. Binding of PAMPs to the respective TLRs leads to the induction of both innate and acquired immune responses. A well-characterized example of such a bacterial structure is lipopolysaccharide (LPS), also referred to as endotoxin, a component of the cell wall of gram-negative bacteria. Interaction of LPS with TLR-4 and CD14 on the surface of monocytes, macrophages and dendritic cells (DCs) causes the production of Interleukin (IL)-12 and subsequently interferon (IFN)- γ [17], thus favoring the induction of Th1 immune responses. Several epidemiological and experimental studies have

shown that the exposure to LPS has bidirectional effects on the elicitation of allergic diseases, depending on the time point of confrontation. Whereas encounter with high endotoxin levels early in life and prior to allergen contact may protect against the development of allergies [18–21], LPS exposure of already allergen-sensitized individuals may aggravate established airway inflammation [19, 21, 22].

Albeit the Th1-promoting effect of infection with intracellular bacteria has often been demonstrated in murine allergy models [8, 9, 13–15], the impact of infection with extracellular bacteria on the induction or maintenance of allergic responses and its immunomodulatory implications have rarely been analyzed. Therefore we investigated the effect of application of gram-positive *Staphylococcus aureus* on the induction of allergen-specific Th2 immune responses. The role of *S. aureus* in the development of AD has been the subject of controversial discussion. Some studies suggest that infection with *S. aureus* is causative for the development of inflammatory reactions, whereas other reports concluded that colonization of skin lesions with *S. aureus* is not an essential prerequisite in the pathogenesis of AD and rather occurs as secondary infection [23, 24]. However, the disease severity of AD is heavily exacerbated by *S. aureus* infection, mainly amplified by the secretion of staphylococcal enterotoxins [25]. These bacterial superantigens vigorously stimulate T cells and might function as allergen. In a subgroup of AD patients specific IgE directed against staphylococcal enterotoxin A (SEA) and SEB were detectable [26] which might contribute to enhanced histamine and leukotriene release in individuals with atopic eczema [27]. The presence of these superantigen-specific IgE antibodies strongly correlated with disease severity [28]. In addition, other components of *S. aureus*, e.g. cell wall constituents like peptidoglycan or lipoteichoic acid, which bind to CD14 [29] and TLR-2 [30], may be effective in increasing the severity of AD. Both lipoteichoic acid and peptidoglycan from *S. aureus* induced IL-5 production in peripheral blood mononuclear cells from patients with AD but not from healthy donors [31]. Furthermore, lipoteichoic acid induced local Th2-prone dermatitis and enhanced allergen-specific IgE production in mice [32]. On the other hand, lipoteichoic acid potentially induced IL-12 production by a human monocytic cell line [33] and immunization of mice with the unique cell wall structure of *S. aureus*, protein A, led to a Th1 response [34]. We herein show that treatment of BALB/c mice with formalin-fixed *S. aureus* particles at the time of sensitization with ovalbumin (OVA) induced a shift from a pronounced Th2 immune response, as observed in un-

treated control animals, towards a more Th1-orientated response, indicated by decreased IL-4 and IL-5 production, but enhanced IFN- γ secretion after stimulation of spleen cells with OVA. Consistently, formation of OVA-specific IgE and IgG1 antibodies (Ab) was efficiently inhibited. The Th1-promoting capacity of *S. aureus* particles was not dependent on the expression of protein A by the inactivated bacteria. Application of *S. aureus* particles during the sensitization phase prevented airway inflammation (airway hyperreactivity, eosinophilia) induced by local challenge with OVA.

Material and Methods

Mice and Immunization Protocol

BALB/c mice were bred and maintained in the Central Animal Facilities of the University of Mainz under specific pathogen-free conditions on a standard diet. The 'Principles of laboratory animal care' (NIH publication No. 85-23, revised 1985) were followed. The experiments were approved by the Ethics Commission according to the German Law on the Protection of Animals. Female BALB/c mice were 6–8 weeks of age at the beginning of the experiments.

Allergen sensitization of mice was induced by repeated intraperitoneal injection of 10 μ g ovalbumin (OVA Grade VI; Sigma-Aldrich, Deisenhofen, Germany) adsorbed to aluminum hydroxide (alum; Pierce, Rockford, Ill., USA) at intervals of 2 weeks [35]. In order to determine the effect of application of *S. aureus* on the OVA-specific immune response, mice were injected with a total volume of 200 μ l containing OVA/alum and 10^9 formalin-fixed *S. aureus* particles, Cowan I strain (SACS; Pansorbin; Calbiochem-Merck Biosciences, Bad Soden, Germany) or protein A-free Wood 46 strain (SAWS; Sigma-Aldrich) as indicated. The endotoxin content of particles was <0.2 EU/ml as determined by Limulus amebocyte lysate assay.

In the model of allergic airway inflammation mice were sensitized by two injections of OVA/alum $\pm$ SACS (days 0 and 10) as described above prior to intranasal provocation with OVA (250 μ g) in a volume of 50 μ l PBS on days 17–19. Control mice received PBS alone intranasally.

Determination of OVA-Specific Ab Levels in the Serum

Mice were bled on the indicated days via the tail vein. Sera were collected and frozen at -20°C until thawed for analysis. Determination of OVA-specific Ab by ELISA was performed as reported [35] with the modification that *o*-phenylenediamine was used as substrate [36]. The optical density (OD) was measured in a microplate reader E_{max} (MWG-Biotech, Ebersberg, Germany) at 490 nm. The antibody (Ab) titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis. Contents of IgG1, IgG2a and IgE in serum samples were standardized using reference sera.

Spleen Cell Cultures

Mice were killed by cervical dislocation, spleens were removed and single cell suspensions were prepared by teasing the tissue

apart. Erythrocytes were lysed by incubating the cells for 1 min in lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 100 μM EDTA-disodium, pH 7.4). After extensive washing splenocytes were passed through a 40- μm cell strainer (BD Biosciences, Bedford, Mass., USA) to remove cell debris. Spleen cells (5×10^6 /well) were cultured for 72 h on 24-well tissue culture plates in a volume of 1 ml culture medium (IMDM supplemented with 10% fetal calf serum [PAN Systems GmbH, Nürnberg, Germany], 50 μM 2-mercaptoethanol [Carl Roth GmbH & Co, Karlsruhe, Germany], 2 mM L-glutamine [Carl Roth GmbH & Co], 100 U/ml penicillin [Gibco, Paisley, UK] and 100 $\mu\text{g}/\text{ml}$ streptomycin [Gibco]) with or without OVA at the indicated concentrations.

Enumeration of IL-4- and Ig-Secreting Cells by ELISPOT Assay

The frequency of IL-4-producing cells in spleen cell cultures was determined by ELISPOT assay as described [36]. The number of OVA-specific Ig-producing B cells among spleen cells was determined ex vivo by ELISPOT assay as described [36] with the modification that OVA (20 $\mu\text{g}/\text{ml}$) was used as antigen to coat ELISPOT microtiter plates.

Determination of Cytokines by ELISA

Cytokines were quantified in culture supernatants using a sandwich ELISA performed according to a standard procedure as previously described [37]. For measurement of TGF- β 1 culture supernatants were activated for 30 min at 37°C by addition of 1 M HCl (pH 3.0) and subsequently neutralized with 1 M NaOH (pH 7.0). Capture Ab (anti-IFN- γ clone R4-6A2, anti-IL-5 clone TRFK5 [both from BD Pharmingen, San Diego, Calif., USA], anti-IL-10 clone JES052A5, rhTGF- β sRII/Fc chimera [341-BR], anti-IL-12p70 clone 9A5 [all from R&D Systems, Wiesbaden, Germany]) and biotinylated detection Ab (anti-IFN- γ clone XMGI.2, anti-IL-5 clone TRFK4, anti-IL-12p70/p40 clone C17.8 [all from BD Pharmingen], anti-IL-10 Ab [BAF417], anti-hTGF- β 1 Ab [BAF240; both from R&D Systems]) were used as recommended by the manufacturer. Recombinant cytokines (mIFN- γ , mIL-5 and mIL-12p70 from BD Pharmingen; mIL-10 from R&D Systems; hTGF- β 1 from PeproTech, Rocky Hill, N.J., USA) were used as standards.

Assessment of Airway Function

Pulmonary function was assessed by methacholine-induced airflow obstruction from non-anesthetized mice 24 h after the last provocation using a single-chamber, non-invasive whole-body plethysmograph (model PLY 3211; Buxco Electronics, Birmingham, UK). Airway reactivity was assessed by analyzing the enhanced pause (Penh) responses of mice at baseline after saline inhalation and following exposure to increasing doses of aerosolized methacholine for 3 min. Data are reported as the mean value of 30 values of Penh recorded every 10 s for 5 min after challenge with increasing concentrations of aerosolized methacholine (Sigma-Aldrich) as indicated. Results are expressed as the fold increase in Penh measured for each concentration of methacholine in relation to baseline Penh.

Bronchoalveolar Lavage and Characterization of Cells

Mice were killed with a lethal dose of ether 36–48 h after the last provocation, the trachea was cannulated and bronchoalveolar lavage (BAL) was performed by gentle flushing with 1 ml PBS

Table 1. Primers used for real-time PCR

Gene and orientation	Sequence
Ubiquitin C	
Forward	5'-GTCTGCTGTGTGAGGACTGC-3'
Reverse	5'-CAGGGTGGACTCTTTCTGGA-3'
IL-12 p35	
Forward	5'-ATGACCCTGTGCCTTGGTAG-3'
Reverse	5'-TCTCCACAGGAGGTTTCTG-3'
IL-12 p40	
Forward	5'-CATCTGCTGCTCCACAAGAA-3'
Reverse	5'-CGCCATTCCACATGTCACTGC-3'
IL-23 p19	
Forward	5'-CAGGGAACAAGATGCTGGAT-3'
Reverse	5'-TGGATACGGGGCACATTATT-3'
IL-27 p28	
Forward	5'-GTTGCTGCTACCCTTGCTTC-3'
Reverse	5'-TGGTAGCGAGGAAGCAGAGT-3'
EBI-3	
Forward	5'-CGGTGCCCTACATGCTAAAT-3'
Reverse	5'-GCGGAGTCGGTACTTGAGAG-3'
IL-10	
Forward	5'-CCAAGCCTTATCGGAAATGA-3'
Reverse	5'-TTTTCACAGGGGAGAAATCG-3'
IL-24	
Forward	5'-GGCCCTTTCTTCAACACAGA-3'
Reverse	5'-AGAAGGCCTCCACAGTTCT-3'
TGF- β 1	
Forward	5'-TTGCTTCAGCTCCACAGAGA-3'
Reverse	5'-TGGTTGTAGAGGGCAAGGAC-3'
Foxp3	
Forward	5'-CTTATCCGATGGGCCATCCTGGAAG-3'
Reverse	5'-TTCCAGGTGGCGGGTGGTTTCTG-3'

supplemented with 10% fetal calf serum. BAL fluid (BALF) was recovered and centrifuged. Cell pellet was resuspended and total cells were counted by means of trypan blue exclusion. Approximately 1×10^5 cells were cytospun onto microscope slides and stained with Diff-Quick (Dade Behring, Marburg, Germany). At least 300 cells were differentiated per slide based on conventional criteria like morphology and staining behavior using a microscope with digital camera unit (BX50WI; Olympus, Hamburg, Germany). To determine the absolute cell number (in cells/ml BALF) of the respective cell population, the percentage of eosinophils, neutrophils, lymphocytes and monocytes counted was multiplied by the total cell number for each sample.

Preparation of Bone Marrow-Derived DCs

Femoral bone marrow from BALB/c mice was isolated. For generation of bone marrow-derived DCs (BM-DCs) red blood cells were removed using lysis buffer and bone marrow cells were cultured on 100-mm Cellstar tissue-culture dishes (Greiner, Frickenhausen, Germany) in culture medium supplemented with rmGM-CSF (20 ng/ml; kindly provided by Dr. F. Seiler, Behringwerke AG, Marburg, Germany) on day 0 as previously de-

scribed with modifications [38, 39]. On days 3, 5, 7 supernatant was removed and cultures were replenished with fresh medium containing rmGM-CSF. On day 10 non-adherent cells were harvested and MHC class II-positive cells were isolated by applying a rat anti-mouse-I-A^d mAb (clone 2G9) [40] and Dynabeads M-450 Sheep anti-Rat IgG (Dyna, Hamburg, Germany) as recommended by the manufacturer. Cells (1×10^6 /well) were seeded on 24-well plates and stimulated for 2 days with 1 μ g/ml lipopolysaccharide (LPS; Calbiochem-Merck Biosciences), 1% w/v SACS or 1% SAWS particles, in the presence of 10 ng/ml rmGM-CSF. Subsequently, culture supernatants of three wells were pooled and analyzed for the content of IL-10 and IL-12. BM-DCs were collected and prepared for mRNA isolation.

Real-Time PCR Analysis

To assess changes in gene expression in spleen cells of SACS-treated mice and differentially stimulated BM-DCs, respectively, total RNA was isolated from 3×10^6 cells per group and real-time (RT)-PCR was performed as described [41]. Primer sequences (table 1) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and primer pairs bridging intron-exon boundaries were chosen. Primers were purchased from MWG Biotech (Ebersberg, Germany). The housekeeping gene ubiquitin C served as control and was used for subsequent normalization.

Statistical Analysis of Data

Statistical evaluation of the experimental data was performed by Student's t test using 'SigmaStat' software. A value of $p < 0.05$ was considered statistically significant.

Results

Co-Application of OVA and *S. aureus* Particles Results in Reduced IgE and IgG1 and Enhanced IgG2a Ab Levels

In order to evaluate the impact of encounter with extracellular bacteria on the intensity of allergic sensitization, we determined the effect of simultaneous intraperitoneal administration of alum-adsorbed OVA protein (OVA/alum) used as allergen and formalin-fixed *S. aureus* particles on OVA-specific Ab production and Th cell recruitment (fig. 1a). The application of SACS particles, which contain protein A as a cell wall constituent, had profound consequences on the humoral immune response: the levels of IgE and IgG1 Ab in the sera of SACS-treated mice were significantly reduced compared with control mice (fig. 1b). On the other hand, IgG2a Ab titers were considerably increased in mice injected with OVA/alum plus SACS (fig. 1b). Significant changes in Ab production were apparent already after the second (for IgE and IgG1) and third (for IgG2a) application of OVA/alum plus SACS and were maintained throughout the sensitization period. The results were verified on the cel-

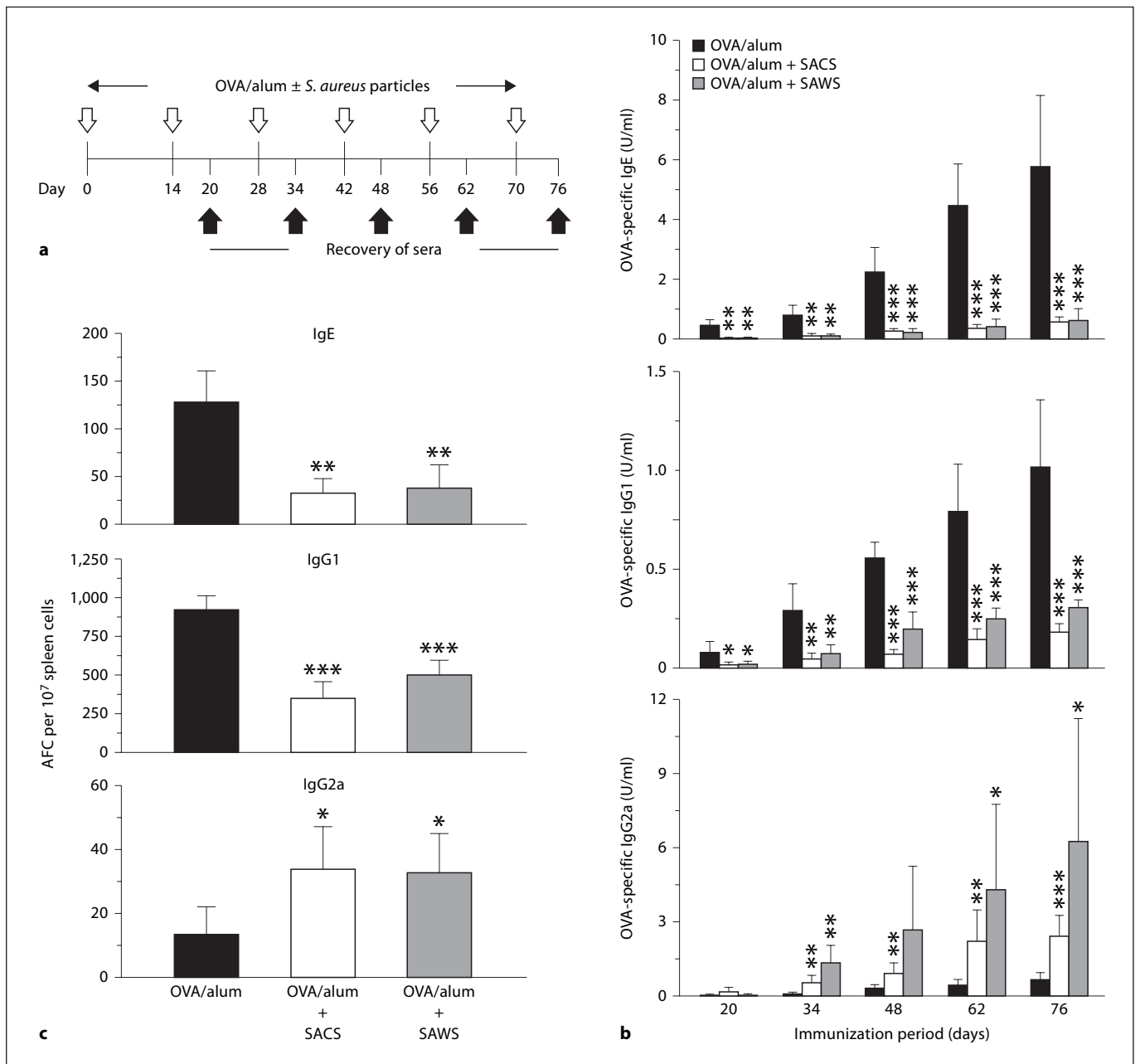


Fig. 1. Administration of *S. aureus* particles during sensitization with allergen inhibits IgE and IgG1 Ab production, but increases IgG2a Ab secretion. **a** BALB/c mice ($n = 5-6$) were immunized with $10 \mu\text{g}$ OVA in alum $\pm 10^9$ SACS or SAWS particles as outlined. **b** On the indicated days sera were recovered and serum levels of OVA-specific IgE, IgG1 and IgG2a Ab were determined by ELISA. Data are presented as mean titers $\pm$ SD of individual sera. **c** On day 77 antibody secreting cells (ASC) among pooled spleen cells were quantified ex vivo using an OVA-specific ELISPOT assay. Data are presented as mean $\pm$ SD of quadruplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus mice immunized with OVA/alum. Data are representative of two (SAWS) to four (SACS) independent experiments.

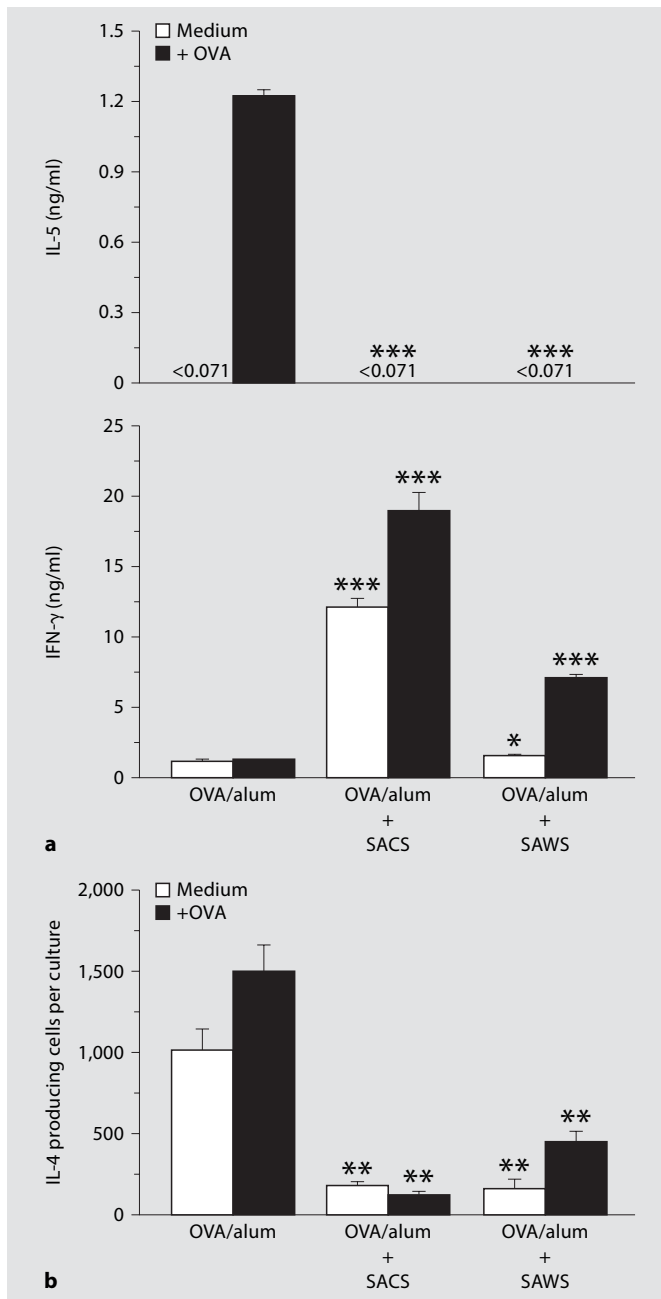


Fig. 2. Administration of *S. aureus* particles during sensitization with allergen inhibits IL-4 and IL-5 production but enhances IFN- γ production. BALB/c mice ($n = 5-6$) were immunized with $10 \mu\text{g}$ OVA in alum $\pm 10^9$ SACS or SAWS particles as shown in figure 1a. On day 77 pooled spleen cells were cultured for 72 h with (■) or without (□) $50 \mu\text{g/ml}$ OVA. **a** IL-5 and IFN- γ were quantified in pooled culture supernatants by sandwich ELISA. Data are presented as the mean $\pm$ SD of duplicates. **b** IL-4-producing cells among splenocytes were enumerated by ELISPOT assay. Data are presented as mean $\pm$ SD of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus mice immunized with OVA/alum. Data are representative of two (SAWS) to four (SACS) independent experiments.

lular level by enumeration of antibody-secreting cells (ASCs) in an ELISPOT assay. Accordingly, the frequency of IgE and IgG1 ASCs among spleen cells ex vivo was significantly decreased in SACS-treated mice, whereas the number of IgG2a ASCs was substantially enhanced (fig. 1c). These effects were partially dependent on the bacterial load, because administration of 10^9 SACS particles caused a stronger inhibition of IgG1 Ab production and induced more IgG2a Ab than a dose of 10^8 SACS particles (data not shown). SACS particles had to be co-applied with OVA at the same time and by the same route to exert regulatory activity for Ab production (data not shown).

Co-Application of OVA and S. aureus Particles Induces a Shift towards a Th1-Biased Immune Response

It is generally accepted that the Ig isotype profile serves as an indicator for the type of T-cell help supporting a murine immune response. Therefore we investigated whether the changes in the Ig pattern, induced by treatment with SACS particles, were due to the recruitment of Th cells with differential polarization in cytokine production. To this end, spleen cells were cultured in vitro with OVA protein, resulting in MHC class II-restricted antigen presentation and, subsequently, stimulation of and cytokine secretion mainly by antigen-specific CD4 $^+$ T cells. Analysis of cytokine release revealed that SACS treatment drastically inhibited the ability of spleen cells to secrete IL-5, but in contrast significantly augmented the production of IFN- γ (fig. 2a). Because levels of IL-4 in the supernatants of spleen cell cultures were below the detection limit of the ELISA system, we used an ELISPOT assay to determine the frequency of IL-4-producing cells among splenocytes. As shown in figure 2b, spleen cells isolated from SACS-treated mice did not produce IL-4 in an OVA-dependent manner, whereas in restimulated cell cultures from untreated mice a substantial population of IL-4-producing cells expanded. In accordance with the ELISA data the activation of IL-5-producing cells in an ELISPOT assay was weak among spleen cells derived from SACS-treated mice (data not shown). Thus, our data indicate a shift in the OVA-specific immune response from a distinct Th2 response (high IL-4 and IL-5, low IFN- γ) towards a Th1-biased response (low IL-4 and IL-5, high IFN- γ) triggered by simultaneous immunization with SACS.

Inhibition of Th2 Sensitization Is Not Dependent on the Expression of Protein A by S. aureus Particles

It has been shown previously that protein A, a cell wall component of most *S. aureus* strains, exhibits immunomodulatory properties, including the induction of Th1 responses after intraperitoneal administration [35]. Therefore, it was tempting to speculate that the expression of protein A might contribute to the Th1-biasing capacity of *S. aureus* particles. We checked this assumption by comparing the ability of protein A-containing SACS particles to suppress the development of an OVA-specific Th2 response with the efficiency of SAWS particles, which are devoid of protein A expression. Figure 1b and c show that co-immunization with OVA/alum and SAWS led to considerable inhibition of OVA-specific IgE and IgG1 Ab responses, being in a comparable range as the inhibition achieved by simultaneous administration of OVA/alum and SACS. Likewise, IgG2a Ab production was significantly enhanced by both SAWS and SACS particles. In addition, the cytokine profile of spleen cells, stimulated in vitro with OVA, indicated a Th1-polarized response after treatment with SAWS and SACS, respectively: secretion of IFN- γ was increased, whereas production of IL-5 was completely abrogated (fig. 2a). Moreover, the frequency of IL-4-producing cells among restimulated splenocytes was significantly reduced (fig. 2b). Thus, these data argue against a major role for protein A as a Th1-inducing determinant in this system.

Modulation of DC Phenotype by S. aureus Particles

To further analyze the Th1-inducing activity of SACS and SAWS particles we determined their ability to modulate the activation of DC. For this reason immature DCs were differentiated from bone marrow precursor cells and subsequently cultured for 48 h with SACS, SAWS, and LPS, respectively. After this period we quantified the expression of different cytokine mRNA by these cells in relation to untreated cells, as well as the release of IL-12 and IL-10 into the culture supernatant. Stimulation with LPS induced strong upregulation of transcript expression for IL-12p35 and a slight increase in mRNA levels for IL-12p40, IL-23p19, IL-10 and IL-24 in comparison with untreated BM-DCs (fig. 3a). Stimulation with SACS or SAWS in addition to IL-12p35 substantially increased mRNA levels for IL-12p40, IL-10 and IL-23p19, and induced moderate enhancement of EBI-3 and IL-24 transcript expression. Bioactive IL-12 is a heterodimeric molecule (p70) composed of covalently linked p35 and p40 chains. The increased expression of the transcripts for both chains by SACS- and SAWS-treated BM-DCs points

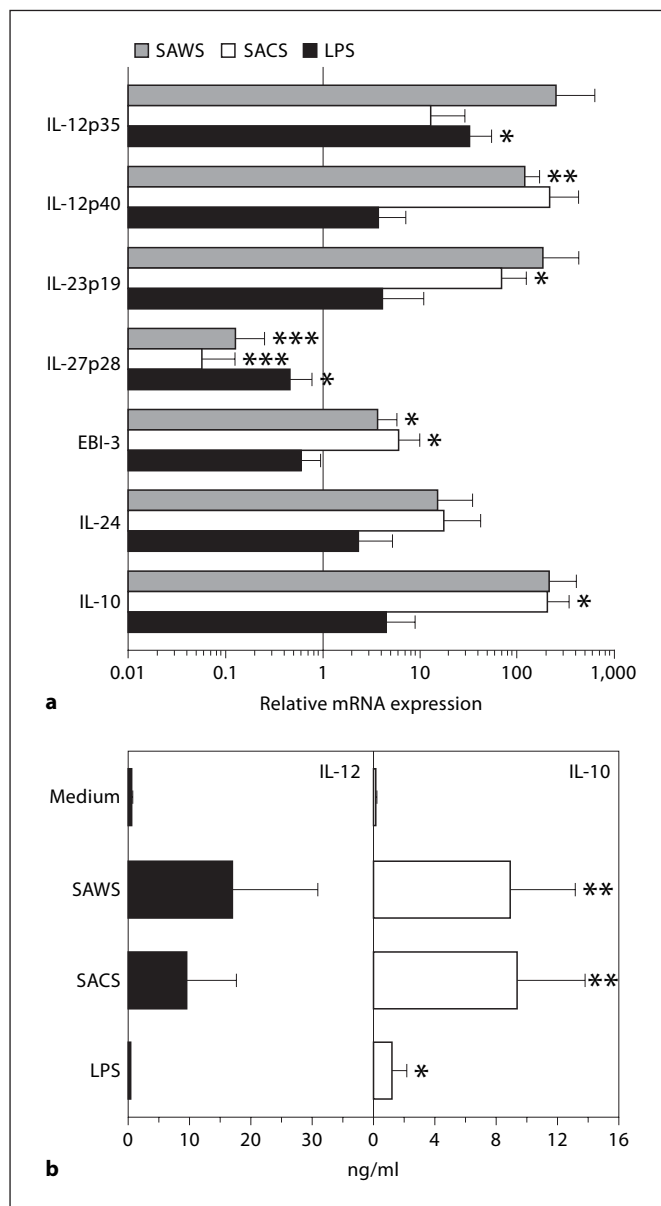


Fig. 3. *S. aureus* particles induce IL-12 and IL-10 production in BM-DC. Immature DC were generated from bone marrow cells by cultivation with rmGM-CSF for 10 days as described. MHC class II-positive cells were purified and stimulated for 48 h with SAWS, SACS and LPS, respectively, or were left untreated. **a** mRNA expression levels of the indicated cytokines from stimulated BM-DC were quantified by real-time RT-PCR and presented relative to mRNA expression levels from unstimulated controls. Data represent mean $\pm$ SD of four independent experiments performed in duplicate. **b** Supernatants of BM-DC cultures were collected and the content of IL-12p70 and IL-10 was measured in duplicates by sandwich ELISA. Data represent mean $\pm$ SD of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus unstimulated BM-DC.

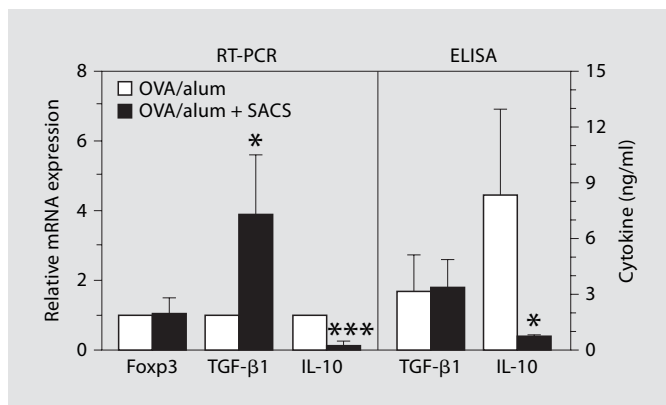


Fig. 4. No induction of regulatory T cells by administration of SACS particles. BALB/c mice ($n = 4-6$) were immunized with 10 μ g OVA in alum $\pm 10^9$ SACS particles. Spleen cells were isolated and cultured for 72 h with 200 μ g/ml OVA. Expression levels of Foxp3, TGF- β 1 and IL-10 transcripts from splenocytes of SACS-treated mice (■) were quantified by real-time RT-PCR and presented relative to mRNA expression levels from splenocytes of untreated controls (□). Contents of TGF- β 1 and IL-10 in supernatants of spleen cell cultures ($n = 3-4$) were measured by sandwich ELISA. Data represent mean $\pm$ SD of three independent experiments. * $p < 0.05$, *** $p < 0.001$ versus spleen cells of control mice.

to an enhanced production of IL-12p70, which we verified by measurement of IL-12 protein levels in culture supernatants (fig. 3b). Likewise, the upregulation of IL-10 mRNA in these cells was reflected by the detection of large amounts of IL-10 protein in the supernatants (fig. 3b).

Treg Are Not Induced by Administration of S. aureus Particles

To determine whether the suppression of IgE Ab production after co-administration of OVA/alum and SACS particles was mainly due to the shift from Th2 towards Th1-biased immune responses or whether Treg played a major role in the modulation of allergic sensitization, synthesis of the immunoregulatory cytokines IL-10 and TGF- β 1, characteristic for the activity of certain subpopulations of adaptive Treg [42], was determined on the mRNA level (by RT-PCR) as well as protein level (by ELISA) after antigenic stimulation of spleen cells in vitro. Expression of IL-10 mRNA as well as release of IL-10 into culture supernatants was largely reduced by SACS treatment of mice (fig. 4). In contrast, transcript expression for TGF- β 1 was significantly augmented in cells originating from SACS-treated animals in comparison

with cells from mice immunized with OVA/alum alone, whereas secretion of soluble TGF- β 1 did not differ (fig. 4). Furthermore, we quantified relative mRNA levels of transcription factor Foxp3, which is crucial for the development and differentiation of the majority of Treg populations [43]. Application of SACS particles together with OVA/alum to mice did not result in an increase in Foxp3 mRNA expression by freshly isolated spleen cells (data not shown) or by spleen cells restimulated in vitro with OVA (fig. 4). Likewise, surface expression of molecules, associated with Treg phenotype or function (CD25, GITR, CTLA-4) did not significantly differ between the two experimental groups as verified by FACS analysis (data not shown). In conclusion, we could not reveal an enhanced systemic activity of conventional Treg populations resulting from administration of SACS particles.

Effect of Administration of S. aureus Particles on an Established OVA-Specific Th2 Immune Response

Mice were initially sensitized by two immunizations with OVA/alum, which gave rise to a substantial Th2 immune response with established IgE Ab production (data not shown). The mice were divided into 2 groups with equivalent mean Ab titers and subsequently immunized in intervals of 2 weeks with either OVA/alum alone (control) or together with 10^9 SACS particles (fig. 5). Already after the second administration of OVA/alum plus SACS particles, IgE Ab titers were significantly reduced compared with mice solely immunized with OVA/alum (fig. 5). Whereas IgE Ab levels rose steadily by continuous immunization with OVA/alum, the increase in IgE Ab production stagnated in SACS-treated mice. Likewise, production of IgG1 Ab was significantly inhibited after the fifth application of OVA/alum together with SACS particles. On the other hand, IgG2a Ab levels in these mice tended to be higher than in control animals, albeit this difference did not reach statistical significance. Analysis of cytokine patterns of splenocytes stimulated with OVA in vitro revealed no significant differences in IL-4, IL-5 or IFN- γ production between spleen cells from SACS-treated mice and control mice (data not shown).

Administration of S. aureus Particles Prevents Allergic Airway Inflammation

To evaluate an effect of systemic SACS application on the elicitation of local allergic reactions we employed the mouse model of experimental allergic lung inflammation, in which mice were initially sensitized by two im-

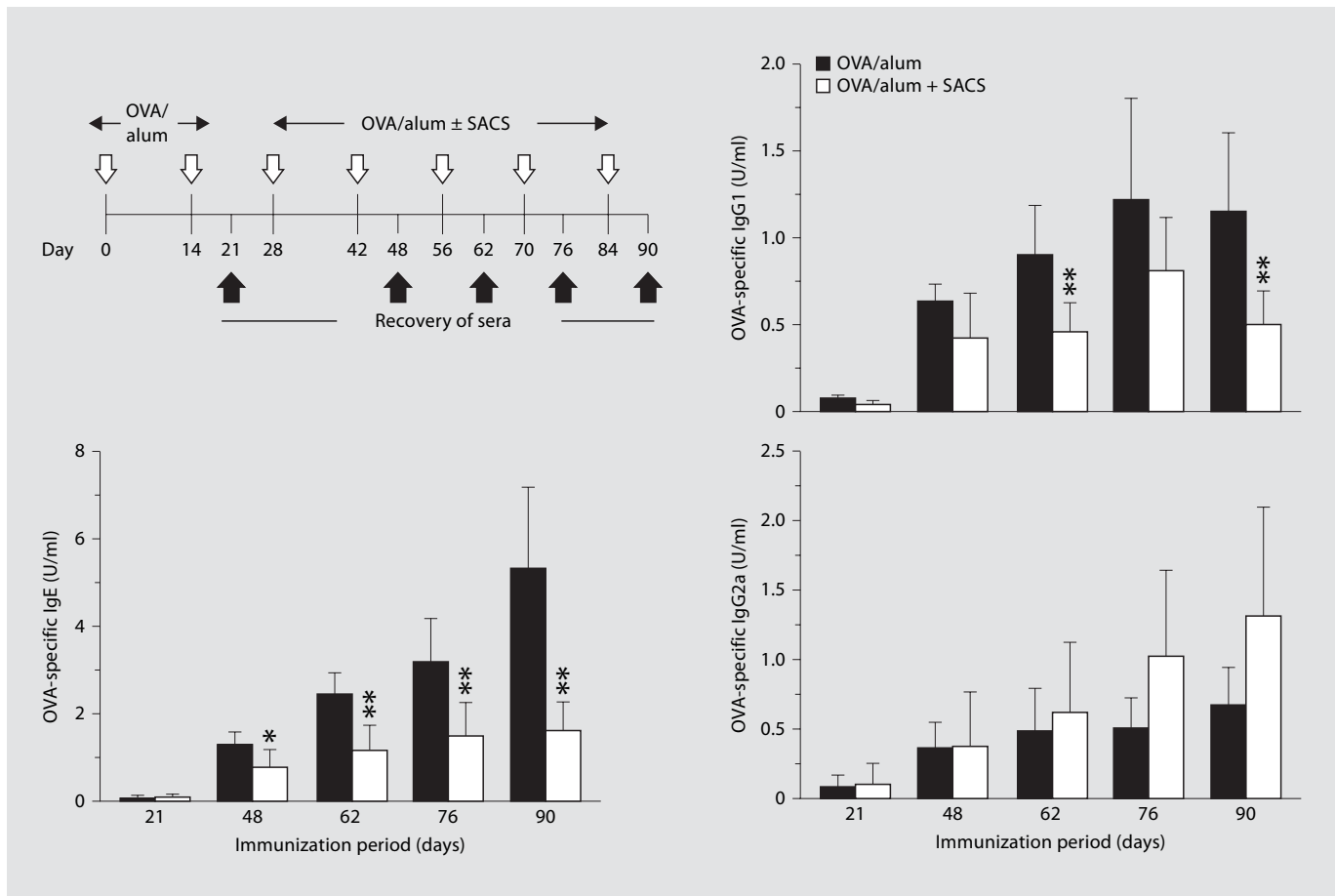


Fig. 5. Administration of SACS particles to allergen-sensitized mice inhibits IgE and IgG1 Ab production. BALB/c mice were sensitized by two intraperitoneal injections of 10 μ g OVA in alum. Subsequently, groups of mice ($n = 6$) were further immunized as shown with 10 μ g OVA in alum $\pm$ 10^9 SACS particles. On the indicated days sera were recovered and serum levels of OVA-specific IgE, IgG1 and IgG2a Ab were determined by ELISA. Data are presented as mean titers $\pm$ SD of individual sera. * $p < 0.05$, ** $p < 0.01$ versus mice immunized with OVA/alum. Data are representative of three independent experiments.

munizations with OVA/alum in the absence or the presence of 10^9 SACS particles. Subsequently, animals were challenged intranasally with OVA in PBS on 3 consecutive days (fig. 6a). Provocation with OVA induced substantial bronchial inflammation in control mice as indicated by the development of airway hyperreactivity (fig. 6b) and by significant infiltration of eosinophils into the airways (fig. 6c). In contrast, treatment with SACS in the sensitization phase considerably suppressed symptoms of lung inflammation after challenge by significantly reducing airway hyperreactivity as well as preventing airway eosinophilia (fig. 6b, c).

Discussion

According to the hygiene hypothesis bacterial infection or the confrontation with bacterial products in early childhood facilitates the development of a balanced T-cell immunity and thus suppresses the induction of allergen-specific Th2 responses promoting the elicitation of type I allergic reactions [2]. Several animal studies have supported this notion by demonstrating that the encounter with a variety of intracellular bacterial species inhibited allergen sensitization and reduced inflammatory symptoms in mouse models of allergic diseases. In this report we provide novel information about the ability of extracellular bacteria to interfere with the development of

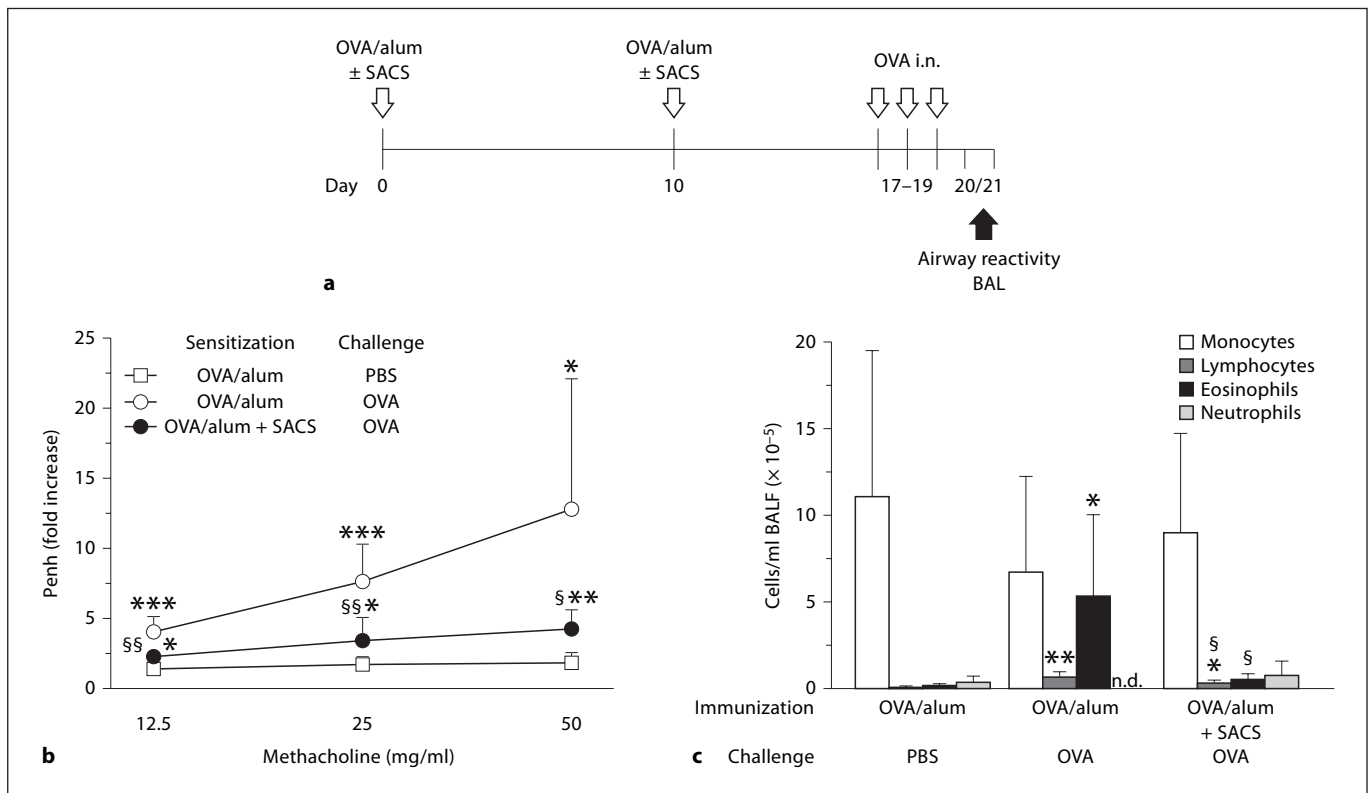


Fig. 6. Administration of SACS particles prevents allergic airway inflammation. **a** BALB/c mice were immunized by two intraperitoneal injections of 10 µg OVA in alum ± 10⁹ SACS particles. Subsequently, mice were challenged by intranasal administration of 250 µg OVA in PBS or treated with PBS alone as indicated. **b** Airway reactivity was measured in response to methacholine as changes in Penh by noninvasive plethysmography 24 h after the last challenge. **c** Absolute numbers of leukocytes in the BALF were determined 36–48 h after the last challenge. Data represent means ± SD of a total of 6–8 mice analyzed in two independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 versus PBS-challenged mice; § p < 0.05, §§ p < 0.01 versus OVA-sensitized/challenged mice.

OVA-specific Th2 immune responses and to inhibit allergic sensitization.

In a well-established mouse model of type I allergy, we show that the administration of formalin-fixed *S. aureus* particles during the sensitization phase with OVA resulted in immune deviation from a Th2-polarized response towards a Th1-orientated response. This shift is evident from a considerably reduced capability of spleen cells to produce Th2 cytokines like IL-4 and IL-5 and a corresponding significant suppression of OVA-specific IgE and IgG1 Ab production. On the other hand, IFN-γ production by spleen cells was markedly increased, probably being responsible for the enhancement of IgG2a Ab formation observed in mice treated with *S. aureus* particles. The inhibitory effect on IgE and IgG1 Ab production was also evident after co-administration of OVA/alum and

SACS particles to mice which had been already sensitized by previous immunizations with OVA/alum alone, thus displaying increased IgE Ab titers. However, the changes obtained by subsequent application of SACS particles were less prominent (fig. 5 compared with fig. 1b) and were not evident on the cytokine level. This observation is in line with previous findings made by us [36, 44] and others [45, 46], revealing that intervention in an established allergen-specific Th2 response with ongoing IgE Ab production is more critical than preventing the development of Th2 cells from naïve T cells by protective manipulation. Most notably, SACS co-application during sensitization very efficiently inhibited the elicitation of inflammatory reactions in the lung following local challenge with OVA, as was verified by reduced eosinophilic infiltration and improved airway reactivity.

The Th1-inducing capacity of *S. aureus* particles did not depend on the expression of the cell wall component protein A. In fact, Sinha et al. [34] demonstrated increased serum levels of IFN- γ , TNF- α and IL-1, but reduced levels of IL-4, IL-6 and IL-10 following intraperitoneal injection of synthetic protein A suggesting the induction of Th1 responses. However, in our study a putative Th1-polarizing effect of protein A was superposed by other structural components of *S. aureus*, because SAWS particles, essentially devoid of protein A, exhibited similar potency to modulate the development of Th2 responses as protein A-containing SACS particles. Presumably these components interact with recognition patterns on APCs, namely macrophages and particularly DCs, to exert their immunomodulatory activity. We show that co-culture of BM-DCs and *S. aureus* particles led to enhanced production of IL-12p70 and IL-10 and to strong upregulation of the respective cytokine mRNA levels. The activation of BM-DCs by SACS and SAWS, respectively, after 48 h was many times higher than following stimulation of BM-DCs with LPS, which as a major component of the outer membrane of gram-negative bacteria has previously been shown to induce the maturation of BM-DCs [39]. However, the capacity of BM-DCs to produce IL-12 was reported to be dependent on the duration of LPS stimulation: expression of mRNA for IL-12p35 and p40 as well as accumulation of IL-12 protein in BM-DC cultures peaked at 4–12 h and declined rapidly thereafter [47, 48]. This might be the reason for the relatively low levels of IL-12p35 and p40 transcripts and IL-12 protein determined in our BM-DC cultures stimulated for 48 h with LPS as was also reported by Harizi et al. [49]. In contrast, activation of BM-DCs by *S. aureus* particles resulted in prolonged and high IL-12 secretion, which might crucially contribute to the efficient polarization of the CD4 $^{+}$ T-cell response into a Th1-orientated response in vivo [50]. A similar pattern of differential secretion of IL-12p40 after stimulation with SACS versus LPS has also been shown by Mitsui et al. [51] using CD11c $^{+}$ splenic DCs. Constituents of the cell wall of *S. aureus* other than protein A, that might be responsible for the activation and the Th1-inducing potential of BM-DCs, might include peptidoglycan or lipoteichoic acid, which are ligands of CD14 [29] and the pattern recognition receptor TLR-2 [30]. Peptidoglycan from *S. aureus* has been shown to trigger IL-12 production in the mouse macrophage cell line J774 [52] and in human CD11c $^{+}$ immature DCs [53]. Likewise, lipoteichoic acid induced IL-12 production by the human monocytic cell line THP-1 [33]. However, it cannot be ruled out that other structural components of

gram-positive bacteria than the above mentioned mediate the Th1-promoting activity.

Another gene, which is highly expressed in BM-DCs following in vitro stimulation with SACS or SAWS, is the gene for the p19 chain. This molecule, together with the IL-12p40 subunit, which is also strongly expressed in SACS- and SAWS-treated BM-DCs, forms the cytokine IL-23 [54]. It has been previously shown that IL-23 stimulates a unique subset of T cells to produce IL-17 (Th17 cells), and that T cells of this lineage, in turn, induce the production of proinflammatory cytokines, which contribute to autoimmunity or protective responses during infection [54]. Therefore, it has to be further investigated, whether SACS treatment in vivo induces the generation of Th17 cells and whether these cells participate in the control of allergic sensitization.

The production of IL-27, another heterodimeric cytokine with Th1-inducing capacity [54], which consists of the two subunits EBI-3 and IL-27p28, appears to be regulated differently from IL-12 and IL-23 in BM-DCs following stimulation with SACS or SAWS. Whereas EBI-3 transcripts were moderately upregulated upon activation of BM-DCs with *S. aureus* particles, IL-27p28 mRNA expression was rather downregulated, implicating that secretion of biologically active IL-27 probably plays a minor role in Th1-inducing activity. In contrast, expression of mRNA for IL-24, a Th1-promoting member of the IL-10 subfamily [55], was significantly enhanced in BM-DCs cultured with *S. aureus* particles, thus possibly contributing to the induction of Th1 responses.

BM-DCs stimulated with SACS or SAWS in addition to IL-12 produced large amounts of IL-10, a cytokine that is essential for the generation and function of a certain Treg subset (Tr1 cells) [56]. IL-10-producing DCs have been shown to induce Tr1 cells [57–59]. Recently, Stock et al. [60] characterized a murine CD8 α^{+} DC population that arose following intraperitoneal injection of OVA together with heat-killed *L. monocytogenes*. These DCs induced Treg which were able to inhibit OVA-induced bronchial hyperreactivity and to suppress allergic airway inflammation. The production of both IL-10 and IL-12 by the DCs was essential to exert their protective effects. However, as judged by secretion of immunomodulatory cytokines IL-10 and TGF- β 1 or expression of transcription factor Foxp3 by antigen-stimulated spleen cells we did not observe enhanced activation or expansion of Tr1 cells or other Treg subtypes (CD25 $^{+}$, GITR $^{+}$, CTLA-4 $^{+}$), which might contribute to the inhibitory effects on OVA-specific Th2 responses. Solely mRNA expression for TGF- β 1 was significantly upregulated in splenocytes

from SACS-treated mice, but this did not correlate with the amount of secreted protein, suggesting post-transcriptional and/or post-translational modulation of TGF- β 1 expression. However, we cannot entirely exclude that Treg subpopulations other than the conventional ones were induced or activated by SACS treatment of mice. Thus, a regulatory function has been ascribed in various experimental systems to CD4 and CD8 double-negative T cells [61], to myeloid suppressor cells [62] and to regulatory natural killer cells [63]. Also it has to be investigated whether local recruitment of Treg in the lung might contribute to the repression of lung inflammation and airway hyperreactivity.

For this study we used well-established models of type I allergy and of allergic airway inflammation which rely on sensitizing mice by intraperitoneal injection of OVA/alum in order to induce strong and persistent systemic Th2 responses. To attain efficient inhibition of Th2 responses we had to co-apply *S. aureus* particles by the intraperitoneal route because separation of time point or route of immunization with OVA/alum and SACS, respectively, abrogated the inhibitory effect, implicating that the particles directly modulate those DCs incorpo-

rating OVA and presenting antigenic peptides to naïve T cells in the draining lymph nodes. Different from our model system, sensitization with allergens in humans usually occurs by various other routes, e.g. by inhalation via the airways or by oral uptake. Nevertheless, our data suggest that early and continuous encounter with extracellular bacteria, similar to intracellular bacteria, by the same route as encountered with allergens might reduce the prevalence of individuals to develop allergies later in life. Identification of the structural components of *S. aureus* responsible for the modulatory effect might facilitate the development of new strategies for allergy treatment.

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