

ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses

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Summary

Background Recently, it has been established that pollen grains contain Th2-enhancing activities besides allergens.

Objective The aim of this study was to analyse whether pollen carry additional adjuvant factors like microbes and what immunological effects they may exert.

Methods Timothy pollen grains were collected and disseminated on agar plates, and the growing microorganisms were cultivated and defined. Furthermore, the immunologic effects of microbial products on DC and T cell responses were analysed.

Results A complex mixture of bacteria and moulds was detected on grass pollen. Besides Gram-negative bacteria that are known to favour Th1-directed immune responses, moulds were identified as being sources of allergens themselves. Herein, we focused on Gram-positive bacteria that were found in high numbers, e.g. *Bacillus cereus* and *Bacillus subtilis*. Contact of immature dendritic cells (DC) from grass pollen allergic donors with supernatants of homogenized Gram-positive bacteria induced maturation of DC as measured by up-regulation of CD80, CD83 and CD86 and by enhanced production of IL-6, IL-12p40 and TNF- α , which was less pronounced compared with effects induced by lipopolysaccharide (LPS). Consequently, stimulation of autologous CD4⁺ T cells with supernatants of homogenized Gram-positive bacteria plus grass pollen allergen-pulsed DC led to an enhanced proliferation and production of IL-4, IL-13, IL-10, IL-17, IL-22 and IFN- γ production compared with T cells that were stimulated with allergen-pulsed immature DC alone, whereas production of the transcription factor for regulatory T cells FoxP3 was not significantly affected.

Conclusions and Clinical Relevance These data indicate that grass pollen is colonized by several microorganisms that influence the immune response differently. Similar to LPS, supernatants of homogenized Gram-positive bacteria may serve as adjuvants by augmenting DC maturation and inflammatory Th1, Th2 and Th17 responses helping to initiate allergic immune responses.

Keywords adjuvants, allergy, dendritic cells, Gram-positive bacteria, Th1/Th2/Th17

Abbreviations DC, dendritic cell; PE, phycoerythrin.

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Introduction

Allergens that elicit type I allergies are mostly relatively small (<70 kD) negatively charged soluble and stable proteins or glycoproteins on the surface of dry particles such as pollen grains and mite faeces [1]. After contact

with mucosal surfaces, they elute from the particle and diffuse into the tissue. Although the immune system is only in contact with small amounts of allergens, many people develop severe allergies against these minimal allergen doses. Pollen allergens are very immunogenic and induce similar numbers of pollen-specific CD4⁺ T cells in healthy and allergic individuals, which differ in their cytokine production profiles [2–4]. The mechanisms leading to the induction of allergic immune

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responses to harmless antigens remain unclear. It is assumed that adjuvants such as lipopolysaccharide (LPS) and other Toll-like receptor (TLR) ligands or pollen associated lipid mediators (PALMs) play an important role [5–7]. It is known for a long time that endotoxin can be used as an essential adjuvant for the induction of antigen-specific memory T cells [8]. LPS activates cells through TLR4 with the accessory proteins, CD14 and LPS-binding protein [9, 10]. In a mouse model, it was shown that no airway inflammatory response was generated, when mice were sensitized with LPS-free ovalbumin [11]. Other studies have also reported that a concurrent exposure to allergen and endotoxin augments the inflammatory response in individuals with asthma [12] and sensitized subjects [13]. Contrarily, the hygiene hypothesis implies that microbial agents protect against allergy and asthma, and indeed many groups have shown a protective effect of TLR2 and/or TLR4 ligands in allergen-induced lung inflammation [14–16].

This study was set out to analyse which microbial factors can be found on pollen grains and which adjuvant effect they exert on the allergen-specific immune response induced. Besides several Gram-negative bacteria and moulds, surfaces of pollen grains were colonized by high numbers of the Gram-positive bacteria, especially of the *Bacillus* type. Supernatants of homogenized Gram-positive bacteria serve as adjuvants contributing to the allergenic potential of allergens.

Materials and methods

Biochemical analysis of grass pollen grains

Pollen was collected from flowering timothy grass from high traffic as well as from rural region. Spatula tips of pollen were cultivated overnight in petridishes with standard culture medium at 37°C. Afterwards, the suspension of the microorganisms was cultivated on agar plates by limiting dilution. Bacterial colonies were determined using Gram staining, and differentiated concerning their biochemical reactions by use of API 20E (BioMérieux, Nürtingen, Germany) and BD BBL Crystal Gram-positive ID kit (Becton Dickinson, Heidelberg, Germany). To identify pathogens, the bacterial strains were cultivated on blood agar (containing 5–10% defibrinated blood from sheep or horses). Moulds were specified according to their morphology on Sabouraud containing agar plates (Sigma-Aldrich, Taufkirchen, Germany).

Bacteria were cultivated in lysogeny broth (LB) medium (Lennox; Roth, Karlsruhe, Germany) at 37°C under agitation, harvested after 6 h and isolated by centrifugation. The sediment containing the bacteria was solubilized in a cell homogenizer (Braun MSK, Melsungen,

Germany) with glass beads (0.1 mm). Then, the glass beads were deposited by sedimentation. After an additional incubation for 30 min, the bacterial suspension was spun down for 30 min at 12 000 g. The supernatant was passed through a 0.2 µm filter. After protein determination, aliquots of the sample were dried using speedvac.

Blood samples

Heparinized blood was obtained from non-allergic healthy donors or atopic donors (mainly with allergic rhinitis) sensitized to grass pollen and who have not undergone specific immunotherapy. Specific sensitization was documented using positive skin prick test to the allergen and detection of allergen-specific IgE in the sera [CAP class ≥ 2 , measured by ImmunoCAP® specific IgE blood test (Phadia AB, Uppsala, Sweden)]. This study was approved by the local ethical committee. Informed consent was obtained from all subjects before the study.

Cell culture reagents

Iscove's modified Dulbecco's medium with L-glutamine and 25 mM Hepes (IMDM, PAA Laboratories GmbH, Pasching, Austria) was supplemented with an antibiotic-antimycotic solution containing 100 µg/mL streptomycin, 100 U/mL penicillin and 250 ng/mL amphotericin B (PAA).

Human recombinant IL-4, IL-1β and TNF-α were purchased from Strathmann Biotech GmbH (Hannover, Germany), GM-CSF (Leukine®) from Immunex Corp. (Seattle, WA, USA) and prostaglandin E₂ from Cayman (Ann Arbor, MI, USA). Grass pollen allergen extract was obtained from ALK Abello (Hamburg, Germany). The endotoxin content of all allergen extracts was tested with QCL-100 chromogenic LAL (Bio-Whittaker, Walkersville, MD, USA) according to the instructions of the manufacturer, and only batches with a contamination of endotoxin <1 ng/mL were used.

Generation of monocyte-derived human DC

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll-Paque 1.077 (PAA) density centrifugation. To enrich CD14⁺ monocytes, 1×10^7 PBMC per well were incubated for 45 min in a 6-well-plate (Costar, Bodenheim, Germany) in IMDM supplemented with 3% autologous plasma at 37°C. After washing the non-adherent cells with pre-warmed phosphate buffered saline, the remaining monocytes (purity >90%) were incubated in 3 mL/well IMDM supplemented with 1% heat-inactivated autologous plasma, 1000 U/mL IL-4 and 200 U/mL GM-CSF.

Cells were fed fresh medium on day 3. On day 6, the resulting immature dendritic cells (DC) were left untreated [negative control (medium)] or were treated with 5 µg/mL grass pollen allergen in the presence or absence of 100 ng/mL LPS (Sigma-Aldrich) or 100 ng/mL lyophilized supernatants of homogenized Gram-positive bacteria *Bacillus cereus* or *Bacillus subtilis* subspecies 1131 and 374. As positive control, DC were treated with the allergen or not, and further stimulated with 1000 U/mL TNF-α, 2000 U/mL IL-1β and 1 µg/mL prostaglandin E₂ to induce their full maturation. Forty-eight hours after stimulation, supernatant of mature DC was collected for determination of IL-1β, IL-6, IL-12p40 and TNF-α. Then, the cells were harvested, washed twice and used for T cell stimulation assays. Mature DC expressed high levels (>90%) of CD80, CD83, CD86 and MHC-class II molecules as controlled using flow cytometry.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMC using antibody-coated paramagnetic MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. Separation was controlled using flow cytometry (purity >98% CD4⁺ T cells).

Co-culture of T cells and autologous allergen-pulsed DC

For proliferation assays, 1×10^5 CD4⁺ T cells were co-cultured in 96-well-plates in triplicates with 1×10^4 autologous immature DC treated with nothing (medium), allergen, allergen plus lyophilized bacterial supernatants or allergen plus LPS in 200 µL IMDM supplemented with 5% heat-inactivated autologous plasma. As positive control, mature DC or mature allergen-treated DC was used. After 5 days, the cells were pulsed with 37 kBq/well of [³H]-thymidine, ICN, Irvine, CA, USA) for 6 h, and [³H]-thymidine incorporation was evaluated using beta counter (1205 Betaplate, LKB Wallac, Turku, Finland).

For cytokine production assays, 5×10^5 CD4⁺ T cells were cultured in 48-well-plates in the presence of 5×10^4 autologous immature or mature DC treated as described above, and in 1 mL IMDM supplemented with 5% heat-inactivated autologous plasma. On day 7, T cells were restimulated with 5×10^4 newly generated autologous DC, treated with the same conditions, and supernatants were collected 24 h later.

For intracellular staining of cytokines, co-cultures were stimulated on day 5 with 50 ng/mL PMA (Sigma-Aldrich), 1 µg/mL ionomycin (Sigma-Aldrich) and 0.7 µL GolgiStopTM (BD Biosciences, Heidelberg, Germany) for 5 h.

Flow cytometry

Surface phenotyping was performed by staining 5×10^5 T cells or 5×10^4 DC with specific anti-human mAbs for 20 min at 4°C. For determination of intracellular cytokines, co-cultures of 5×10^5 T cells and 5×10^4 DC were fixed using Fix/Perm Buffer (eBioscience, Inc., San Diego, CA, USA) for 30 min at 4°C. Then, cells were permeabilized using Permeabilization Buffer (eBioscience) for 5 min, and staining was performed in Permeabilization Buffer for 30 min at 4°C.

The following antibodies were used: AlexaFluor 647-conjugated CD4 (MT310, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), FITC-conjugated FoxP3 (PCH101, eBioscience), PE-conjugated IL-17A (eBio64-DEC17, eBioscience), PE-conjugated CD80 (L307.4), CD83 (HB15e), CD86 [2331 (FUN-1)], IL-4 (MP4-25D2), IL-10 (JES3-19F1), FITC-conjugated HLA-DR (L243), IFN-γ (4S.B3) and mouse or rat IgG isotype controls (all from BD Biosciences). After incubation, the cells were washed and analysed in a FACSCalibur (BD Biosciences) equipped with CELLQuest Software.

Quantification of cytokine production by ELISA

Human IL-1β, IL-6, IL-17, IL-22, TNF-α (R&D Systems, Wiesbaden, Germany), IL-4, IL-10, IL-12p40, IL-13 and IFN-γ (BD Biosciences) were measured using ELISA according to the instructions of the distributors of the employed pairs of antibodies.

Statistics

ANOVA was used for analysis of variance between different experimental groups. Student's *t*-test and the Mann-Whitney *U*-test were used to test the statistical significance of the results; $P \leq 0.05$ was considered to be statistically significant.

Results

Isolation of Gram-positive bacteria from grass pollen grains

To identify the microorganisms colonizing grass pollen, we investigated fresh pollen from flowering timothy grass collected from different regions (near a high traffic street and a rural garden). The pollen was cultivated on agar plates, and the growing microorganisms were selected and classified. Table 1 shows the bacteria and moulds identified.

It is obvious that the habitat in the rural countryside contained a wide spectrum of microorganisms. Besides Gram-negative bacteria, which are known to cause a Th1-directed immune shift, we identified the moulds

Table 1. Microorganisms colonizing timothy grass pollen. Pollen samples were drawn from two different sources

Pollen source	High traffic region	Rural region
Bacteria – Gram-positive	<i>Bacillus brevis</i> <i>Micrococcus luteus</i>	<i>Bacillus subtilis</i> <i>Bacillus cereus</i> <i>Bacillus sphaericus</i> <i>Bacillus brevis</i> <i>Paenibacillus alvei</i> <i>Corynebacterium aquaticum</i> <i>Oerskovia spec.</i> <i>Enterococcus casseliflavus</i> <i>Staphylococcus sanguis</i> <i>Staphylococcus haemolyticus</i>
Bacteria – Gram-negative		<i>Acinetobacter lwoffii</i> <i>Escherichia adecarboxylata</i> <i>Rahnella aquatilis</i> <i>Pseudomonas maltophilia</i>
Moulds		<i>Penicillium spec.</i> <i>Botrytis spec.</i>

Penicillium and *Botrytis*, which are allergenic sources by themselves. Importantly, a lot of Gram-positive bacteria were also identified, especially of the *Bacillus* type. Interestingly, the bacterial strains *B. cereus* and both *B. subtilis* subspecies showed double zone haemolysis on blood agar. This identifies these ubiquitous environmental microorganisms as facultative pathogens.

Supernatants of homogenized Gram-positive bacteria induce maturation of immature human DC

Next, we investigated whether bacterial components being present in the lyophilized supernatants of three selected homogenized Gram-positive bacteria (*B. cereus*, *B. subtilis* subspecies 1131, and *B. subtilis* subspecies 374) might induce DC maturation. In Fig. 1a and b, it is shown that treatment of immature DC from grass pollen allergic donors with endotoxin free grass pollen allergen extract did not lead to DC maturation, whereas addition of supernatants from all selected homogenized Gram-positive bacteria to the allergen extract induced up-regulation of the DC maturation marker CD83 as well as the co-stimulatory molecules CD80 and CD86 (evaluating mean fluorescence intensity). The MHC-class II molecule HLA-DR was already expressed up to 95% by immature DC and not further enhanced. Compared with LPS, which induced a strong and significant up-regulation of all measured CD markers, a significantly enhanced CD80, CD83 and CD86 expression was only observed for *B. subtilis* subspecies 374. However, fully mature DCs

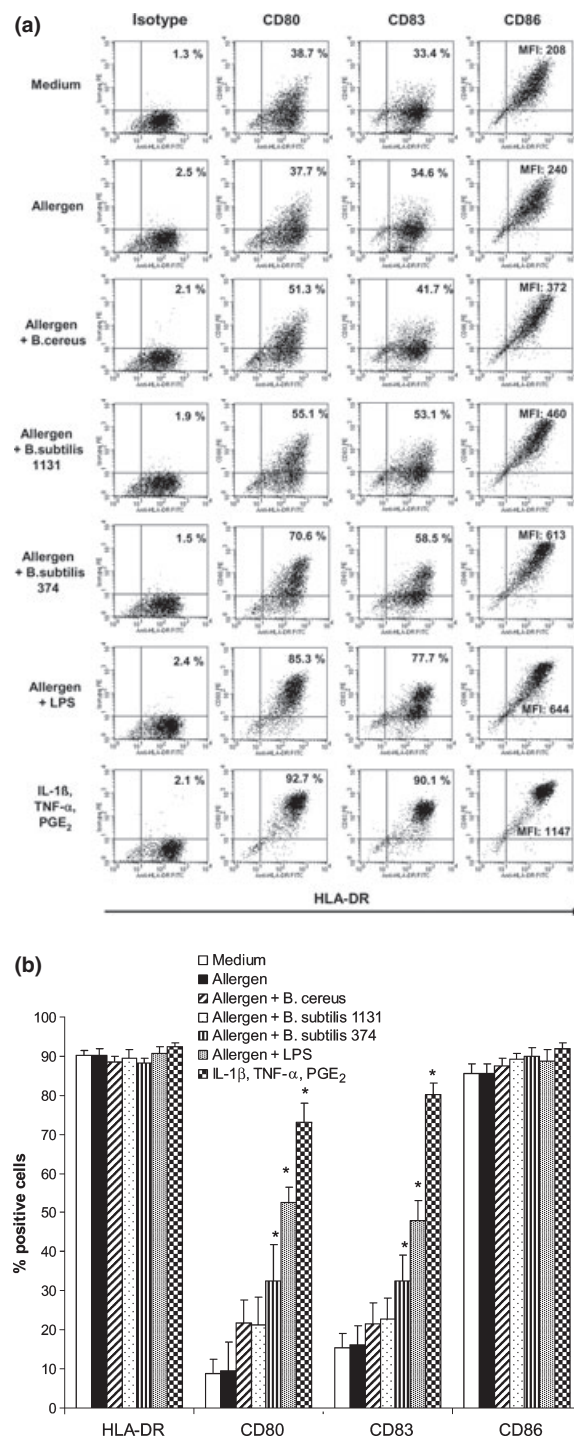


Fig. 1. Supernatants of homogenized Gram-positive bacteria induce CD80, CD83 and CD86 expression in immature dendritic cells (DC). (a) Immature DC from grass pollen allergic donors on day 6 of culture were left untreated [negative control (medium)] or were treated with grass pollen allergen $\pm$ supernatants of different homogenized Gram-positive bacteria, LPS or the positive control IL-1 β , TNF- α , and PGE $_2$ to induce maximal maturation. After 48 h, surface marker expression was analysed using flow cytometry as indicated. Shown are one representative result (a) and the mean $\pm$ SEM (b) of nine independent experiments/donors, * $P \leq 0.05$ when compared with immature allergen-treated DC, MFI, mean fluorescence intensity.

were only obtained by the presence of the pro-inflammatory cytokines IL-1 β and TNF- α together with PGE₂ (Fig. 1a and b), and this maturation was not further augmented by adding LPS or supernatants from homogenized Gram-positive bacteria (data not shown). Besides the enhanced expression of DC maturation markers, production of IL-6, IL-12p40 and TNF- α by immature DC was also induced by supernatants of almost all homogenized Gram-positive bacteria (Fig. 2). Again, this was less pronounced than that observed with LPS reaching significance only for *B. subtilis* subspecies 374 regarding TNF- α production. A combination of *B. subtilis* subspecies 374 and *B. cereus*, however, increased DC maturation similar to LPS (data not shown).

Treatment of immature DC from non-allergic healthy donors with LPS, or with supernatants of the three homogenized Gram-positive bacteria led to similar results regarding surface marker expression and production of pro-inflammatory cytokines (data not shown).

DC treated with supernatants of homogenized Gram-positive bacteria or with LPS enhances allergen-specific proliferation in CD4⁺ T cells

To analyse whether treatment of DC with supernatants of homogenized Gram-positive bacteria or LPS enhance allergen-specific proliferation in T helper cells from

grass pollen allergic donors or non-allergic healthy donors, we performed co-cultures of CD4⁺ T cells with DC from the same donor treated with grass pollen allergen in the presence or absence of either supernatants of homogenized Gram-positive bacteria or LPS. Fully mature DC, treated with the allergen or not, was used as positive control. As expected, only mature allergen-treated DC induced a strong proliferation in autologous CD4⁺ T cells compared with stimulation with untreated mature DC, untreated immature DC or allergen-treated immature DC. Proliferation of CD4⁺ T cells from allergic donors was higher than that from non-allergic donors (Fig. 3). In both groups, addition of LPS or supernatants from homogenized *B. subtilis* subspecies 374 to immature allergen-treated DC led to a significantly increased proliferative response of autologous CD4⁺ T cells, but less pronounced as observed for fully mature allergen-pulsed DC (Fig. 3). A combination of *B. cereus* and *B. subtilis* subspecies 374 further increased T cell proliferation. In non-allergic donors, this up-regulation was even comparable to fully mature allergen-treated DC. Supernatants of homogenized Gram-positive bacteria or LPS added together with the allergen during DC maturation did not further enhance proliferation (data not shown).

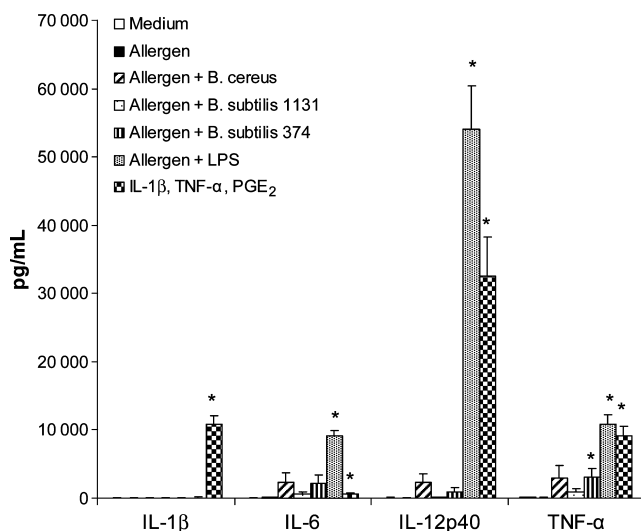


Fig. 2. Induction of proinflammatory cytokines in immature dendritic cells (DC) by supernatants of homogenized Gram-positive bacteria. Immature DC from grass pollen allergic donors on day 6 of culture were treated as described in Fig. 1. After 48 h, supernatants were collected for determination of IL-1 β , IL-6, IL-12p40 and TNF- α using ELISA. Shown are the means $\pm$ SEM of eight independent experiments/donors, * $P \leq 0.05$ when compared with immature allergen-treated DC.

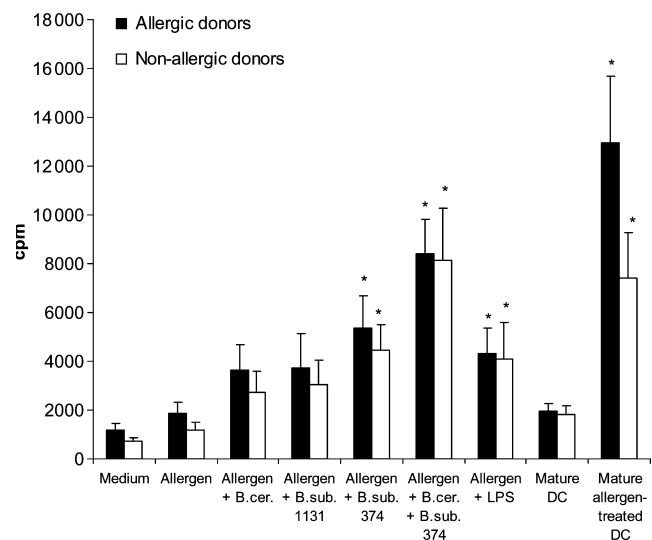


Fig. 3. Dendritic cells (DC) treated with supernatants of homogenized Gram-positive bacteria increase allergen-specific proliferation of autologous CD4⁺ T cells. CD4⁺ T cells from grass pollen allergic donors or non-allergic healthy donors were stimulated with autologous immature DC that had been treated with medium (negative control), grass pollen allergen or grass pollen allergen plus supernatants of different homogenized Gram-positive bacteria or LPS. Fully mature DC, treated with the allergen or not, were used as positive control. After 5 days of co-culture, proliferation was measured using [³H]-thymidine incorporation. Shown are the means $\pm$ SEM of nine independent allergic and six non-allergic donors, * $P \leq 0.05$ when compared with immature allergen-treated DC.

DC treated with supernatants of homogenized Gram-positive bacteria or with LPS enhances allergen-specific Th1, Th2 and Th17 cytokine production in CD4⁺ T cells from grass pollen allergic donors

To investigate whether supernatants of homogenized Gram-positive bacteria or LPS-treated DC alter the cytokine profile of CD4⁺ T cells, we stimulated T cells from grass pollen allergic donors with autologous DC treated as described above and analysed intracellular cytokine expression after additional stimulation with PMA and ionomycin in the presence of a protein transport inhibitor for 4 h on day 5. In Fig. 4a, it is shown that intracellular allergen-induced cytokine production of CD4⁺ T cells after stimulation with immature DC was only detectable for the cytokines IL-4, IL-17 and the transcription factor FoxP3, whereas intracellular detection of the cytokines IL-10 and IFN- γ did not differ between stimulation with immature untreated or allergen-treated DC. Intracellular cytokine and FoxP3 production was not significantly affected by supernatants of homogenized Gram-positive bacteria or LPS added together with the allergen to immature DC, whereas allergen-treated mature DC led to significantly enhanced intracellular cytokine and FoxP3 levels except for IL-10 and IL-17.

To analyse cytokine secretion into the supernatants, the co-cultures were further restimulated with freshly generated DC after 1 week for an additional time of 24 h, which was necessary to accumulate the cytokine content. As shown in Fig. 4b, allergen-induced production of IL-4, IL-10, IL-13, IL-17, IL-22 and IFN- γ was detectable after stimulation with immature allergen-treated DC, and significantly enhanced after stimulation with mature allergen-treated DC compared with untreated control DC. In supernatants of co-cultures of CD4⁺ T cells and immature DC treated with allergen plus supernatants of homogenized Gram-positive bacteria or LPS, increased amounts of all mentioned cytokines could be detected, reaching significance for both *B. subtilis* subspecies regarding IL-4 and IL-10 production and for *B. subtilis* subspecies 374 regarding IFN- γ production. Again, addition of Gram-positive bacterial components or LPS together with the allergen during DC maturation did not further enhance cytokine production (data not shown).

In co-cultures from non-allergic donors, treatment of immature DC with allergen plus supernatants of homogenized Gram-positive bacteria or LPS led to similar results. However, no IL-4 and only little IL-13 production was detectable (data not shown).

Discussion

Allergic immune responses are not only triggered by the allergen itself but also by cofactors released from

the allergen carrier or exposed together with the allergen [17]. In this study, we analysed the surface of grass pollen grains for the presence of microorganisms which may influence the antigen-specific immune response by directly activating receptors of the innate immune system, e.g. TLR. We could demonstrate that grass pollen grains contain high numbers of Gram-negative and Gram-positive bacteria and several moulds. This is in accordance with other groups also describing several anaerobic and aerobic microorganisms, coliforms and microscopic fungi on pollen, but without further analysing their functional role in pollen allergen-driven immune responses [18, 19]. Gram-negative bacteria, e.g. *Acinetobacter lwoffii*, have been shown to cause a Th1-directed immune response, due to LPS and the activation via TLR4 [20]. The moulds *Penicillium* and *Botrytis* have already been shown to possess allergenic potency [21]. As the potential role of the isolated Gram-positive bacteria *B. cereus* and *B. subtilis* in allergic immune responses has not been analysed so far, we focused on these Gram-positive aerobic bacteria. Lyophilized supernatants of homogenized *B. cereus* or *B. subtilis* subspecies 1131 and 374 induced maturation of monocyte-derived immature DC from grass pollen allergic donors by up-regulation of the co-stimulatory molecules CD80 and CD86 as well as the DC-specific maturation marker CD83, but not as strong as LPS. In addition, the production of IL-6, IL-12p40 and TNF- α by immature DC were also less enhanced after addition of supernatants of homogenized *B. cereus* or *B. subtilis* subspecies 1131 and 374 in comparison to LPS, whereas a combination of the two *Bacillus* strains led to DC maturation similar to LPS. In co-cultures with autologous CD4⁺ T cells, addition of supernatants of homogenized Gram-positive bacteria or LPS plus allergen-treated DC led to an enhanced proliferation as well as Th1, Th2 and Th17 cytokine secretion into the supernatant, but not on the single cell level compared with immature allergen-pulsed DC alone. Experiments with non-allergic donors led to similar results except the well-known fact that allergen-specific proliferation was lower and no or only little Th2 cytokines could be detected.

The fact that not only LPS but also supernatants of homogenized Gram-positive bacteria can induce maturation of human monocyte-derived DC is in line with a recent study showing that whole human blood cells are activated to produce IL-6 by both, Gram-negative and Gram-positive bacteria [22]. Another report by Kim et al. demonstrated that *B. subtilis*-specific poly-gamma-glutamic acid promoted the development of Th1 and Th17 cells, but inhibited the development of Th2 cells [23]. This discrepancy to our results may be due to the fact that they have used only one component of *B. subtilis*, whereas we have used the whole supernatant of homogenized *B. subtilis* subspecies 1131 and 374. Nevertheless,

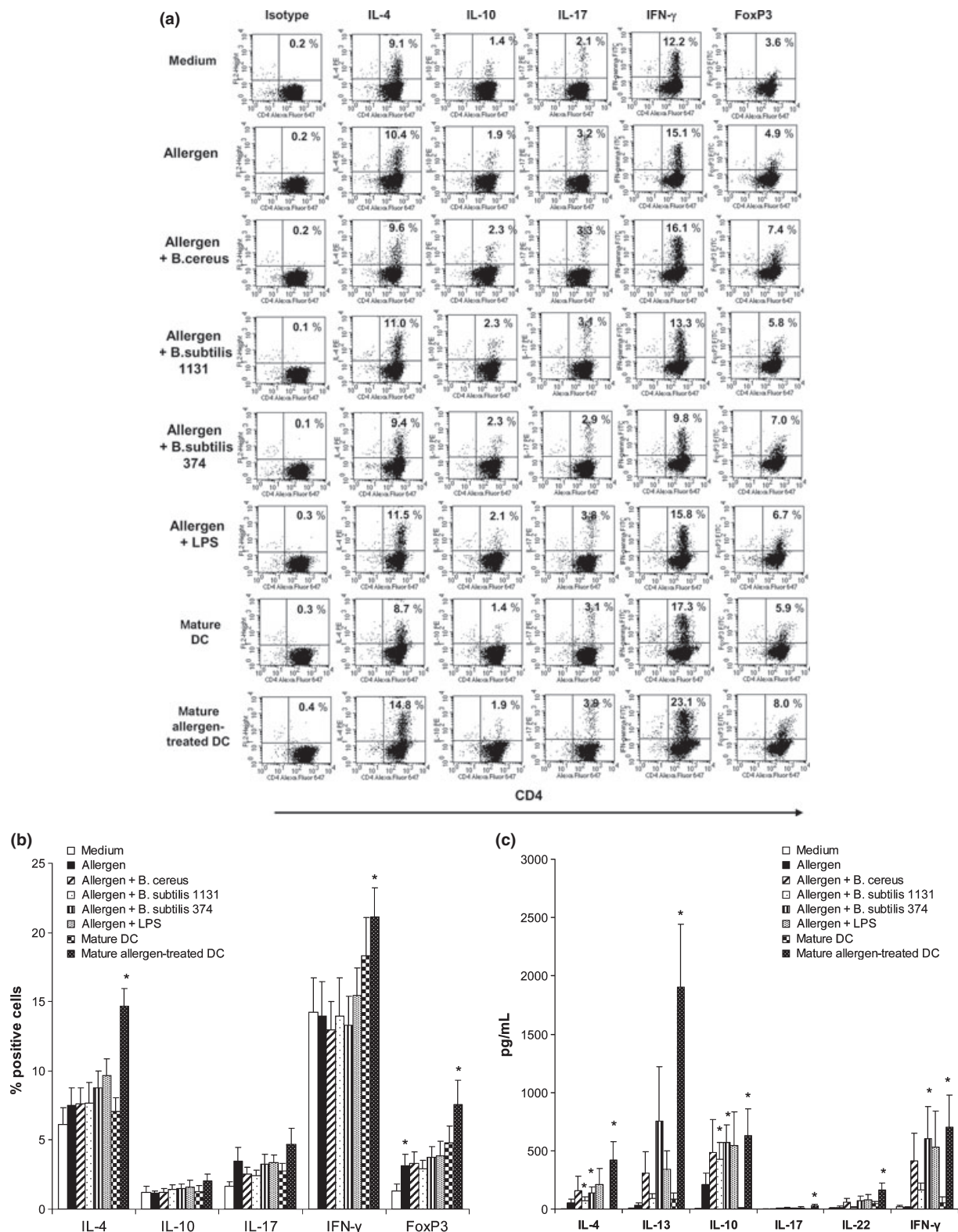


Fig. 4. Dendritic cells (DC) treated with supernatants of homogenized Gram-positive bacteria enhance allergen-specific production of Th1, Th2 and Th17 cytokines in autologous CD4⁺ T cells. CD4⁺ T cells from grass pollen allergic donors were stimulated as described in Fig. 3. (a and b) After 5 days of co-culture, production of intracellular IL-4, IL-10, IL-17, IFN- γ and FoxP3 was analysed as described in Materials and methods. (c) After 1 week, co-cultures were restimulated with freshly isolated mature DC treated with the same stimuli, and supernatants were collected 24 h later for determination of IL-4, IL-10, IL-13, IL-17, IL-22 and IFN- γ production using ELISA. Shown are one representative result (a) and the means $\pm$ SEM (b and c) of seven independent experiments/donors, * $P \leq 0.05$ when compared with immature allergen-treated DC.

similar to our study, they have also described that polygamma-glutamic acid induced the production of IL-12p40, CD80 and CD86 in DC and macrophages in a TLR4-dependent manner, and the effect on Th1/Th2 development was dependent on the presence of antigen-presenting cells (APC), whereas Th17 cells developed regardless of the presence or absence of APC [23]. These results suggest that microbial products from Gram-positive or -negative bacteria, being present in ambient air and dust or on pollen grains, may not only protect from Th2-mediated airway hypersensitivities on the one hand but may also contribute to the immunogenic potential of pollen by enhancing Th1-, Th2- and Th17-mediated inflammation on the other hand, which has been recently reviewed for LPS [24]. Notably, pollen grains themselves release substances that induce activation and maturation of DC together with Th2-inducing adjuvant activity [25, 26]. For example, PALMs induce chemotaxis and the activation of neutrophils and eosinophils, and birch pollen phytoprostanes inhibit IL-12 production and augment migratory together with Th2 cell-attracting capacity in DC [27, 28].

Besides the enhancement of Th1, Th2 and Th17 cytokine production, we observed an increased production of IL-10, but no effect on the transcription factor FoxP3 by supernatants of homogenized Gram-positive bacteria or LPS plus allergen-pulsed DC. The immune response to allergens leads to allergic responses or not, depending on environmental factors and on the genetic susceptibility of the individual, [14, 29]. Although allergic

immune responses are characterized by a dominance of Th2 and Th1 cells and by reduced frequencies of allergen-specific Tr1 cells, which are enhanced during successful immunotherapy, in healthy individuals, IL-10- and TGF- β -producing allergen-specific Tr1 cells represent the dominant subset [3, 30, 31]. However, IL-10 production is also found in subsets of Th17 and Th1 cells negatively regulating their function [32, 33]. Thus, the production of IL-10 induced by pollen-derived substances might be derived from FoxP3-negative Tr1 cells or from such effector T cells. Further investigations might resolve this subject as well as the question of whether or not the inhibitory function of Treg is affected by Gram-positive bacteria.

In summary, pollen grains are colonized by several Gram-positive and Gram-negative bacteria that possess adjuvant activity by greatly enhancing DC maturation and induction of Th1-, Th2- and Th17-mediated allergic inflammation.

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