

Original article

The allergy-protective properties of *Acinetobacter lwoffii* F78 are imparted by its lipopolysaccharide

Background: An increasing number of epidemiological studies show that exposure to farming environment during early childhood strongly influences the development of allergic reactions later in life ('hygiene hypothesis'). Also, it had been shown that certain bacteria from this environment may have allergy-protective properties. In the present study, we further characterized one of these bacteria, namely *Acinetobacter lwoffii* F78, with regard to the bacteria-induced signaling and possible mechanisms of allergy protection.

Methods: The impact of *A. lwoffii* F78 on human monocyte-derived dendritic cells especially with respect to their T_HHelper cell polarization capacity was investigated by ELISA and real-time PCR experiments as well as confocal microscopy. The responsible molecule for these effects was further characterized and identified using blocking experiments.

Results: It was shown that *A. lwoffii* F78 induced a T_H1-polarizing program in human dendritic cells which led to T_H1 differentiation. In addition, a positive influence on the TBet/GATA3 level could be detected. Blocking experiments revealed that the lipopolysaccharide (LPS) of *A. lwoffii* F78 was the responsible molecule promoting these effects.

Conclusion: We found evidence that the allergy-protecting effects of *A. lwoffii* F78 are because of the activation of a T_H1-polarizing program in human dendritic cells, and that the LPS of *A. lwoffii* F78 is responsible for these beneficial effects.

**J. Debarry¹, A. Hanuszkiewicz²,
K. Stein¹, O. Holst², H. Heine¹**

Divisions of ¹Innate Immunity and ²Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany

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PD Dr H. Heine
Division of Innate Immunity
Research Center Borstel
Leibniz-Center for Medicine and Biosciences
Parkallee 22
D-23845 Borstel
Germany

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According to the World Health Organization, asthma is one of the most prominent chronic infections in children worldwide, and, moreover, a strong increase in the incidence of asthma and allergies in the last few decades was reported (1). In contrast, the incidence of infectious diseases like mumps, measles, tuberculosis, or hepatitis A is decreased (2). These findings led to the hygiene hypothesis which states that the tremendous increase in atopic diseases correlates with less contact to microbes and fewer infections in childhood (3). In this context, a number of epidemiological studies showed that children who grow up in a farming environment will develop less atopic disorders later in life (4–7). This positive effect of the farming environment is currently discussed as a result of the activation of the innate immune system by microbial compounds (8). For instance, an inverse correlation between the microbial burden [measured by the content of lipopolysaccharide (LPS) or CpG motifs] and the development of atopic disorders in children could be shown (9, 10). Furthermore, LPS and CpG motifs seem to be potent in preventing an allergic outcome in mouse allergy models (11, 12).

As it is known that the immune response of newborns is T_HHelper 2 (T_H2) dominated and the first year of life is important for the establishment of a normal T_H1/T_H2/T_{reg} (regulatory T cells) balance (13, 14), it seems plausible that the stimulation of the innate immune system plays an important role in this process by suppressing the allergy-associated T_H2 response by inducing either a regulatory response (T_{reg}) or a T_H1 immune response. Indeed, at present, both explanations (lack of immune deviation and lack of immune suppression) are discussed and also supported by a number of studies (15, 16). Both models have in common that the induction of either T_H1 or T_{reg} cells suppresses T_H2 responses and thereby might prevent from developing atopic diseases. Important for the polarization and consequently for the developing of a normal T_H1/T_H2/T_{reg} balance are antigen presenting cells (APCs) which process antigens, present them to T cells, and provide certain differentiation signals (17, 18). In atopic diseases, dendritic cells (DCs) are the most important APCs (19, 20). The kind of differentiation signal is dependent on the primary stimuli, and therefore environmental factors like

microbial compounds in the farming environment can influence the differentiation state of T cells.

In a previous study, we showed that two bacteria namely *Lactococcus lactis* G121 and *Acinetobacter lwoffii* F78 isolated from farming environment have the ability to prevent an allergic outcome in a mouse allergy model (21). Here, we focused on *A. lwoffii* F78 and were interested which polarization signals human DCs furnished after activation with *A. lwoffii* F78 and which receptors are involved. Our data provided evidence that *A. lwoffii* F78 induces T_H1 differentiation, and that LPS is the responsible molecule promoting this effect. Thus, T_H1 induction might be the underlying mechanism of *A. lwoffii* F78-induced allergy protection, and its LPS is thought to be the initiating active principle.

Methods

Bacteria

Growth conditions and characterization of the strain were described elsewhere (21). LPS was isolated by hot phenol/water (22) and further purified by enzymatic treatment (RNase A, Sigma-Aldrich, USA; DNase I, Roche, Germany; Proteinase K, Roche, Germany), ultracentrifugation (105 000 g, 4°C, 24 h), and re-extraction with PCP III (phenol/chloroform/light petroleum 1 : 1 : 1, by vol.) (23).

Isolation and generation of different human cell types

For all experiments, human cells were isolated from heparinized blood of healthy donors. Atopy status was determined by specific IgE responses against the timothy grass pollen major antigen Phl p1, which has been tested by a specific immunoblot using a mixture of three mAb (Bo14, IG12, HB7) against Phl p1 (24). Because we could not detect any statistical significant differences (Two-way ANOVA and Bonferroni posttests, $P > 0.05$) in the cytokine release of atopic (4) vs nonatopic (6) individuals in our experiments, results are representative for both groups.

Generation and stimulation of human monocyte-derived DCs (moDCs)

Dendritic cells were generated as described elsewhere (21). Immature moDCs were harvested and recultured in 24-well plates with complete medium (RPMI 1640 (Cambrex, East Rutherford, NJ, USA) supplemented with 10% heat-inactivated FCS (Biochrom AG, Berlin, Germany), 100 U/ml penicillin (PAA Laboratories GmbH), and 100 mg/ml streptomycin (PAA Laboratories GmbH) at a density of 10^6 cells/ml. After 2–4 hours, cells were stimulated with respective stimuli in complete medium, and analysis was performed as indicated for the different experiments. The synthetic lipid A structure compound 406 was a kind gift of Prof. Kusumoto from Osaka, Japan. In human cells, it possesses no stimulatory activity, but it is a strong lipid A antagonist (25).

Isolation and purification of human naïve T cells

Heparinized blood of healthy donors was prepared by Ficoll (PAA Laboratories GmbH, Pasching, Austria) separation method (26), and peripheral blood mononuclear cells (PBMCs) were harvested.

Lymphocytes were isolated by counter flow elutriation centrifugation, and naïve T cells were purified with the *Naïve CD4⁺ T Cell Isolation Kit* (Miltenyi Biotec GmbH, Germany) according to manufacturer's instructions.

Co-culture of moDCs with autologous naïve T cells

Differentially stimulated moDCs (16 h) were harvested and washed to remove free stimulants. Subsequently, the moDCs were co-cultured with autologous naïve T cells in fresh medium in round-bottom microtiter plates at moDC/T cell ratio of 1 : 10. At day 5 of co-culture, cells were restimulated with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, CalBiochem, Germany) and ionomycin (500 ng/ml, Molecular Probes, USA), and after additional 20 h, the supernatant was collected, and the content of IFN- γ was measured using an ELISA. For the analyses of T cell-specific gene expression, supernatants of stimulated moDCs (16 h) were harvested and used to stimulate naïve T cells. After additional 24 h, T cells were collected for RNA isolation.

Real-time quantitative RT-PCR

Total RNA was extracted from moDCs or T cells using Absolutely RNA kit from Stratagene. Total RNA was then reverse transcribed into cDNA using Oligo(dT)_{12–18} and Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). According to the manufacturer's instructions, different target genes were quantified by real-time quantitative PCR on LightCycler 2.0 using the FastStart DNA Master^{PLUS} SYBR Green kit (Roche Applied Science, Mannheim, Germany). Specific mRNA levels were normalized to the level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA in the same sample and are expressed in arbitrary units. Primers used in this study are as follows: HPRT-for 5'-GTCAGGCAGTATAATCCAAAGA; HPRT-rev 5'-GGGCATATCCTACAAACAACT; IFN- γ -for 5'-GTTTGGGTTCTCTTGGCTGTTA; IFN- γ -rev 5'-TTTGGCTCTGCATTATTTTTCTGT; TIM-3-for 5'-TGTTTTTCACATCTTCCCTTTGA; TIM-3-rev 5'-GACCTCCGCTCTGTATTCCA; TBet-for 5'-GACTCCCCCAACACAGGAG; TBet-rev 5'-GGGACTGGAGCACAATCATC; GAT A3-for 5'-TCAGACCACCACAACCACAC; and GATA3-rev 5'-TTCATAGTCAGGGGTCTGTAAAT.

Western blots

Protein lysates of moDCs were generated with Laemmli buffer (60 mM Tris pH 6.8, 100 mM DTT, 5% glycerol, 2% SDS, 0.001% bromophenol blue) and were denatured at 95°C for 5 min. SDS-PAGE was performed using NuPAGETM 4–12% Bis-Tris gels (Invitrogen, Germany). Subsequently, proteins were blotted on nitrocellulose membranes using Novex Mini-Cell (Invitrogen, Germany) according to manufacturer's instructions. The proteins p38 and phospho-p38 were detected using rabbit anti-human p38 (Cell Signaling, USA) and mouse anti-human phospho-p38 (Cell Signaling, USA) with the secondary antibodies goat anti-mouse IRDye800 (Molecular Probes, USA) and goat anti-rabbit Alexa-Fluor680 (Molecular Probes, USA). Blots were scanned using the Odyssey[®] Infrared Imaging System (Li-Cor, USA) at 700 and 800 nm and analyzed using ODYSSEY[®] software v1.2 (Li-Cor, USA).

Measurement of cytokines

Cytokine concentrations were determined in moDCs or co-culture supernatants using commercial ELISAs specific for IFN- γ IL-8

(both BioSource, Solingen, Germany), and IL-12p70 (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

Confocal microscopy

After differentiation, moDCs were recultured and incubated with FITC-labeled latex beads (Polysciences, USA) in μ -slide VI (Ibidi GmbH, Germany). Cells were fixed with 4% PFA, permeabilized with 0.25% Triton X-100, and dyed with *mouse* anti-human TLR2 (Genentech, USA) and TOTO-3-iodid (Invitrogen, Germany). Slides were analyzed using the laser scanning microscope TCS SP and the software TCS NT (Leica, Germany).

Expression of costimulatory molecules on human moDCs

Stimulated moDCs were harvested and stained with fluorochrome-conjugated mAbs for CD40 (PE-labeled, BeckmanCoulter, USA), CD80 (PE-labeled, BeckmanCoulter, USA), CD86 (PE-labeled, BD PharMingen, USA), or MHC class II (PE-labeled, BeckmanCoulter, USA) and were fixed with 1.5% paraformaldehyde. Isotype controls IgG1 (PE-labeled, BeckmanCoulter, USA) and IgG2a (PE-labeled, BeckmanCoulter, USA) were used in parallel. Data analysis was performed with the WinMDI 2.9 software developed by Joseph Trotter (Scripps Institute, La Jolla, CA, USA).

Statistical analysis

Statistical analysis (paired *t*-test, one-tailed *P*-value) was performed with GraphPad Prism5. Data presented in the Figs 1A, B and 5A, C are presented as group data from the indicated number of subjects. Significant differences compared to control cells are indicated by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.005$). All other figures are representative for the indicated number of experiments and depicted as mean $\pm$ SD.

Results

Acinetobacter lwoffii F78 induces a T_H1 -polarizing program in human dendritic cells

Recently, we could show that bacteria strains such as *A. lwoffii* F78 and *L. lactis* G121 isolated from cowsheds that were part of the ALEX study (6) protected mice from OVA-induced allergic symptoms (21). Additionally, we analyzed the effect of these bacteria on human dendritic cells and could demonstrate the induction of IL-12 release as well as upregulated expression of the T_H1 -polarizing Notch ligand Delta 4. For *A. lwoffii* F78, dose-response experiments revealed best efficiency at a concentration of 10^6 CFU/ml, wherefore all described experiments in the present study were performed with the optimal concentration. To further investigate the induction of a T_H1 -polarizing program by *A. lwoffii* F78, we analyzed the regulation of other known T_H1 -driving cytokines and molecules and showed that *A. lwoffii* F78 induced the upregulation of such T_H1 -driving cytokines (e.g., ICAM-1, IL-23p19, IFN- β , and CCL5, data not shown) comparable to LPS from *Salmonella enterica* sv. Friedenau which is an inducer of T_H1 polarization (Debarry/Heine, unpublished data). Not only did *A. lwoffii* F78 induce the same genes as pure LPS but also with the very same kinetic. To evaluate the effect of this stimulation on the capacity of the DCs to directly polarize T cells, we performed co-culture experiments of stimulated DCs with autologue naïve T cells. After 5 days, T cells were reactivated with PMA and ionomycin, and the release of IFN- γ was determined. As depicted in Fig. 1A, *A. lwoffii* F78 strongly induces the release of IFN- γ from

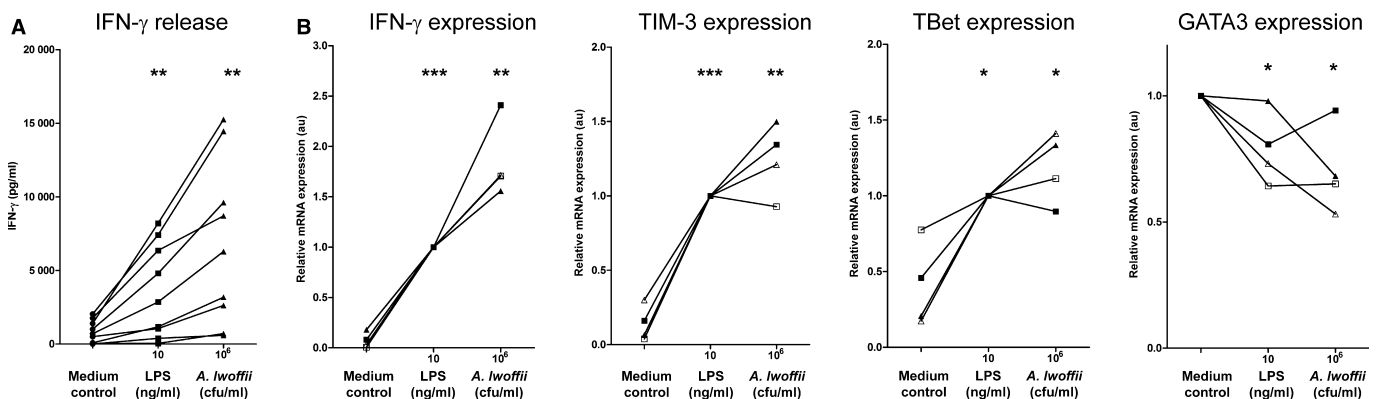


Figure 1. *Acinetobacter lwoffii* F78-treated DCs induce T_H1 differentiation of naïve T cells. (A) For 16 h, stimulated human DCs were co-cultured with autologous naïve T cells and restimulated after 5 days of co-culture. The induced IFN- γ release was measured in the supernatant after additional 20 h. (B) After 16 h of stimulation, the supernatant of DCs were harvested and used to activate allogenic naïve T cells. After 24 h, indicated target mRNAs were measured. The mRNA expression level after stimulation with lipopolysaccharide (LPS) (IFN- γ , TIM-3, TBet) or the unstimulated control (GATA3) was set to 1.0 for each subject. Data are presented as mean values of 9 (1A) or 4 (1B) independent experiments with moDCs and naïve T cells from different healthy donors. Statistical analysis was performed by paired *t*-test using one-tailed *P*-values. Significant differences to control cells are indicated by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.005$).

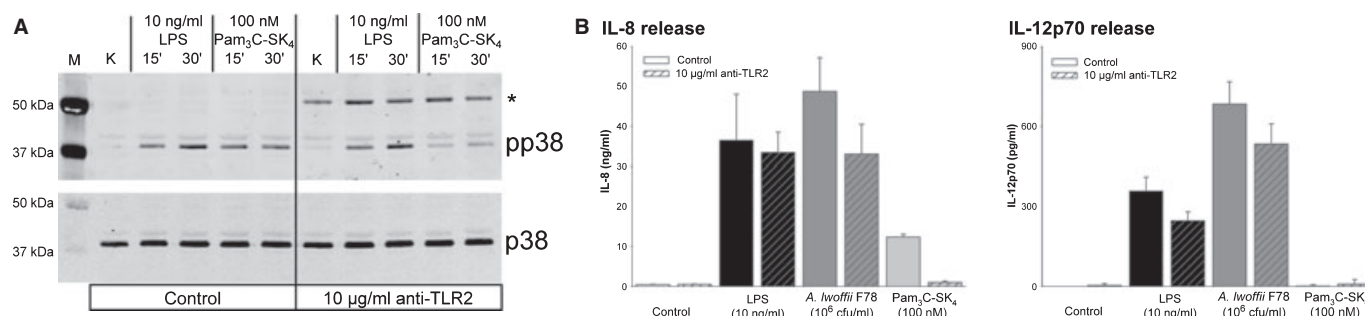


Figure 2. TLR2 ligands are not important for the T_H1-polarizing effects of *Acinetobacter lwoffii* F78. Human moDCs were stimulated with the indicated stimuli in the presence or absence of the blocking TLR2 antibody. (A) Immunoblotting experiments were performed to analyze the phosphorylation of the MAP kinase p38 (pp38). The asterisk indicates the TLR2 antibody heavy chain. (B) After 16 h, the concentration of IL-8 and IL-12 was measured in the supernatant. Data (mean values $\pm$ SD) are representative for at least 4 independent experiments with moDCs from different healthy donors.

autologous T cells, again demonstrating a clear shift to a T_H1 response. This effect does not require direct cell-to-cell contact between DCs and T cells, because the supernatant of *A. lwoffii* F78-stimulated DCs was able to drive T_H1 polarization in allogenic naïve T cells as well (Fig. 1B). Not only was the expression of IFN- γ and TIM-3 mRNA upregulated in these T cells but also the ratio of the mRNA expression of the transcription factors TBet and GATA3 was shifted toward a T_H1 profile. To exclude the possibility that the stimuli themselves, which are still present in the supernatant of the DCs, induce this T_H1 shift, we also stimulated T cells directly using LPS and *A. lwoffii* F78. Those cells showed no significant differences in expression levels of the investigated genes compared to the control cells (data not shown).

Together, stimulation of human dendritic cells with *A. lwoffii* F78 clearly induced a full T_H1-polarization program.

TLR4 mediates *A. lwoffii* F78-induced T_H1-polarization of T cells

We next investigated which innate immune receptors are responsible for the T_H1-promoting effect of *A. lwoffii* F78. In our recent study, we demonstrated that *A. lwoffii* F78 was able to activate HEK293 cells upon transfection with the innate immune receptors NOD1, NOD2, TLR2, and TLR4. First, the importance of TLR2 was examined using a blocking anti-TLR2 antibody. The synthetic and pure TLR2 agonist Pam₃C-SK₄ induced phosphorylation of the MAP kinase p38 in human dendritic cells with a similar kinetic as LPS (Fig. 2A, left panel). Preincubation with the anti-TLR2 antibody clone 1392 (Genentech) selectively blocked p38 phosphorylation induced by Pam₃C-SK₄ but not by LPS (Fig. 2A, right panel). Under the same conditions, the induction of IL-8 release by 100 nM Pam₃C-SK₄ was completely abrogated indicating that indeed the TLR2 pathway was totally blocked (Fig. 2B). Whereas LPS was not affected at all, the *A. lwoffii* F78-induced IL-8 release

showed a minor reduction upon antibody pretreatment. However, more importantly, neither was the TLR2 agonist Pam₃C-SK₄ able to induce IL-12 release by itself nor did the antibody pretreatment significantly blocked *A. lwoffii* F78-induced IL-12 release (Fig. 2B). This strongly argues against the involvement of TLR2 in the *A. lwoffii* F78-induced T_H1 polarization capacity.

NOD1 and NOD2 are intracellular cytosolic pattern recognition receptors (27). Thus, it appears likely that cellular uptake is a prerequisite for activation of cells by NOD ligands. To analyze if signaling through these receptors is required for the induction of T_H1-promoting factors, we blocked phagocytosis in human dendritic cells with cytochalasin D (CytD, see Fig. 3). Although uptake of FITC-labeled latex beads was completely abrogated at 1 μ M cytochalasin D, the LPS- and *A. lwoffii* F78-induced IL-12 release was only little affected. Because pure NOD ligands (similar to TLR2 ligands) were not capable of inducing IL-12 secretion by themselves in our system (data not shown), this data indicated that cellular uptake of *A. lwoffii* F78 was not required for the T_H1-polarizing effects and clearly pointed to TLR4 as the most important receptor involved in T_H1 polarization by *A. lwoffii* F78.

To directly address the TLR4 importance, we used the TLR4/lipid A antagonist compound 406 (C406) (25). Preincubation of human dendritic cells with compound 406 completely abrogated not only the LPS-induced but also the *A. lwoffii* F78-induced IL-12 release, whereas the IL-12 release induced by the Gram-positive bacterium *L. lactis* G121 was not reduced (Fig. 4). Furthermore, when C406 was used in co-culture experiments with autologous naïve T cells, LPS- as well as *A. lwoffii* F78-driven IFN- γ release of T cells was completely blocked.

The LPS of *A. lwoffii* F78 mediates T_H1-polarizing effects

Finally, we wanted to address the question whether the LPS of *A. lwoffii* F78 is capable of promoting all

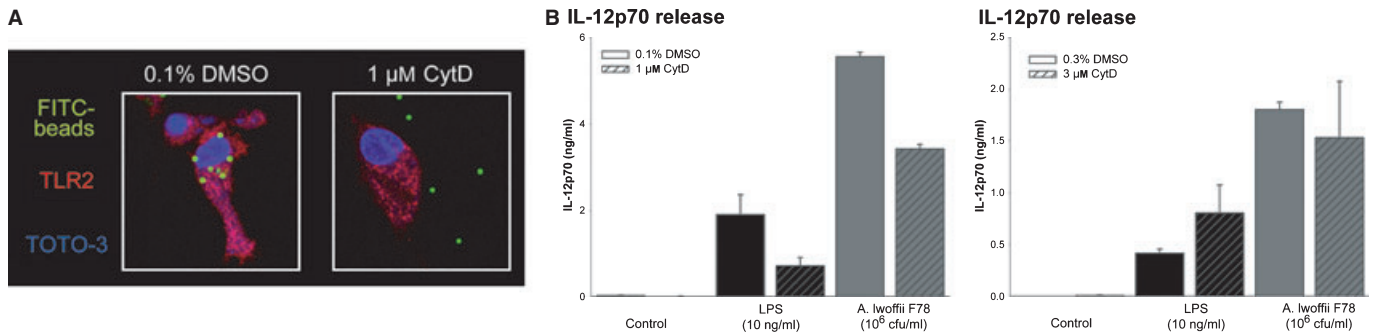


Figure 3. Cellular uptake of *Acinetobacter lwoffii* F78 is not required for the T_H1-polarizing effects. Human moDCs were incubated with FITC-labeled latex beads (A) or the indicated stimuli (B) in the presence or absence of CytD. (A) Afterward, cells were stained for confocal microscopy using TLR2 antibody and TOTO-3. (B) After 16 h, the concentration of IL-12 was measured in the supernatant. Data (mean values $\pm$ SD) are representative for at least 3 independent experiments with moDCs from different healthy donors.

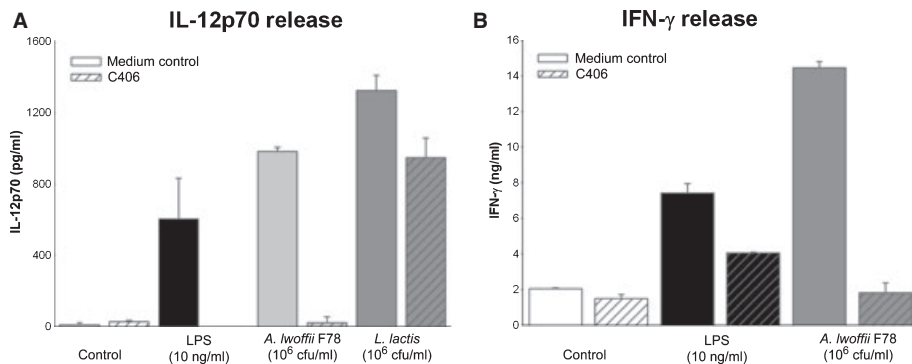


Figure 4. TLR4/lipid A antagonist compound 406 blocks the T_H1-polarizing effects of *Acinetobacter lwoffii* F78. Human moDCs were stimulated with indicated stimuli in the presence or absence of C406. (A) After 16 h of stimulation, the concentration of IL-12 was measured in the supernatant. (B) After 16 h of stimulation, these DCs were co-cultured with autologous naïve T cells and were restimulated after 5 days. The induced IFN- γ release was measured in the supernatant after additional 20 h. Presented data (mean values $\pm$ SD) are representative for at least 3 independent experiments with moDCs from different healthy donors.

the T_H1-polarizing effects that we had seen so far. Therefore, the LPS of *A. lwoffii* F78 was isolated and characterized (28). First, we tested whether *A. lwoffii* F78 LPS activated human dendritic cells (Fig. 5A) and induced maturation (Fig. 5B). As presented in terms of IL-8 and TNF- α induction, the LPS of *A. lwoffii* F78 possessed a very similar activity as the whole bacteria preparation as well as a control LPS preparation from *S. enterica* sv. Friedenau. Furthermore, upregulation of costimulatory molecules such as CD40, CD80, CD86, and MHC class II was induced by *A. lwoffii* F78 LPS as well.

The last set of experiments was designed to analyze whether *A. lwoffii* F78-LPS could also induce the hallmarks of T_H1 polarization: the release of IL-12 (Fig. 5A) and the production of IFN- γ (Fig. 5C) in co-culture experiments. As shown, both indicators were induced in a comparable manner to *S. enterica* LPS and the whole bacteria preparation of *A. lwoffii* F78. To evaluate the purity of the isolated LPS, we also

performed all experiments in the presence of the TLR4/lipid A antagonist compound 406. Both the activation of DCs (IL-8 release) and the T_H1-polarizing effects were blocked under these conditions (data not shown).

In conclusion, we could show that the potential of the Gram-negative bacterium *A. lwoffii* F78 to induce T_H1 polarization was mediated by the LPS and signaling through TLR4.

Discussion

Recently, we showed that the reduced risk of farm children to develop allergies later in life might be because of the micro-organisms in this environment (21). We have isolated and characterized two bacteria that are able to protect mice in an allergy model. In the present study, we further characterized one of the cowshed strains, namely *A. lwoffii* F78, and present evidence that the underlying

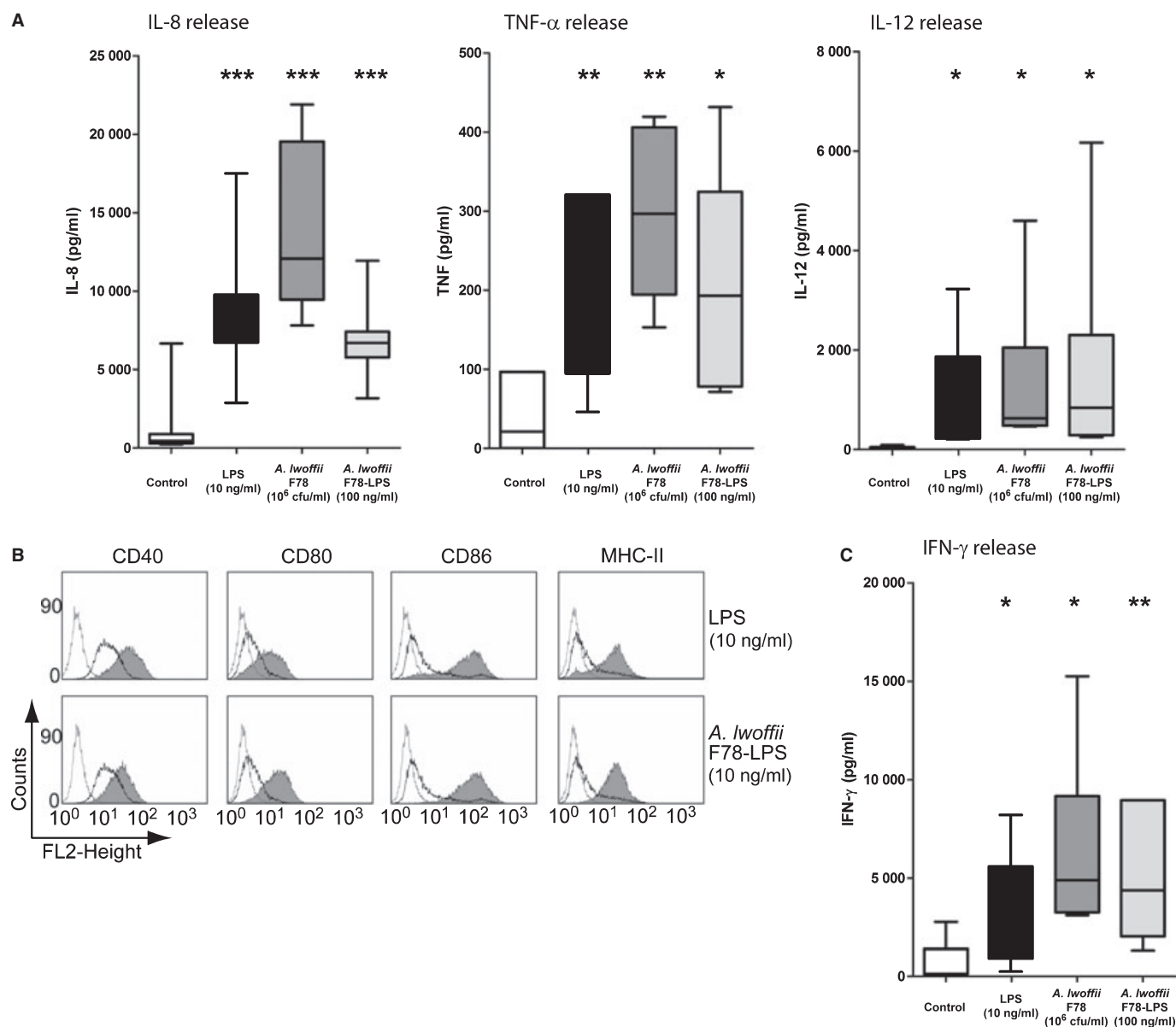


Figure 5. Lipopolysaccharide (LPS) isolated from *Acinetobacter lwoffii* F78 is responsible for the T_H1 polarization. Human moDCs were stimulated with indicated stimuli. (A) After 16 h, the concentration of IL-8, TNF- α , and IL-12 was measured in the supernatant. Group data are presented from at least 6 independent experiments with moDCs from different healthy donors. (B) Analysis of CD40, CD80, CD86, and MHC class II expression in human moDCs after treatment (shaded histograms) with indicated stimuli for 24 h is shown. Gray open histograms indicate the isotype control, and black open histograms indicate untreated moDCs. Presented data are representative for three experiments. (C) After 16 h of stimulation, DCs were co-cultured with autologous naïve T cells and were restimulated after 5 days. The IFN- γ release was measured in the supernatant after additional 20 h. Group data are presented from at least 9 independent experiments with moDCs from different healthy donors. (A + C) Data are plotted as boxes with whiskers representing min to max values. Statistical analysis was performed by paired *t*-test using one-tailed *P*-values. Significant differences to control cells are indicated by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.005$).

mechanism might be the strong induction of T_H1 -type immune response by *A. lwoffii* F78, and that the LPS from this bacterium is responsible for the bacteria-induced effects.

As dendritic cells are key players in the modulation of the immune system and are also known to be important

in the development of allergies (17, 29), we investigated the polarizing capacities of *A. lwoffii* F78-stimulated DCs on naïve T cells. Consistent with our first study (21), where we detected the induction of a T_H1 -polarizing program in DCs (IL-12 release and T_H1 -polarizing Notch ligand expression), we showed that a number of

T_H1 -polarizing genes (e.g. ICAM-1 and IFN) are induced in DCs by *A. lwoffii* F78, and that these DCs are capable of inducing T_H1 differentiation (IFN- γ release). Here, the induction of the costimulatory molecule CD40 in combination with the high amount of IL-12 induced by *A. lwoffii* F78 might be important, because CD40 on one hand positively regulates the IL-12 production after ligation with CD40L and IL-12 and on the other hand activates, via the IL-12 receptor on T cells, the transcription factor STAT4 which in turn leads to high IFN- γ production. Further on, we were interested whether also the ratio of the transcription factors TBet and GATA3 was altered by the bacteria as it is known that these transcription factors are associated with T_H1 and T_H2 cells, respectively (30, 31). We proved that *A. lwoffii* F78-treated DCs positively regulate the TBet/GATA3 ratio in naïve T cells and as a result led to a more T_H1 -driven immune response. In the last few years, a number of studies described an altered or imbalanced TBet/GATA3 ratio with low levels of TBet and high levels of GATA3 in patients with asthma (32). Therefore, the increased TBet/GATA3 ratio by *A. lwoffii* F78 might be one of the mechanisms in allergy protection by the farming environment and could partly explain the hygiene hypothesis. This would proof the theory of "lacking immune deviation" (33) as an explanation for the hygiene hypothesis and is supported by a number of studies which showed a lack of T_H1 differentiation in allergic patients and allergy-protective effects by the induction of T_H1 responses (12, 16). However, in the last few years, a number of studies came up reporting positive effects of regulatory T cells which led to the idea of 'lacking immune suppression' as an explanation for the hygiene hypothesis (15, 34). We therefore tested the induction of regulatory parameters such as IL-10 and TGF- β , but we did not detect any significant gene expression upon stimulation with *A. lwoffii* F78. Also, the high amounts of induced IL-12 argued against regulatory mechanisms, because it is known that IL-10 counteracts the production of IL-12. In contrast to our data, Peters et al. (35) showed allergy-protective effects of dust samples from cowsheds which seemed to be the result of immune suppression and regulatory mechanisms. Therefore, we suggest that both mechanisms contribute to allergy protection by the farming environment and both might be important in the modulation or conditioning of the children's immune responses. For that reason, in future studies, we plan to investigate the importance of regulatory T cells *in vivo* with respect to the protective effects of *A. lwoffii* F78.

Another aim of the present study was to characterize and identify the cell wall components or other structures from *A. lwoffii* F78 which are responsible for the

protective effects. In an overexpression system with HEK293 cells, we detected a number of possible ligands for different pattern recognition receptors. However, with different blocking experiments, we showed that the ligands for TLR2, NOD1, and NOD2 seemed not to be important for the activation and polarization of human dendritic cells. In contrast, the TLR4 antagonist C406 completely blocked the activation of the cells by *A. lwoffii* F78. Therefore, we also tested the isolated LPS from *A. lwoffii* F78 for its capacity to activate and polarize DCs and to induce T_H1 differentiation and detected a similar pattern of activation and polarization as with the whole bacteria. Hence, the LPS of *A. lwoffii* F78 seemed not only to be responsible but also sufficient for the observed protective effects. That TLR4 is important in the development of allergic diseases was shown by an epidemiological study where an association between a TLR4 polymorphism and lower incidence for atopic diseases was found (36). Furthermore, a number of studies showed T_H1 -polarizing and allergy-protective properties for endotoxin (37), but this was clearly dependent on concentration, time of treatment, and biological activity of the used LPS. For example, in an ongoing allergic inflammation, LPS rather activates the inflammation than attenuates it. Therefore, *in vivo* studies with the isolated LPS from *A. lwoffii* F78 are subject of our ongoing studies, especially with respect to the concentrations of LPS necessary for the protective effects, as Peters et al. (35) could show that *E. coli* LPS had apparently no protective properties in concentrations comparable to a farming environment. However, the *A. lwoffii* F78 LPS might be structurally different to the *E. coli* LPS used in the study of Peters et al. Also, synergistic effects with components of other bacteria (e.g., NOD2 ligands) might occur *in vivo* which would also reduce the needed concentrations of the single structures. In conclusion, our data strongly indicate that the allergy protecting effects of *A. lwoffii* F78 are attributed to the activation of a T_H1 -polarizing program in human dendritic cells, and that the LPS of *A. lwoffii* F78 is the responsible molecule triggering these beneficial effects.

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