

***Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties**

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Background: Children who grow up in a farming environment show lower levels of atopic sensitization, hay fever, and asthma than children of the same age not living in such an environment. A number of investigations provided good evidence that this is due to an early-life contact with cowsheds, farm animals, and/or consumption of products like raw milk. Also, it had been indicated that microorganisms might have an important effect on the development of allergies, and thus the question arose of which farm microbial organisms, their products, or both might induce or influence allergy-protective mechanisms.

Objective: We sought to gain further insight into the potential allergy-protective properties of microbes isolated from the farming environment.

Methods: Of a number of bacterial species identified in cowsheds of farms, 2 were selected, isolated, and characterized, namely *Acinetobacter lwoffii* F78 and *Lactococcus lactis* G121. The isolates were investigated with regard to their activation of pattern-recognition receptors, the maturation of human monocyte-derived dendritic cells, the upregulation of

inflammatory cytokines, the T_H1-polarizing Notch ligand expression, and their influence on the allergic phenotype.

Results: It is shown that both bacterial isolates were able to reduce allergic reactions in mice, to activate mammalian cells *in vitro*, and to induce a T_H1-polarizing program in dendritic cells.

Conclusion: Our data strongly support the hygiene hypothesis, which states that an environment rich in microbiologic structures, such as a farming environment, might protect against the development of allergies.

Clinical implications: This work provides the first data on a potential application of cowshed bacteria in allergy protection. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Hygiene hypothesis, *Acinetobacter lwoffii*, *Lactococcus lactis*, cowshed, allergy protection, ovalbumin mouse model

Children who grow up in a farming environment are showing lower levels of atopic sensitization, hay fever, and asthma than children of the same age living in the same village but not in a farming environment.¹ There is good evidence that an early-life contact with cowsheds, farm animals, and/or consumption of products like raw milk might protect against the development of allergic diseases later in life.² Peters et al³ showed that inhalation of an extract obtained from dust collected in farm cowsheds protected mice against the development of allergic reactions. The same extract has also been shown to influence human dendritic cell (DC) functions *in vitro*. Although the extract is not yet fully characterized, the study indicates that the allergy-protecting effect of farming environment might be directly related to inhalation of cowshed dust, which contains a variety of microbial factors.

The exposure to microbes and their products in early childhood might be responsible for shifting the T_H lymphocyte balance from T_H2 domination in newborns to T_H1 domination later in life.¹ T_H2 cells are known to be responsible for evoking allergic reactions. It has been shown that some bacterial infections might exacerbate asthma.⁴⁻⁶ However, microorganisms colonizing human intestines are necessary for a correct development of the immune system^{7,8} and play an important role in preventing pulmonary allergic responses as well.⁹ These observations indicate that microorganisms might have an

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Abbreviations used

BAL:	Bronchoalveolar lavage
DC:	Dendritic cell
HPRT:	Hypoxanthine-guanine phosphoribosyltransferase
moDC:	Monocyte-derived dendritic cell
NOD:	Nucleotide-binding oligomerization domain
OVA:	Ovalbumin
PAMP:	Pathogen-associated molecular pattern
PE:	Phycoerythrin
TLR:	Toll-like receptor

important influence on the development of allergies. Because it is also known that contact with farm livestock can modulate the allergy-protective effect of a farming environment,¹⁰ it is an intriguing question as to which microbial organisms, their products, or both typical for farms and their surroundings might induce or influence strong allergy-protective mechanisms. In pilot experiments we have identified a number of bacterial species occurring in cowsheds of farms included in the German arm of the Allergy and Endotoxin study,¹ antibodies against which were found in sera of children living on these farms. For further basic investigations concerning the potential allergy-protective properties of microbes isolated from the farming environment, we have chosen 2 isolates because of their relative abundance in cowshed microflora, namely *Acinetobacter twoffii* F78 (Gram-negative) and *Lactococcus lactis* G121 (Gram-positive). Here we report on the isolation and characterization of these 2 bacterial species and show that both were able to reduce allergic reactions in mice, to activate mammalian cells *in vitro*, and to induce a T_H1-polarizing program in DCs. Therefore it is postulated that both strains might play a role in allergy protection.

METHODS

Bacteria

For growth conditions, 16S rDNA analyses, and strain characterization, see the [Methods](#) section in the Online Repository at www.jacionline.org.

Transfection of HEK293 cells

HEK293 cells were transfected with the expression plasmids, as described previously.¹¹ After 24 hours of transfection, cells were washed and stimulated for 18 hours with live *L. lactis* G121 and live *A. twoffii* F78. Finally, supernatants were collected, and the IL-8 content was quantified by using a commercial ELISA (Biosource, Solingen, Germany).

Generation and stimulation of human monocyte-derived DCs

Heparinized blood of healthy donors was prepared by using the Ficoll (PAA Laboratories GmbH, Pasching, Austria) separation method,¹² and PBMCs were harvested. Subsequently, monocytes were isolated by means of counterflow elutriation centrifugation.¹³ Monocyte-derived DCs (moDCs) were then prepared as described by Sallusto and Lanzavecchia.¹⁴ In brief, monocytes (>95% purity)

were cultured at 10⁶ cells/mL in RPMI 1640 (Cambrex, East Rutherford, NJ) supplemented with 10% heat-inactivated FCS (Biochrom AG, Berlin, Germany), 100 U/mL penicillin (PAA Laboratories GmbH), 100 µg/mL streptomycin (PAA Laboratories GmbH), 500 U/mL GM-CSF (Strathmann Biotec GmbH, Hamburg, Germany), and 500 U/mL IL-4 (Strathmann Biotec GmbH), exchanging half of the medium, including cytokines, every 2 to 3 days. At day 7, cells were harvested and recultured in complete medium at a density of 10⁶ cells/mL in 24-well plates. Cells were either left untreated or stimulated with 10 ng/mL LPS from *Salmonella enterica* sv. Friedenau and the indicated concentration of live *L. lactis* G121 and *A. twoffii* F78.

Expression of costimulatory molecules on human moDCs

After 24 hours of incubation with the indicated stimuli, cells were harvested and stained with fluorochrome-conjugated mAbs for CD40, CD80, CD86, or MHC class II and were fixed with 1.5% paraformaldehyde. The mAb CD40-phycoerythrin (PE) and CD80-PE, MHC class II-PE, IgG1-PE and IgG2a-PE were purchased from Beckman Coulter (Fullerton, Calif), and the CD86-PE mAb was obtained from BD PharMingen (San Diego, Calif). Cells were analyzed by means of flow cytometry on a FACSCalibur by using the CellQuest program (Becton Dickinson, San Jose, Calif). Data analysis was performed with the WinMDI 2.8 software developed by Joseph Trotter (Scripps Institute, La Jolla, Calif).

Real-time quantitative RT-PCR

Total RNA was extracted from moDCs after 3, 6, and 12 hours of incubation with the indicated stimuli by using the Absolutely RNA kit from Stratagene (La Jolla, Calif) and reverse transcribed into cDNA by using Oligo(dT)₁₂₋₁₈ and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif). Transcripts were quantified by means of real-time quantitative PCR on LightCycler 2.0 with the FastStart DNA MasterPLUS SYBR Green Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. For each sample, specific mRNA abundance was normalized to the amount of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA and was expressed in arbitrary units (for primers used, see [Table E1](#) in the Online Repository at www.jacionline.org).

Measurement of cytokines

Supernatants of moDCs were harvested after 24 hours of incubation with the indicated stimuli. Cytokine concentrations in the supernatants were analyzed by using an ELISA specific for human IL-12p70 (eBioscience, San Diego, Calif), according to the manufacturer's instructions.

Animals

Pathogen-free 6- to 8-week-old female BALB/c mice were obtained from Harlan Winkelmann (Borchen, Germany). Mice were kept under specific pathogen-free housing conditions. Water and ovalbumin (OVA)-free diet were supplied *ad libitum*. All animal experiments were performed in accordance with German and international guidelines and were approved by local authorities (Regierungspraesidium Giessen).

Sensitization, treatment, and local allergen provocation

Mice were sensitized to OVA by means of 3 intraperitoneal injections of 10 µg of OVA grade VI (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg Al(OH)₃ (Pierce, Rockford, Ill) diluted in 200 µL of PBS on days 0, 7, and 14. An allergen challenge was performed on 3 consecutive days (days 19, 20, and 21) by means

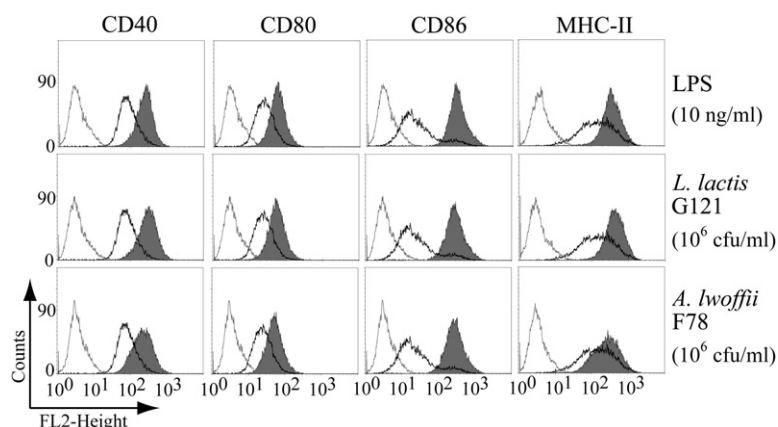


FIG 1. Both bacteria induce upregulation of costimulatory molecules in human moDCs. Analysis of CD40, CD80, CD86, and MHC class II expression in human moDCs after treatment (shaded histograms) with indicated stimuli for 24 hours is shown. Gray open histograms indicate the isotype control, and black open histograms indicate untreated moDCs. Data are representative for 3 independent experiments with moDCs from different healthy donors.

of application of an OVA aerosol (1% wt/vol OVA [grade V, Sigma] in PBS) through the airways for 20 minutes each day, as previously described,¹⁵ to induce a local inflammatory response in the lungs. As a negative control, groups of mice received PBS instead of OVA during sensitization and challenge.

As treatment, anesthetized mice received intranasally 10^8 cfu lyophilized bacteria of *L. lactis* G121 or *A. lwoffii* F78, respectively, or PBS as a control 3 times per week starting 10 days before the first sensitization over the whole sensitization and challenge process (see also Fig E1 in the Online Repository at www.jacionline.org).

Analysis of OVA-specific immunoglobulin titers

Blood samples were taken 1 day after the last challenge, and serum concentrations of OVA-specific IgE, IgG1, and IgG2a were determined by means of ELISA, as described previously.¹⁶

Differential cell counts in bronchoalveolar lavage fluid

Forty-eight hours after the last allergen challenge, bronchoalveolar lavage (BAL) was performed, as previously described.¹⁷ Total leukocyte counts were determined with an automated Casy TT cell counter (Schaerfe Systems, Reutlingen, Germany). BAL cells were placed in a cytospin at 300g and differentially stained with Diff-Quick (DADE Diagnostics, Unterschleissheim, Germany) after fixation with methanol. BAL cell differentiation was performed by using light microscopy with standard morphologic criteria. Differential cell counts of 100 leukocytes were performed in duplicates. Remaining BAL fluids were centrifuged at 300g, and cell-free supernatants were stored at -20°C for cytokine analysis.

Lung histology

Lungs were fixed *ex situ* by means of intratracheal instillation of a 4% (wt/vol) formaldehyde solution and were stored in 4% formaldehyde. Lung tissues were embedded into paraffin, and 3- μm sections were stained with hematoxylin and eosin or periodic acid-Schiff. For Congo Red staining, sections were stained for 20 minutes in a 0.5% Congo Red (Sigma) solution in 50% aqueous ethanol. After rinsing with aqua dest, slides were 10 times dipped in alkaline ethanol, washed with water, and finally counterstained with Weigerts hematoxylin (Merck, Darmstadt, Germany).

Analysis of airway reactivity

Noninvasive measurement of midexpiratory airflow in response to increasing concentrations of β -methacholine was performed by using head-out body plethysmography, as previously described.¹⁵ The methacholine concentration that caused a 50% reduction in baseline midexpiratory airflow is reported.

Statistics

Numeric data are presented as mean values $\pm$ SD. One-way ANOVA or the Student unpaired *t* test were performed to determine the level of significance between the different animal groups. Differences were considered significant at a *P* value of less than .05.

RESULTS

Activation of pattern-recognition receptors by cowshed bacteria

To define the interaction of the bacterial strains with innate immune cells, we first investigated in a HEK293 cell system which pattern-recognition receptors are involved in the recognition of the isolated bacteria (see Table E2 in the Online Repository at www.jacionline.org). No activity could be detected in HEK293 cells transfected with either Toll-like receptor (TLR) 3, 5, 7, 8, or 9. Although *L. lactis* G121 activated the cells through nucleotide-binding oligomerization domain2 and TLR2, *A. lwoffii* F78 activated the cells through TLR2, TLR4, NOD1, and NOD2.

Maturation of human moDCs

Because both isolated bacteria preparations provided innate immune receptor agonists, we investigated whether stimulation of moDCs with the bacteria leads to maturation (Fig 1). Both *L. lactis* G121 and *A. lwoffii* F78, as well as the positive control LPS, were able to induce maturation, which is clearly seen in the upregulation of CD40 and CD86. Furthermore, moDCs stimulated with the bacteria showed a higher expression of the antigen-presenting

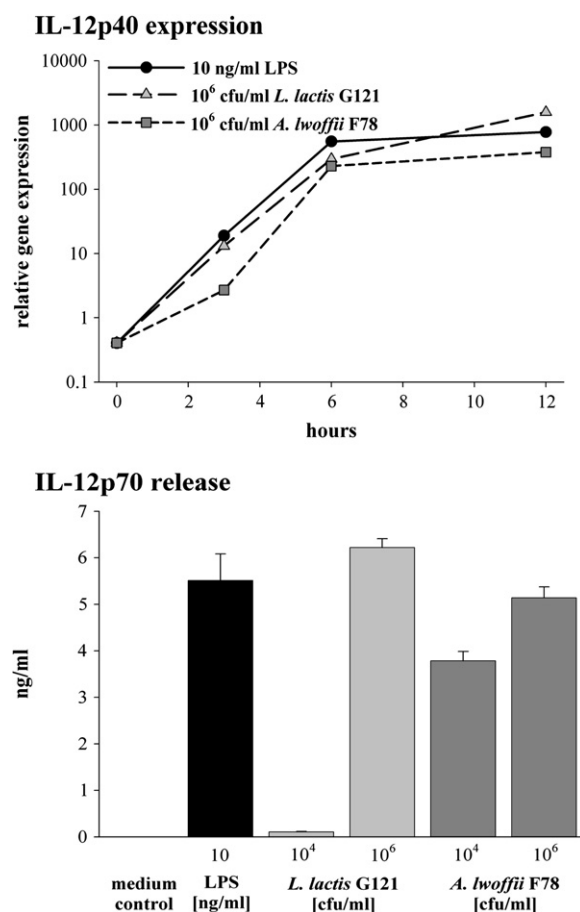


FIG 2. Bacteria-induced IL-12 production in human moDCs. Human moDCs were stimulated with the indicated stimuli. IL-12p40 mRNA relative to HPRT was determined by means of quantitative real-time RT-PCR. The release of IL-12p70 was measured after 24 hours of incubation in culture supernatants. Data are representative of 3 independent experiments with moDCs from different healthy donors.

MHC class II molecules. However, the Gram-negative *A. lwoffii* F78 induced upregulation of the costimulatory molecules at very low concentrations (approximately 10^2 – 10^3 cfu/mL), whereas the Gram-positive *L. lactis* G121 was active only at higher concentrations (approximately 10^4 cfu/mL, data not shown).

Upregulation of inflammatory cytokines

We investigated the expression and release of inflammatory cytokines in DCs after treatment with the bacteria because the pattern of cytokine release is, among other signals, important for the T_H cell polarization.^{18,19} We analyzed the expression of the T_H1-polarizing cytokine IL-12,^{20–22} which is a heterodimeric cytokine consisting of the 2 subunits p35 and p40.²³ The mRNA levels for IL-12p40 were upregulated in the bacteria-treated moDCs in a similar manner as in the LPS-stimulated DCs (Fig 2, upper panel). As expected, these upregulated mRNA levels resulted in the release of the cytokine (Fig 2, lower panel). Again, comparable with the upregulation of costimulatory molecules, *A. lwoffii* F78 activated the cells at lower concentrations than *L. lactis* G121.

T_H1-polarizing Notch ligand expression

To further investigate the capacities of the bacteria to modulate the T_H cell polarization, we examined the expression of the Notch ligands on DCs after stimulation with the bacteria. Two groups of Notch ligands are expressed by DCs, and the ratio is important for T-cell polarization: the Delta group directs T-cell polarization toward T_H1, whereas the Jagged group induces T_H2 responses.^{24,25} We could show that *L. lactis* G121 and *A. lwoffii* F78 upregulated the expression of Delta-4 mRNA, whereas the mRNA levels of Jagged-1 were strongly downregulated after 24 hours (Fig 3). Taken together, both bacteria induced a T_H1-polarizing Notch ligand expression on DCs.

Influence of *A. lwoffii* F78 and *L. lactis* G121 application on the allergic phenotype

Because our data on human DCs strongly supported the idea that particular cowshed bacteria are able to modulate allergic reactions, we tested this hypothesis in an established mouse allergy model. To mimic the most probable means of exposure, we treated mice intranasally every second day with 10^8 cfu of the respective bacteria starting 10 days before sensitization and continuing over the whole sensitization and challenge process. Interestingly, despite the repeated local treatment with lyophilized bacteria, only a very weak inflammatory response was induced, as shown by infiltration of quite low numbers of neutrophils and lymphocytes (Fig 4). In sensitized animals no effects were observed on OVA-specific IgE and IgG2a serum titers, whereas for OVA-specific IgG1 titers, a decrease was observed after treatment with both bacterial strains (see Table E3 in the Online Repository at www.jacionline.org). However, both bacterial preparations were able to markedly suppress the local allergic inflammatory reaction. This could be demonstrated by a significant decrease in the number of eosinophils in the BAL fluid. In contrast, the numbers of BAL macrophages or lymphocytes were not or were only slightly influenced (Fig 4). In addition, lung histology revealed a reduction in peribronchial and perivascular inflammatory cell infiltration in animals that had received either *L. lactis* G121 or *A. lwoffii* F78 through the intranasal route, and remaining tissue infiltrates were characterized by a decreased appearance of eosinophils (Fig 5). Moreover, both treatments almost completely hindered the development of mucus-producing goblet cells, resulting in a lung histology almost comparable with that of nonallergic control mice (Fig 5). Finally, a significant improvement of allergy-induced airway hyperreactivity could be observed after local treatment with each of the bacterial preparations, as indicated by significantly higher β -methacholine concentrations necessary to induce a 50% midexpiratory airflow reduction (Fig 6).

DISCUSSION

Although there are several epidemiologic studies suggesting that exposure to a farming environment reduces the risk of development of allergic diseases later in

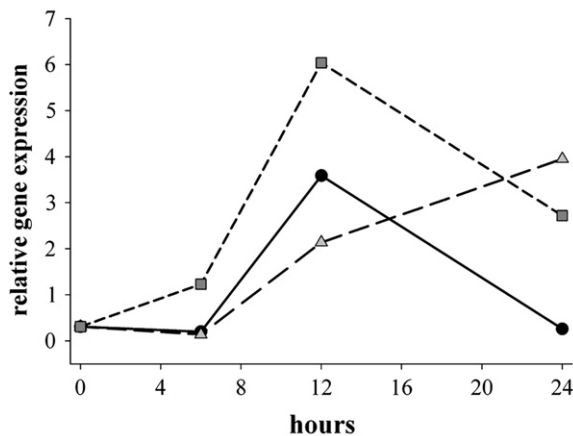
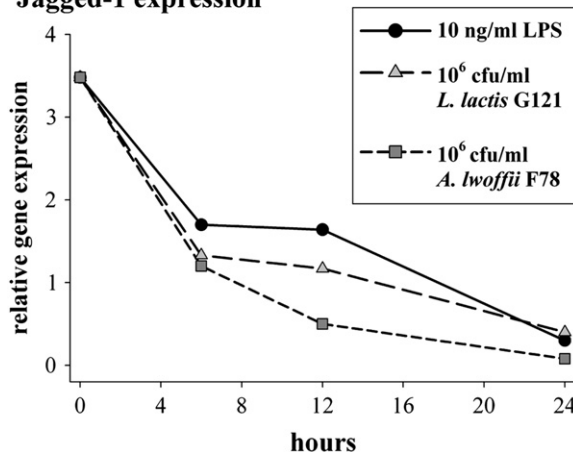
Delta-4 expression**Jagged-1 expression**

FIG 3. Both bacteria induce a T_H1 -polarizing Notch ligand expression in human moDCs. Human moDCs were stimulated for the indicated times with indicated stimuli. Delta-4 and Jagged-1 mRNA was measured by using quantitative real-time RT-PCR, and data are presented as mRNA levels of both genes relative to HPRT. Data are representative for 3 independent experiments with moDCs from different healthy donors.

life, there is little known about the underlying mechanisms.^{1,26} In the present study we provide evidence that bacteria isolated from farm cowsheds are able to protect mice against the development of an allergic airway inflammation that might be due to the T_H1 -promoting effects of these bacteria. We have chosen *L. lactis* G121 and *A. lwoffii* F78 from a number of identified bacterial species of Bavarian farms because of their relative abundance in cowshed microflora.

Because the innate immune system plays an essential role in the initial immune activation and modulates subsequent adaptive immune responses,²⁷ we investigated which pattern-recognition receptors are involved in the recognition of the bacteria. As expected, preparations of the Gram-negative bacteria *A. lwoffii* F78 activated cells through TLR2 and TLR4, as well as NOD1 and NOD2, whereas the Gram-positive *L. lactis* G121 preparation

signaled through NOD2 and TLR2. DCs express a wide range of these pattern-recognition receptors and are involved in the communication between the innate and the adaptive immune systems.²⁸ Therefore we asked whether *L. lactis* G121 and *A. lwoffii* F78 are able to activate DCs. Immature DCs upregulate costimulatory molecules, including CD40, CD80, and CD86, after stimulation through their innate immune receptors in a maturation process.²⁹⁻³¹ These molecules help to activate T cells in the context of antigen presentation by MHC class II and thereby become able to prime T_H cells toward T_H1 or T_H2 , depending on the stimulus.³⁰ Activation of DCs with *L. lactis* G121, as well as with *A. lwoffii* F78, resulted in maturation of the DCs. In addition to the maturation, DCs express after stimulation a stimulus-dependent pattern of cytokines and cell-surface markers that determine the T_H1 - or T_H2 -promoting capacities. One of the most prominent cytokines released by DCs is the proinflammatory cytokine IL-12, which plays a central role in T_H1 responses by inducing the production of IFN- γ , thus favoring the differentiation of T_H1 cells.^{21,22,32} Both bacterial strains we have isolated from the cowsheds were able to induce the expression and release of IL-12 and thereby promote a T_H1 response. In addition to IL-12, contact-dependent mechanisms might also contribute to T_H cell polarization. One of these mechanisms is the expression profile of Notch ligands.³³ Recently, Napolitani et al³⁴ have shown an altered level of Notch ligand expression in T_H1 -promoting DCs with a high level of Delta-4 and a decreased expression of Jagged-1. *L. lactis* G121 and *A. lwoffii* F78 were able to decrease the level of Jagged-1 mRNA and upregulated Delta-4 mRNA, leading to an increased level of T_H1 -promoting and a reduced level of T_H2 -promoting Notch ligands on the cell surface of the DCs. This altered ratio is important for the capacity of DCs to polarize either T_H1 or T_H2 responses, and with respect to the bacterial preparations from cowsheds, both induce a shift in a T_H1 -polarizing direction. Together, bacteria-induced matured DCs might be key regulator cells for the protective effect of the farming environment because they promote a T_H1 phenotype in contrast to the T_H2 phenotype in allergic subjects^{35,36} and thereby might contribute to the establishment of a normal T_H1/T_H2 balance in early childhood.

Because a shift from T_H2 toward T_H1 reactions in response to a specific allergen has been shown to prevent the development of allergies, we asked whether *in vivo* application of the cowshed-derived bacterial preparations would be able to modulate allergic inflammatory immune reactions in a mouse model of experimental allergic asthma. It was of special interest that repeated intranasal application of both bacterial preparations, either Gram-positive *L. lactis* G121 or Gram-negative *A. lwoffii* F78, resulted in a significantly reduced allergic airway inflammation and improvement of airway responsiveness, despite the fact that the systemic allergic sensitization, as shown by OVA-specific IgE levels, was not influenced. This observation indicates that the immunomodulating effects are limited to T-lymphocyte activities, that local effector

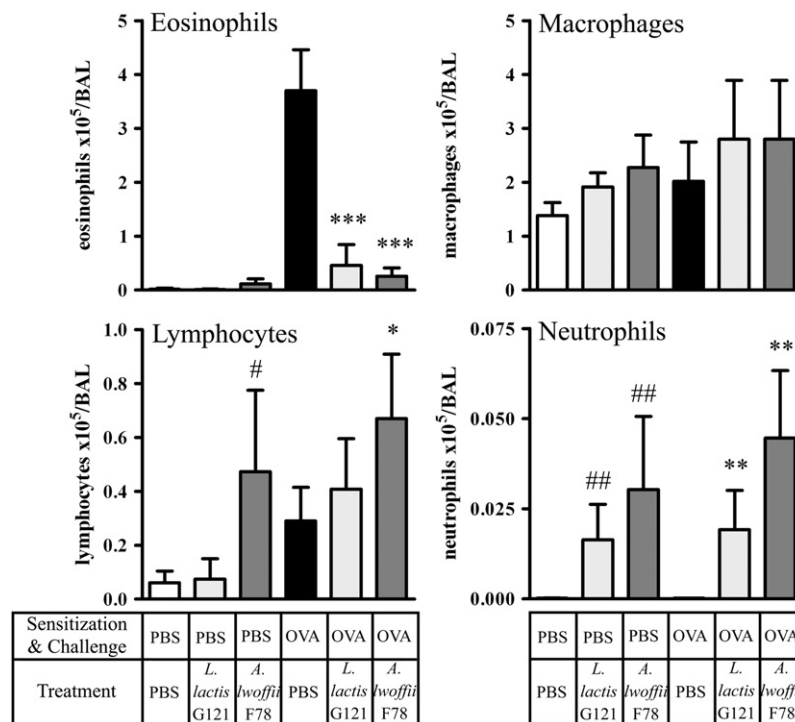


FIG 4. Inhibition of eosinophilic infiltration by means of intranasal treatment of mice with *L. lactis* G121 and *A. lwoffii* F78. Groups of mice ($n = 6$) were intranasally treated with PBS, *L. lactis* G121, or *A. lwoffii* F78 and were partially sensitized and challenged to OVA. Differential cell numbers were analyzed in the BAL fluid. #Significant differences from the nonsensitized control group; *significant differences from the sensitized PBS-treated group: $P < .05$ (#, *), $P < .01$ (##, **), or $P < .001$ (***).

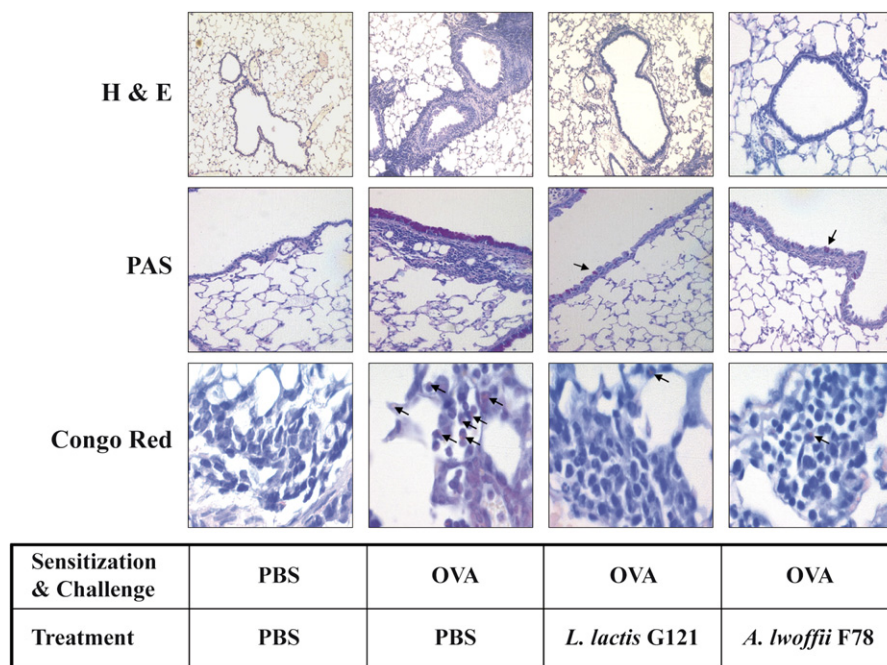


FIG 5. Intranasal treatment with *L. lactis* G121 and *A. lwoffii* F78 inhibits development of airway inflammation and goblet cell hyperplasia. Mice were treated as described in Fig 4. Shown are representative microphotographs of airways from mice after hematoxylin and eosin staining, periodic acid–Schiff staining of goblet cells (arrows point to individual goblet cells), and Congo Red staining of eosinophils (arrows).

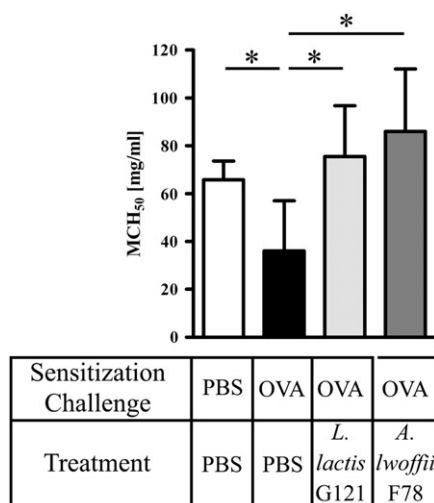


FIG 6. Intranasal treatment with *L. lactis* G121 and *A. lwoffii* F78 improves airway reactivity. Groups of mice ($n = 8$) were treated as described in Fig 4, and lung function was analyzed by using head-out body plethysmography. Presented are means $\pm$ SDs of the methacholine concentration that caused a 50% reduction in baseline midexpiratory airflow (MCH_{50}). *Significance with a P value of less than .05.

mechanisms of the allergic immune response are directly influenced by bacterial components, or both. In contrast to our findings, intranasal application of LPS only during allergen challenge resulted in an exacerbation of the allergic inflammatory response,³⁷ which was, however, not the case when LPS treatment started during sensitization.³⁸ Similar results were also obtained after *Mycoplasma pneumoniae* infection, which reduced the allergic phenotype when occurring 3 days before allergic sensitization and augmented the allergic reaction when occurring after the last allergen challenge.³⁹ Therefore either the duration of accessibility or a certain time window before allergen challenge determines whether bacterial components provide allergy-protective effects.

Taken together, our data support the concept by Herrick and Bottomly⁴⁰ that maturation of DCs in the presence of pathogen-associated molecular patterns (PAMPs) leads to a balanced development of T_H1 and T_H2 activities. In contrast, allergens themselves induce DC activation without IL-12 production, which then induces the development of T_H2 responses. However, concomitant signals of PAMPs and allergens or the preactivation of local DCs by PAMPs favors the development of T_H1 responses that might control T_H2 -driven allergic immune responses.^{37,41,42} Interestingly, only negligible signs of a T_H1 -driven inflammatory response, minimal influx of neutrophils and lymphocytes, were observed after repeated intranasal treatment of the mice (either naive or OVA sensitized) with rather high doses of the bacterial preparations, suggesting that the chosen protocol induced tolerance against these nonpathogenic microorganisms. In addition, this low inflammatory response argues for the safety of novel immunotherapeutic strategies to induce immune deviation.

In conclusion, our data strongly support the hygiene hypothesis, which states that an environment rich in microbiologic structures, such as a farming environment, might protect against the development of allergies, even if the presence of high amounts of allergens argues against the allergen hypothesis.⁴³ Certain microbes, such as those present in the environments of children living in rural or less-developed areas, act by promoting an immune deviation from T_H2 to T_H1 in the allergen-specific response rather than by stimulating T regulatory cell activity. We propose that the lack of immune deviation rather than the reduced immune regulation is the major immunologic mechanism explaining the increased prevalence of allergy based on the hygiene hypothesis.

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REFERENCES

- Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;358:1129-33.
- von Mutius E. Paediatric origins of adult lung disease. *Thorax* 2001;56:153-7.
- Peters M, Kauth M, Schwarze J, Körner-Rettberg C, Riedler J, Nowak D, et al. Inhalation of stable-dust extract prevents allergen-induced airway inflammation and hyperresponsiveness. *Thorax* 2006;61:134-9.
- Kraft M, Cassell GH, Pak J, Martin RJ. *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in asthma: effect of clarithromycin. *Chest* 2002;121:1782-8.
- Park SJ, Lee YC, Rhee YK, Lee HB. Seroprevalence of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in stable asthma and chronic obstructive pulmonary disease. *J Korean Med Sci* 2005;20:225-8.
- Yasuda H, Suzuki T, Zayasu K, Ishizuka S, Kubo H, Sasaki T, et al. Inflammatory and bronchospastic factors in asthma exacerbations caused by upper respiratory tract infections. *Tohoku J Exp Med* 2005;207:109-18.
- Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 2004;4:478-85.
- Rastall RA. Bacteria in the gut: friends and foes and how to alter the balance. *J Nutr* 2004;134(suppl):2022S-6S.
- Noverr MC, Noggle RM, Toews GB, Huffnagle GB. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect Immun* 2004;72:4996-5003.
- Kabesch M, Lauener RP. Why Old McDonald had a farm but no allergies: genes, environments, and the hygiene hypothesis. *J Leukoc Biol* 2004;75:383-7.
- Zähringer U, Lindner B, Knirel YA, van den Akker WM, Hiestand R, Heine H, et al. Structure and biological activity of the short-chain lipopolysaccharide from *Bartonella henselae* ATCC 49882T. *J Biol Chem* 2004;279:21046-54.
- Boyum A. Separation of white blood cells. *Nature* 1964;204:793-4.
- Turpin J, Hester JP, Hersh EM, Lopez-Berestein G. Centrifugal elutriation as a method for isolation of large numbers of functionally intact human peripheral blood monocytes. *J Clin Apheresis* 1986;3:111-8.
- Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994;179:1109-18.

15. Wegmann M, Fehrenbach H, Fehrenbach A, Held T, Schramm C, Garn H, et al. Involvement of distal airways in a chronic model of experimental asthma. *Clin Exp Allergy* 2005;35:1263-71.
16. Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992;89:1127-38.
17. Neuhaus-Steinmetz U, Glaab T, Daser A, Braun A, Lommatzsch M, Herz U, et al. Sequential development of airway hyperresponsiveness and acute airway obstruction in a mouse model of allergic inflammation. *Int Arch Allergy Immunol* 2000;121:57-67.
18. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 1999;20:561-7.
19. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* 2003;3:984-93.
20. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;3:133-46.
21. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993;260:547-9.
22. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 1995;154:5071-9.
23. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* 1989;170:827-45.
24. Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H, et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 2003;19:549-59.
25. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 2004;117:515-26.
26. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;347:869-77.
27. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-80.
28. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
29. Cayabyab M, Phillips JH, Lanier LL. CD40 preferentially costimulates activation of CD4+ T lymphocytes. *J Immunol* 1994;152:1523-31.
30. Somoza C, Lanier LL. T-cell costimulation via CD28-CD80/CD86 and CD40-CD40 ligand interactions. *Res Immunol* 1995;146:171-6.
31. Borriello F, Sethna MP, Boyd SD, Schweitzer AN, Tivol EA, Jacoby D, et al. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 1997;6:303-13.
32. Manetti R, Parronchi P, Giudizi MG, Piccinini MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;177:1199-204.
33. Rutz S, Mordmuller B, Sakano S, Scheffold A. Notch ligands Delta-like1, Delta-like4 and Jagged1 differentially regulate activation of peripheral T helper cells. *Eur J Immunol* 2005;35:2443-51.
34. Napolitani G, Rinaldi A, Berton F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005;6:769-76.
35. Romagnani S. The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000;105:399-408.
36. Kon OM, Kay AB. T cells and chronic asthma. *Int Arch Allergy Immunol* 1999;118:133-5.
37. Delayre-Orthez C, de Blay F, Frossard N, Pons F. Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin Exp Allergy* 2004;34:1789-95.
38. Delayre-Orthez C, Becker J, de Blay F, Frossard N, Pons F. Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *Int Arch Allergy Immunol* 2005;138:298-304.
39. Chu HW, Honour JM, Rawlinson CA, Harbeck RJ, Martin RJ. Effects of respiratory *Mycoplasma pneumoniae* infection on allergen-induced bronchial hyperresponsiveness and lung inflammation in mice. *Infect Immun* 2003;71:1520-6.
40. Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. *Nat Rev Immunol* 2003;3:405-12.
41. Vandenbulcke L, Bachert C, Van Cauwenberge P, Claeys S. The innate immune system and its role in allergic disorders. *Int Arch Allergy Immunol* 2006;139:159-65.
42. Yang IA, Fong KM, Holgate ST, Holloway JW. The role of Toll-like receptors and related receptors of the innate immune system in asthma. *Curr Opin Allergy Clin Immunol* 2006;6:23-8.
43. Marks GB. Environmental factors and gene-environment interactions in the aetiology of asthma. *Clin Exp Pharmacol Physiol* 2006;33:285-9.