INVESTIGATIVE REPORT

Differential Immunomodulating Effects of Inactivated Probiotic Bacteria on the Allergic Immune Response

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Bacterial stimulation plays an important role in modulating the allergic immune response. The aim of this study was to investigate the effects of inactivated probiotic Lactobacillus acidophilus and non-pathogenic Escherichia coli strain Nissle on the phenotype and function of T- and B-cells. Peripheral blood mononuclear cells from patients with grass-pollen allergy (n=10) and non-allergic patients (n=19) were co-stimulated with inactivated bacteria and grass-pollen allergen. Expression of CD23, CD80, CD86 and CD69 were analysed, and the intracellular production of interleukin-4 and interferon-γ was measured by direct ex vivo flow cytometry after stimulation. Both bacteria induced a significant up-regulation of CD69 expression on T lymphocytes (p=0.001). CD23 expression was significantly increased following stimulation with allergen (p=0.008), but reduced after stimulation with Lactobacillus and significantly reduced with E. coli plus allergen (p=0.029). CD80 expression was reduced after stimulation with Lactobacillus in the allergic group only (p=0.021). By contrast, CD86 expression was significantly increased after stimulation with Lactobacillus (p=0.049) and distinctly increased with E. coli in both groups (p=0.001). The cytokine patterns of CD69-positive T lymphocytes from allergic patients showed a T-helper2-dominated response after allergen stimulation (interferon-γ/interleukin-4-ratio 0.2), directed into a T-helper1-like response by stimulation with both types of bacteria (interferon-γ/interleukin-4-ratios 1.5–2.0 in both groups). These data show that both types of bacteria modulate the allergic immune response by the alteration of CD23 and co-stimulatory molecule expression. Regarding cytokine production, the data suggest a differential response to both bacteria depending on the atopic state, but a clear promotion of T-helper1-dominated response in allergic donors. Key words: allergy; T-cells; Escherichia coli Nissle 1917; Lactobacillus acidophilus.

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of peripheral lymphocytes, representing systemic bacterial administration and their impact on gut-associated lymphoid tissue lymphocytes.

Previous studies have shown that a T-helper (TH2)-dominated response via the increased production of interleukin (IL)-4 can be arrested by cross-regulatory cytokines such as interferon gamma (IFNγ) (12). Focusing on the allergen-specific immune response, the ability of allergen (A) and of the above-mentioned bacterial components (LC-lactobacilli, EC-coli Nissle) to modulate the peripheral TH1/TH2 balance was investigated in adult allergic (a) and non-allergic (na) individuals. In addition, the state of activation and the expression of important co-stimulatory molecules in the presence of the bacteria was analysed by direct ex vivo flow cytometry.

MATERIALS AND METHODS

Patients

The study included 29 age- and sex-matched adult patients from the Department of Otorhinolaryngology (Bundeswehrkran-kenhaus, Berlin, Germany), who gave written consent. The study was approved by the local ethics committee. Ten allergic patients suffering from seasonal allergic rhinitis were diagnosed as being sensitized to grass pollen according to skin prick testing (ALK, Hamburg, Germany) and detection of specific IgE (Pharmacia, Hamburg, Germany) and allergen (A) and of the above-mentioned bacterial com-
ponents (Lc-lactobacilli, EC-coli Nissle) to grass pollen (CAP-class ≥2). Nineteen non-allergic, non-grass-pollen sensitized healthy individuals were included as controls. Exclusion criteria were: administration of systemic and local steroids within 14 days prior to the study; or current specific immunotherapy or other immunomo-
dulating medication. None of the patients had allergic symptoms at the time of investigation, as the study was performed during the winter season, and none had any other severe diseases.

Cell separation and culture

Heparinized blood samples were taken once from patients and control individuals. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized whole blood by Ficoll density gradient centrifugation and washed repeatedly in phosphate buffered saline (PBS) before being resuspended and cultured in RPMI 1640 medium (Sigma, Munich, Germany) supplemented with 10% foetal calf serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). PBMCs were incubated in flat-bottomed 24-well plates at 37°C in a water-saturated atmosphere containing 5% CO₂.

Stimulants

PBMCs were stimulated with grass pollen extract (500 SQ/ml, provided by ALK Scherax, Hamburg, Germany), −80°C chilled-inactivated E. coli Nissle (Mutaflor, Ardeypharm, Herdecke, Germany) at a 1:1 ratio, or L. acidophilus (Paidoflor, Ardey-
pharm) at a 2:1 ratio (13). The grass pollen extract additionally contained anti-CD28 (0.5 µg/ml, BD PharMingen, Heidelberg, Germany) for co-stimulation. As a positive control, PBMCs were incubated with staphylococcal enterotoxin B (SEB) (1 µg/ml, Sigma). The negative control contained unstimulated cells. PBMCs were collected after 18 h for surface staining and after 16 h for intracellular staining. Grass pollen extract was analysed with regard to endotoxin content, measured by the chromogenic Limulus amebocyte lysate assay (Bio Whittaker) and showed 222 pg/ml in the allergen dilution of 100,000 SQ/ml. Accordingly, 500 SQ/ml of allergen extract contains a negligible amount of endotoxin, which has no modulating effect.

Flow cytometry

The expression of surface CD20, CD23, CD80, CD86, CD4 and CD69, and of intracellular IL-4 and IFNγ were assayed by flow cytometry (all antibodies were "mouse-anti-human" from BD/PharMingen). PBMCs (1×10⁶) were incubated with the specific antibodies and the appropriate isotype controls (BD/PharMingen) for 30 min at 4°C in 100 µl staining buffer (2% bovine serum albumin in PBS, 0.002% sodium azide) following a 10-min-blocking with 5 µl Beringlobin® (Aventis-Behring, Marburg, Germany). Cells for intracellular staining were additionally incubated with Brefeldin A (1 µl/ml, Sigma) for 3 h after 13 h of incubation, and permeabilized with 500 µl FACS-permeabilizing solution (BD Bioscience, Heidelberg, Germany) before staining. Finally, the cells were washed and fixed in PBS with 2% paraformaldehyde.

Flow cytometry of 4-fold labelled cells was performed with a FACScalibur (Becton Dickenson, Heidelberg, Germany) flow cytometer and evaluated using Cell Quest software (Becton Dickenson). Lymphocytes were gated by anti-CD20 or anti-CD4 fluorescence, the percentage of the double-positive cells after deduction of the isotype control values was determined.

Preceding analysis

Dose-response experiments were initially carried out using increasing bacterial concentrations (i.e. 0.25×10⁶, 0.5×10⁶, 1×10⁶ and 2×10⁶ E. coli / L. acidophilus per 1×10⁶ cells) for stimulation. Subsequently, a time course analysis covering 6, 18, 24 and 48 h of stimulation was performed, using the determined bacterial concentration. PBMCs were stained with CD4 and CD69 Al-
lophycocyanin, respectively, CD20 and CD23, CD54 or CD86 to

Statistical analysis

Analysis of the statistical significance of within-group changes was carried out using Brunner’s analysis and the Wilcoxon test for non-parametric data. Descriptive statistical analysis, table calculations and diagrams were produced using Excel 2000 and SPSS 11.0. The values in Table I represent the mean ± standard error of the mean or ratio of means.

RESULTS

Mitochondrial activity assay

With respect to cell viability, the addition of bacteria caused a reduced mitochondrial activity strongest after stimulation with L. acidophilus (OD₄₅₀ 0.18; OD₆₅₀ 0.19) in comparison with SEB (OD 0.4) and allergen-stimu-
lated (OD, 0.29) as well as unstimulated cells (OD U 0.28). Incubation of bacteria alone did not show any effect on the mitochondrial activity measured.
Increased T-cell activation by microbacterial stimulation

First, the T-lymphocytes were investigated with regard to the state of activation via surface CD69 expression (14). A significant up-regulation of CD69 expression was detected following allergenic (median: A, 3.9%; p = 0.004; A, 2.9%; p = 0.001) and bacterial stimulation with L. acidophilus in both groups (median: LC, 5.0%; LC+A, 5.7%; p = 0.001; LC, 3.7%; LC+A, 4.4%; p = 0.001) in comparison with basal CD69 expression (median: U, 2.1%; U, 2.0%). In the presence of E. coli, CD69 expression showed a distinct up-regulation, which was highly significant with or without the addition of allergen (median: EC, 29.2%; EC+A, 36.0%; p = 0.001; EC, 23.8%; EC+A, 29.5%; p = 0.001).

Table I. Intracellular measurement of cytokine production by CD4-positive T lymphocytes in allergic (a) and non-allergic (na) individuals. Expression of interleukin (IL)-4 and interferon (IFN)γ on CD4+ T-lymphocytes, determined by flow cytometry in unstimulated cells (U) and following incubation with grass pollen allergen (A), E. coli Nissle (EC) and L. acidophilus (LC). Each value represents the mean ± SEM as a percentage of CD4+ T-lymphocytes

<table>
<thead>
<tr>
<th>CD4+</th>
<th>IL-4</th>
<th>IFNγ</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td>na</td>
</tr>
<tr>
<td>U</td>
<td>0.27±0.14</td>
<td>0.55±0.09</td>
</tr>
<tr>
<td>A</td>
<td>0.34±0.18</td>
<td>0.66±0.08</td>
</tr>
<tr>
<td>LC</td>
<td>0.65±0.28*</td>
<td>0.97±0.23*</td>
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<tr>
<td>LC+A</td>
<td>0.91±0.22**</td>
<td>1.08±0.22**</td>
</tr>
<tr>
<td>EC</td>
<td>0.17±0.14</td>
<td>0.88±0.19*</td>
</tr>
<tr>
<td>EC+A</td>
<td>0.37±0.19</td>
<td>0.91±0.20**</td>
</tr>
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*p < 0.05 compared with U; **p < 0.05 compared with U, A; ***p < 0.05 compared with U, A, EC.

Triggering of TH1-dominated T-cell responses by L. acidophilus, lactobacilli and E. coli

As the balance of TH1/TH2 response plays a central role for the development of allergic disease (15), intracellular IL-4 and IFNγ production was measured on CD4-positive T-lymphocytes by intracellular staining (Fig. 1). Stimulation with grass pollen extract led to an increase in IL-4 production (median: U, 0.27%; A, 0.34%) and, to a lesser extent in IFNγ production in the allergic group (median: U, 0.1%; A, 0.16%).

Analysis of the IFNγ/IL-4 ratio of allergen-specific T-lymphocytes is presented in Fig. 2 and shows a TH2-dominated response after allergen exposure, especially in the allergic group (CD69+ IFNγ/IL-4-ratio, 0.2; 0.75). In contrast to these findings, TH1-dominated responses were observed following the incubation with L. acidophilus in both groups (CD69+ IFNγ/IL-4-ratio, 0.67; 0.84). However, stimulation with lactobacilli plus allergen resulted in a TH2-like response in allergic and non-allergic individuals (CD69+ IFNγ/IL-4-ratio, 0.67; 0.84). As seen in Table I, this may be due to a significant increase in IL-4 production (median: LC+A, 0.91%; LC+A, 1.08%, p = 0.041), whereas IFNγ production remained basically unchanged in the allergic group (median: LC, 0.48%; LC+A, 0.49%; LC, 0.87%; LC+A, 0.74%).

A significant increase in IFNγ production was present after stimulation with E. coli and allergen (median: EC+A, 0.57%; p < 0.007; EC+A, 0.80%; p < 0.001), whereas single E. coli-stimulation caused a strong up-regulation of IFNγ in non-allergic individuals only (median: EC, 0.14%; EC, 0.70%; p < 0.001, see Table I). Finally, E. coli also had little effect on the production of IL-4 in the allergic group. Non-allergic subjects showed a significant increase (median: EC, 0.17%; EC, 0.88%; p < 0.001). As shown in Fig. 2, the IFNγ/IL-4 ratio of allergen-specific T-lymphocytes reflects these findings of a TH1-dominated response following exposure to E. coli (CD69+ IFNγ/IL-4-ratio, 2.0; 1.75) as well as E. coli and allergen (CD69+ IFNγ/IL-4-ratio, 4.0; 2.66).

E. coli stimulation enhanced CD80 and CD86 expression

The importance of co-stimulatory molecules, especially the up-regulation of CD86 but not CD80 following
allergenic or IL-4 stimulation in the development of TH2-cells in patients with atopic diseases has been described repeatedly (16, 17). Our results show that grass pollen extract induced a small, but significant, increase in CD86 expression only in the allergic subjects (median: U 4.26%; A 5.6%; p = 0.025) and had no impact on the expression of CD80 on B-lymphocytes (median: U 10.6%; A 10.4%).

We then investigated the influence of the above-mentioned bacteria on the expression of co-stimulatory molecules. As shown in Fig. 3, stimulation with *L. acidophilus* reduced the percentage of B-cells positive for CD80 in the allergic group (median: lC 7.4%, p = 0.021). *E. coli* caused an up-regulation of CD80 in both groups, which was statistically significant only in the non-allergic subjects (median: EC 13%; EC 16%; p = 0.001) and similarly after stimulation with *E. coli* and allergen (median: EC+A 13%; EC+A 16%, p = 0.001).

As shown in Fig. 4, CD86-expression on B lymphocytes following bacterial stimulation showed a significant increase in both groups (median: EC 21%; EC+A 21%; EC 20.7%; EC 24.8%, p = 0.007, p = 0.001; LC 12.6%; LC 9.9%, p = 0.049, p = 0.001), except for the increase following *L. acidophilus* plus allergen, which did not reach statistical significance (median: LC+A 15%; LC+A 6.5%).

### Modulation of CD23 expression by inactivated bacteria

CD23, the IL-4-induced and IFNγ-modulated low-affinity receptor for IgE, plays a crucial role in regulating T- and B-cell interactions within the allergic response (18). Therefore we analysed the effect of bacterial and allergenic stimuli on the expression of CD23 on peripheral B-lymphocytes in allergic and non-allergic subjects. We observed higher basal expression and significantly higher up-regulation of CD23 after exposure to allergen within the allergic group (median: U 11.9%; A 15.0%, p = 0.012) than the non-allergic control (median: U 5.1%; A 5.4%, p = 0.012). *E. coli* reduced the expression of CD23 compared with allergenic stimulation (median: EC 8.2%; EC+A 5.7%, p = 0.029).

Incubation with *L. acidophilus* led to decreased expression of surface CD23 in the allergic group in
and non-pathogenic bacteria demonstrates the possible therapeutic potency especially of non-pathogenic E. coli Nissle. Although some studies indicate that human cytokine activities fail to exhibit an exclusively pro-TH1 or -TH2 pattern (19), our results show rather a reduction in one pathway.

Moreover, allergic patients showed decreased CD23 expression in the presence of bacteria, especially E. coli. These observations are closely related to the modulating effects of IL-4 and IFNγ on the expression of CD23. As previous studies describe increased CD23 expression caused by IL-4 and a decreased CD23 expression caused by IFNγ, the above-mentioned cytokine patterns following bacterial stimulation explain our findings (18). The in vivo relevance has been shown, but could not be confirmed clinically (20, 21). Increased CD23 expression in patients suffering from atopic dermatitis and allergic rhinitis has been shown previously by our group (22). Furthermore, former studies show that IFNγ inhibits CD23 mRNA and sCD23 production on human B-cells (18). sCD23, which was not measured in this study, has cytokine properties alone, but is also able to elicit strong inflammatory action by stimulating the production of TNFα and IL-1β and co-stimulates IL-2-induced INFγ production (23). Decreased shedding of sCD23 results in decreased IgE production (24).

In addition, the co-stimulatory molecules CD80 and CD86 were modulated by both types of bacteria. Non-pathogenic E. coli resulted in up-regulation of CD80 and CD86, indicating a non-specific stimulation of the B-cell system. This was consistent with an increased production of specific antibodies and proliferative activity, as described previously by Cukrowska et al. (25). By contrast, incubation with probiotic L. acidophilus led to inhibition of CD80 but up-regulation of CD86 especially in allergic individuals. Thus, our findings may support the hypothesis of CD80 functioning as a more neutral differentiation signal instead of a TH1-maintaining one and – also explaining the E. coli-derived results – of both molecules directing the development of TH1 and TH2 in a less contrasting pattern (17, 26).

**DISCUSSION**

This study investigated the immunomodulatory effects of inactivated probiotic L. acidophilus and non-pathogenic E. coli in an ex vivo model on peripheral lymphocytes from sensitized allergic individuals and healthy controls.

The results show that stimulation with allergen caused a TH2-like response with enhanced IL-4 production in both groups and up-regulation of CD86 and CD23 on B-lymphocytes in allergic individuals. This immune response was profoundly modulated by the bacterial components L. acidophilus and E. coli. Single bacterial stimulation resulted in a TH1-dominated response in both groups, as reported in previous clinical studies (5). Furthermore, the expression of CD23, but also the co-stimulatory molecules CD80 and CD86 were modulated by both L. acidophilus and E. coli.

The shift in the TH1/TH2-balance measured via IL-4 and IFNγ following stimulation with non-pathogenic bacteria demonstrates the possible therapeutic potency especially of non-pathogenic E. coli Nissle. Although some studies indicate that human cytokine activities fail to exhibit an exclusively pro-TH1 or -TH2 pattern (19), our results show rather a reduction in one pathway.

Moreover, allergic patients showed decreased CD23 expression in the presence of bacteria, especially E. coli. These observations are closely related to the modulating effects of IL-4 and IFNγ on the expression of CD23. As previous studies describe increased CD23 expression caused by IL-4 and a decreased CD23 expression caused by IFNγ, the above-mentioned cytokine patterns following bacterial stimulation explain our findings (18). The in vivo relevance has been shown, but could not be confirmed clinically (20, 21). Increased CD23 expression in patients suffering from atopic dermatitis and allergic rhinitis has been shown previously by our group (22). Furthermore, former studies show that IFNγ inhibits CD23 mRNA and sCD23 production on human B-cells (18). sCD23, which was not measured in this study, has cytokine properties alone, but is also able to elicit strong inflammatory action by stimulating the production of TNFα and IL-1β and co-stimulates IL-2-induced INFγ production (23). Decreased shedding of sCD23 results in decreased IgE production (24).

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As it is shown that T-cell activation does not necessarily need antigen presenting cell signalling for activation (27), differences between the bacteria may be based on toll-like receptor (TLR)-mediated regulation of pathogen-related responses, since there are different cascades of activation mechanisms of Gram-positive and Gram-negative bacteria (28, 29). Without focusing on TLR-mediated cascades, the basic activity of lymphocytes plays a crucial role in the distinct TH1-like responses on bacterial stimulation in the presence of allergen, especially the allergic group. If one assumes preventive or even maturing effects of *L. acidophilus* and even non-pathogenic *E. coli* beyond childhood, these may be also explained via the TLR model. As the hygiene hypothesis focuses only on the maturation of the immune system towards TH1 phenotype in childhood (30), TLR pathways may also be involved in the maturation of the normal adult immune system. It remains unclear whether the above-mentioned TH1-responses following stimulation with probiotic and apathogenic bacteria as seen in our study show prolonged effects on driving the maturation of the adult immune system. However, the current study does not lead to precise conclusions about the comparative potency of both types of bacteria.

The strong TH1-like responses following bacterial stimulation also seen in the non-allergic controls suggest that priming signals may exist and consequently prevent over-expression of TH2-dominated responses. These effects might play a role not only in childhood (31). Previous studies using murine models indicate that such immunomodulatory effects are derived from long-term administration of mutated bacterial agents. Such effects may also result in long-term down-regulation of hypersensitivity reactions secondary to antigen-specific TH1/TH2 imbalances (32). As it is even hypothesized that TH1- and TH2-mediated conditions might frequently coexist and that the contrasting TH1/TH2-model might be an oversimplification (33), we assume, that long-term immunomodulating is possible beyond priming in infancy.

Another interesting observation is the shift of the allergen-specific TH2 response towards a TH1-like response following stimulation with *E. coli* Nissle plus allergen. This effect was not seen with *L. acidophilus*. Whether these observations reflect the clinical findings of a poor reduction in symptoms after oral *L. acidophilus* treatment requires further investigation (34). On the other hand, oral *E. coli* application has been reported to reduce the incidence of IgE-dependent allergies, namely respiratory and skin manifestations, in children when administered after birth (6, 34).

In summary, our observations indicate that inactivated non-pathogenic *E. coli* and probiotic *L. acidophilus* have immunomodulating capacities, as we determined profound changes in the TH balance. Clinical studies also indicate the applicability of probiotic agents for preventive use (35, 36).

The promotion of a TH1-dominated immune response by long-term application of bacteria, and especially *E. coli*, may be critical, as autoimmune diseases are characterized by a TH1-dominated immune response with high levels of TH1-type cytokines, e.g. IFNγ. On the other hand, it has recently been shown in a clinical trial that oral administration of *E. coli* Nissle maintains the remission of ulcerative colitis, an autoimmune disease of the gut (37).

*E. coli* Nissle, but not *lactobacilli*, were shown to induce high levels of IL-10 and IL-12, which might be due to the content of lipopolysaccharides in Gram-negative bacteria only. Therefore IL-12-promoted induction of IFNγ may be the cause of the differences in modulation of TH1/TH2 balance seen when comparing the types of bacteria. Finally, changes in the expression of surface molecules, such as CD86 and CD23, might also originate from this pro-inflammatory immune response (38).

We conclude that probiotic *L. acidophilus* and non-pathogenic *E. coli* Nissle exhibit immunomodulatory effects on lymphocytes by influencing the cytokine pattern, indicating a potential for preventive and therapeutic efficacy in the treatment of TH2-dominated allergic diseases. *E. coli* in particular increases the production of IFNγ under allergen challenge, which might also result in reduced symptoms in allergic individuals. However, in order to elucidate the role of non-pathogenic *E. coli* Nissle in modulating allergic symptoms and its mechanism of action, further investigation are needed.

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