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Allergen-stimulated expression of CD154 (CD40 ligand) on CD3+ lymphocytes in atopic, but not in nonatopic individuals. Modulation by bacterial lipopolysaccharide

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The intention of this study was to mimic a naturally occurring stimulation by allergens and bacterial infection in order to determine whether specific allergen-induced, inflammatory responses may be changed or modified by bacterial products. Blood leukocytes from six atopic and six nonatopic individuals were examined for their surface expression of CD154, CD11a, and HLA-DR molecules and for secretion of IgE, eosinophil cationic protein (ECP), and the cytokines interleukin (IL)-4 and IL-5. Signals through CD154 are required for activation and proliferation of effector cells associated with the allergic, inflammatory response. HLA-DR and CD11a/CD18-mediated interactions are also involved in T- and B-cell functions. Birch-pollen (BP) allergens induced CD154 expression on CD3-positive lymphocytes only in atopic individuals. In nonatopics, the expression of CD154 could be induced only after exposure to BP and subsequent lipopolysaccharide (LPS) stimulation. Levels of CD154 expression were always higher in atopics than nonatopics. CD11a and HLA-DR expressions were upregulated, irrespective of atopic state, after BP and/or LPS stimulation. The increased secretion of IL-5 and total IgE in BP-supplemented cell cultures indicated that an allergic response had occurred. In conclusion, the results of this report do not support the hypothesis of a changed inflammatory response stimulated by the combined action of bacteria and allergens, as compared to allergen provocation alone.

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Epidemiologic studies indicate that bacterial infection may strongly affect the allergic response (1, 2), and there are indications that very early in life, the development of atopy may be overridden by high levels of microbial stimulation that may immunomodulate allergic reactions (3–6). CD154

is a receptor present on CD4-positive T-cells (7, 8). The physical interaction between CD154 on T cells and CD40 on B cells plays an important role in the development of humoral immunity (9, 10), in the induction of IgE synthesis, and in T-cell-dependent B-cell growth and differentiation (7, 11, 12). HLA-DR/CD3 and CD11a/ICAM-1 also participate in many T-cell-dependent B-cell responses (see ref. 13 for review). Bacterial lipopolysaccharide (LPS) represents the toxic moiety of endotoxin (14) and is an integral part of the outer membrane of Gram-negative bacteria. It is continuously shed into the surrounding environment and is related to both the risk of developing nonatopic chronic obstructive pulmonary diseases (15, 16) and the severity of domestic asthma (17). LPS is also a highly potent proinflammatory substance (18–20).

In this study, therefore, in order to mimic a naturally occurring exposure to allergens (birch pollen) and subsequent stimulation by bacteria or bacterial products, i.e., LPS, we examined blood leukocytes from atopics and nonatopics. These were incubated in their natural habitat (whole blood) that was pre-exposed to purified allergen extracts before LPS stimulation, and analyzed for their expression of CD154 on CD3+ T cells; the lymphocyte-specific antigens CD3, CD4, CD8, and CD19; and the leukocyte integrins CD11a and HLA-DR – and for their secretion of IgE, eosinophil cationic protein (ECP), and the Th2-associated cytokines interleukin (IL)-4 and IL-5.

Material and methods

Subjects

Twelve volunteers (five men and seven women, age range 26–46 years), six nonatopics and six atopics, were symptomless at the time of experiments (outside the pollen season) and not taking any kind of medication. All atopics were clinically allergic to birch pollen (BP) and skin-prick positive (>3 mm) to BP. Two atopics had been hyposensitized to allergens of house-dust mite. Atopics ($n=4$), but not nonatopics ($n=6$), had elevated levels of ECP (>16 ng/ml) and specific IgE (>0.35 IU/ml) against BP, whereas nonatopics had undetectable levels of IgE.

Antibodies and reagents

LPS from *E. coli* O26:B6 was obtained from Difco Laboratories, Detroit, MI, USA. Aquagen allergen extracts (ALK, Hørsholm, Denmark) from BP had been prepared from

highly purified allergen sources and were totally devoid of enzyme activity, whereas the LPS concentrations in these extracts were less than 10 pg/ml, as measured by *Limulus* amoebocyte lysate (Bio-Whittaker, Walkersville, MI, USA) assay. The monoclonals phycoerythrin (PE)-conjugated anti-human CD154, fluorescein isothiocyanate (FITC)-conjugated anti-CD11a, anti-HLA-DR-PE/anti-CD3-FITC, anti-CD3-FITC, anti-CD19-PE/anti-CD3-FITC, and anti-CD4-FITC/anti-CD8-PE were all from Becton Dickinson (San Jose, CA, USA). Isotype-matched controls (Sigma, St Louis, MO, USA) were run in parallel to all monoclonal antibodies. ECP was analyzed with the Pharmacia CAP System ECP (Pharmacia AB, Uppsala, Sweden) (sensitivity 4 ng/ml). Specific and total IgE in sera were estimated with the Pharmacia CAP System RAST and total IgE fluoroimmunoassay (sensitivities 0.35 IU/l, respectively).

Preparation of cell cultures

Whole-blood cultures

Peripheral blood anticoagulated with EDTA (for flow cytometry) or citrate (for cytokine analyses) was incubated in polystyrene tubes (Falcon, Oxnard, CA, USA, no. 2057) in the presence or absence of BP or LPS, in humidified air with 5% CO₂ at 37°C. After completion of incubation, citrate plasma was harvested by centrifugation at 2000 g for 12 min and frozen at –70°C before analysis for cytokines.

Experimental procedure

After a 2-h exposure of leukocytes to BP allergens (1000 IU/ml), cells were subsequently stimulated with or without LPS (100 ng/ml) for different time periods before preparation of leukocytes for flow cytometry, and isolation and freezing of plasma for cytokine and IgE analysis. The choice of sampling times was based on preliminary studies for each parameter, with 8–10 different time points ranging up to 6 days. The time points chosen in the figures were those of maximal responses for each parameter. CD154 responded maximally after 6 and 24 h in BP- and BP-plus LPS-supplemented cultures, respectively, whereas CD11a and HLA-DR expression was maximal after 4 and 24 h, respectively. Total IgE concentrations were measured at the end of the culture period. LPS concentrations ranging from 1 ng/ml to 10 µg/ml were analyzed in a preliminary stage. The only concentration used in this study (100 ng/ml) was chosen because it gave a nearly maximal LPS response *in vitro* and is likely to be obtained in plasma during severe endotoxemia (21).

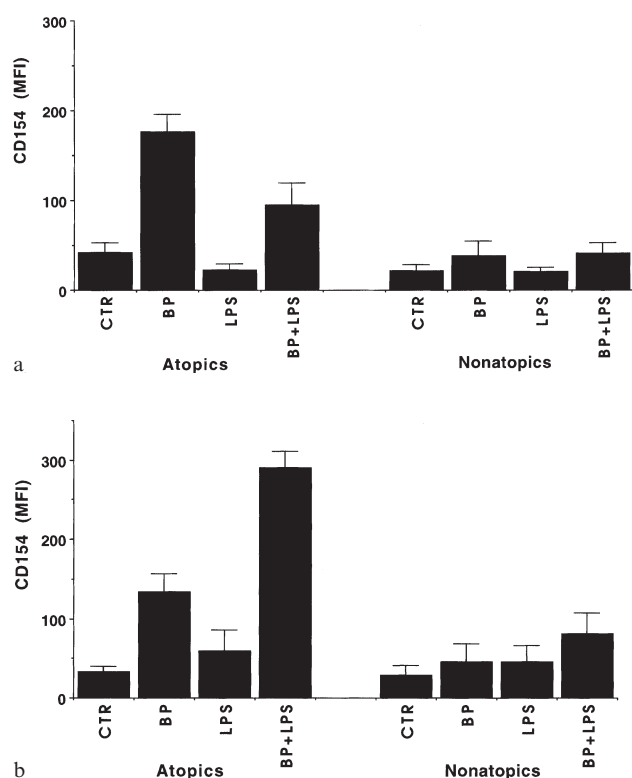


Figure 1. CD154 expression on CD3+ lymphocytes in atopics ($n=6$) and nonatopics ($n=6$) after birch pollen (BP) and/or lipopolysaccharide (LPS) stimulation. Values given are means $\pm$ SEM. Incubation times: a) 6; b) 24 h.

Cytokine measurements

Cytokines IL-4 and IL-5 in plasma or cell-culture medium were quantified with commercially available ELISA kits from Genzyme (Cambridge, MA, USA) and R&D systems (Abingdon, UK), respectively. The sensitivities were 45 pg/ml for IL-4 and 3 pg/ml for IL-5.

Flow cytometry

Volumes of 50 μ l of whole blood (unstimulated or stimulated with BP or LPS) in 12 $\times$ 75 polystyrene tubes (Falcon no. 2052) were incubated for 30 min on ice with the monoclonal antibodies indicated in the Antibodies and reagents section above, or with isotype-matched controls. After erythrocyte lysis, washing, and fixation in 1% paraformaldehyde, cells were analyzed in a FACSortTM flow cytometer (Becton Dickinson), equipped with an argon laser operating at 488 nm and filter settings for fluorescein (FITC) and phycoerythrin (PE). Ten thousand events were collected from each sample and analyzed by CellQuestTM software (Becton Dickinson). Electronic analysis gates were set on lymphocytes, granulocytes, or monocytes according

to their forward (FSC) and side-scatter (SSC) properties. Results were expressed as mean fluorescence intensity (MFI) or percentage of positive cells.

Statistics

Results are expressed as mean $\pm$ SEM or as median (range). Wilcoxon's rank sum test and the signed rank test for paired data were used to estimate significance. A P value of <0.05 was considered statistically significant.

Results

Leukocyte characterization

Cell distributions were not significantly changed during the 4-day incubation period, and no significant differences in cell distributions were seen between atopics and nonatopics, nor did the type of stimulant influence the cell distribution. In the leukocyte fraction of whole blood, differential counting by electronic gating in the flow cytometric FSC/SSC plots showed that lymphocytes (Ly) accounted for 22–44% (median 33%), monocytes (Mo) 4–11% (median 7%), and granulocytes (Gr) 45–72% (median 61%). No significant differences ($P<0.05$) were observed between atopics and nonatopics.

The fraction of CD3+ Ly among all lymphocytes ranged from 55% to 81%, independent of atopic state. CD4/CD8 or CD3/CD19 ratios were not significantly different between atopics and nonatopics, nor was the fraction of CD3-positive Ly, which did not significantly change during the incubation period or after stimulation with BP or LPS.

CD154, CD11a, and HLA-DR expression on blood leukocytes and IL-4 and IL-5 secretion after BP stimulation – comparison between nonatopics and atopics

In atopics, BP alone induced a maximally fourfold increase in CD154 expression on CD3+ Ly (whole-blood cultures, Fig. 1). The maximal expression was seen after 6 h of BP stimulation. This expression slightly declined with prolonged incubation up to 24 h. In nonatopic donors, CD154 expression did not change upon BP challenge.

BP allergens induced no changes in CD11a expression on Ly and Gr. However, a small, but significant ($P<0.01$) upregulation (maximal after 3 h) was demonstrated on Mo, 1.6-fold in both atopics and nonatopics (Fig. 2), declining after this time point.

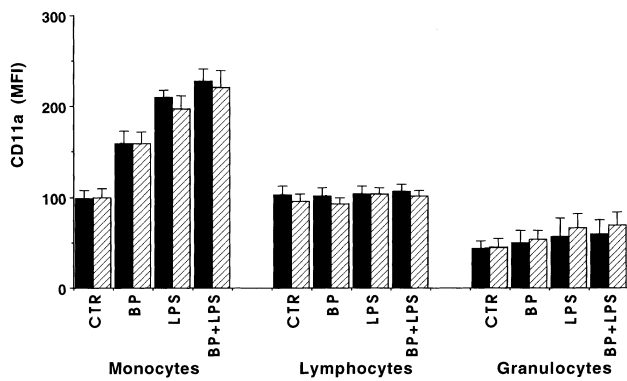


Figure 2. CD11a expression on blood monocytes, lymphocytes, and granulocytes of nonatopics ($n=5$) (filled bars) and atopics ($n=5$) (hatched bars) after birch-pollen (BP, 3 h) and/or lipopolysaccharide (LPS, 1 h) stimulation. Values given are means $\pm$ SEM.

HLA-DR expression on Gr and Ly was unresponsive to BP stimulation, whereas HLA-DR expression on Mo increased gradually up to maximal levels after 24 h of BP stimulation, 3.5- and 3.6-fold, respectively, for nonatopics and atopics (Fig. 3).

In atopics, but not in nonatopics, BP induced secretion of detectable plasma IL-5 levels (4.0 ± 1.0 pg/ml, mean $\pm$ SEM) after 4 days. IL-4 was undetectable at all time points. The plasma levels of total IgE increased during a 4-day span of incubation in both atopics (from 0.68 ± 0.19 IU/m in unstimulated to 0.93 ± 0.22 IU/m in BP-stimulated cultures) and nonatopics (from undetectable levels to 0.40 ± 0.18 IU/m; all values mean $\pm$ SEM).

CD154, CD11a, and HLA-DR expression and IL-4 and IL-5 secretion after LPS stimulation – comparison between nonatopics and atopics

The expression of CD154 on CD3+ Ly did not increase above basal levels (Fig. 1) after LPS stimulation for up to 4 days. A small difference in Ly CD154 expression between atopics and nonatopic individuals was statistically insignificant.

CD11a was equally expressed in atopics and nonatopics, both on unstimulated and LPS-stimulated blood leukocytes (Fig. 2). Unstimulated Ly and Mo expressed similar surface density of CD11a, while Gr expressed significantly less CD11a (0.4-fold [median], $P < 0.01$) than Ly and Mo. The expression of CD11a on Ly could not, at any time point, be increased with LPS. Monocytic CD11a expression, maximal after 3 h of incubation, increased 2.0-fold upon incubation with LPS, irrespective of atopic state.

Irrespective of atopic state, basal HLA-DR expression was higher on freshly drawn blood Mo and Ly than Gr, 4.3-fold

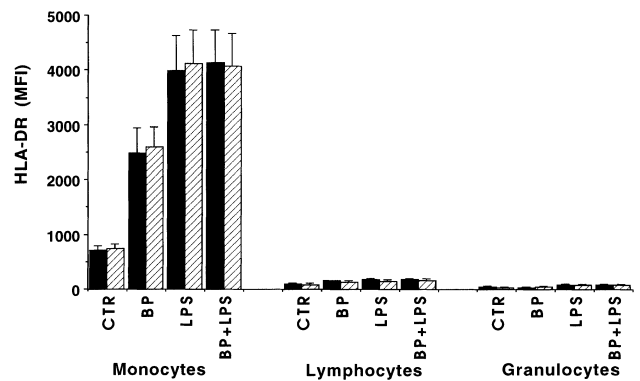


Figure 3. HLA-DR expression on blood monocytes, lymphocytes, and granulocytes of nonatopics ($n=5$) (filled bars) and atopics ($n=5$) (hatched bars) after incubation with birch pollen (BP) for 24 h and/or lipopolysaccharide (LPS) for 22 h. Values given are means $\pm$ SEM.

and 1.9-fold, respectively. Furthermore, HLA-DR was equally expressed in atopics and nonatopics in whole-blood cultures (Fig. 3) ($P > 0.05$). Unstimulated (after 24-h cultivation) Mo and Ly demonstrated a much higher (30-fold and 12-fold, respectively) expression of HLA-DR than Gr. The HLA-DR expression on Mo could be raised maximally 5.5-fold (2 h of preincubation with no stimulant followed by 22 h of LPS stimulation) in both atopics and nonatopics. The HLA-DR expression on Ly and Gr could not be further raised by incubation with LPS.

LPS stimulation did not induce any detectable secretion of IL-4 or IL-5. However, detectable but insignificant increases of IgE concentrations were observed after a 4-day span of incubation in both atopics (from 0.68 ± 0.19 IU/ml in unstimulated to 0.70 ± 0.13 IU/ml in LPS-stimulated cultures) and nonatopics (from undetectable levels to 0.37 ± 0.14 IU/ml).

CD154, CD11a, and HLA-DR expression on blood leukocytes and IL-4 and IL-5 secretion after exposure to BP and subsequent LPS stimulation – comparison between nonatopics and atopics

In samples pre-exposed (2 h) to BP and subsequently costimulated with LPS, CD154 expression on CD3+ Ly increased above levels found with BP stimulation alone. In atopics, CD154 expression increased twofold over the level obtained with BP stimulation alone after prolonged incubation for 24 h (Fig. 1). In nonatopics who were unresponsive to BP alone, pre-exposure to BP before addition of LPS and further costimulation with BP and LPS induced a moderate CD154 expression on CD3+ cells after a 24-h incubation period ($P < 0.05$).

In both atopic and nonatopic donors, CD11a expression on Mo could be further slightly increased (1.4-fold) after costimulation with BP plus LPS, compared to stimulation by BP alone. This additive stimulatory effect was not seen in Ly and Gr. The maximal expression of CD11a on Mo was seen after 2 h of BP exposure and a subsequent 1 h of BP plus LPS costimulation.

Irrespective of atopic state, BP plus LPS costimulation did not induce a higher expression of HLA-DR in any cell type compared to LPS stimulation alone, whereas BP plus LPS stimulation induced a 1.6-fold higher Mo HLA-DR expression in both atopics and nonatopics than BP stimulation alone.

In atopics, IL-5 was detectable (2.3 ± 0.8 pg/ml, mean $\pm$ SEM) in plasma after 4 days of costimulation with BP and LPS, whereas in nonatopics IL-5 was undetectable at any time point. Irrespective of atopic state, IL-4 was undetectable in all experiments. Compared to unstimulated cultures, total IgE concentrations increased nonsignificantly in atopics (to 0.73 ± 0.24 IU/ml) and in nonatopics (to 0.54 ± 0.15 IU/ml) during a 4-day span of incubation with BP and LPS in combination.

Discussion

The hypothesis of this study was based on epidemiologic data indicating that postnatal environmental factors may reduce the risk of developing allergy and allergic diseases (1, 2, 5). Exposure to different allergens early in life, possibly intrauterine (22–24), may influence primary sensitization and the development of asthma and allergic disease (25). Susceptibility to tolerance induction is probably markedly influenced by environmental factors that function as adjuvants in the airway mucosa. The reduced risk of developing allergic diseases in the second and subsequently born children in families (1) and the difference in manifestation of allergic disease between eastern and western German areas (2) may be explained if infections early in life protect against sensitizing responses to allergens. At the immunologic level, exposure to antigen in combination with cytokines induced by infectious diseases seems to switch or modulate the T-cell responses (from T-helper (Th)₂ cell to Th₁ responsiveness), thereby influencing the further evolution of allergic disease (3, 4, 6, 24).

The molecule CD154, which is a TNF receptor family member expressed on activated, but not on resting T cells (8, 10), may be a decisive factor in tolerance as opposed to an immune response (reviewed in ref. 26). The costimulation

pathway of CD154 and CD40, the CD154 ligand on B cells, may allow for selective expression of the CD4⁺ T cells (27). In this study, however, no selective expansion of the CD4⁺ T cells was seen, probably due to a small memory-cell population in the cell cultures studied. No difference between atopics and nonatopics was seen in the B-cell population, a finding which is in accordance with Levy et al. (28), but in contrast to Davidsson et al. (29), who demonstrated a relative increase of CD19⁺ B cells in peripheral blood during allergen provocation. As also demonstrated by Davidsson et al., CD154 expression may be induced after allergen stimulation in atopics, but not in nonatopics. In this study, BP induced CD154 expression in lymphocytes in atopic patients, but not in nonatopics. After exposure to BP and subsequent costimulation by BP and LPS, a small but significantly increased expression of CD154 was seen in both atopics and nonatopics. In atopic patients, costimulation with BP and LPS did cause a delayed and even higher expression of CD154 compared to BP stimulation alone. However, these responses after BP and LPS stimulation indicate that induction of inflammatory events may be potentiated by bacterial products after priming or prestimulation by allergens, indicating, for instance, that endotoxin, as a contaminant in airborne dust, may cause an adjuvant effect on allergen sensitization (17), thereby indirectly making lymphocytes more responsive to allergen stimulation.

This difference between atopics and nonatopics in their expression of the CD154 molecule has also been reported by Markert et al. (30), who demonstrated that the capability of B-cell-dependent CD40 ligand expression on T cells from allergic patients was increased compared to healthy individuals. Other groups (31, 32) did not demonstrate CD154 upregulation in cell cultures, a finding which might be due to an ineffective *in vitro* system to induce functional activation of resting lymphocytes (33). The apparent lack of induction of CD154 could also be due to a rapid induction of CD154 expression, followed by a downregulation, as also seen in other studies (34), suggesting release of soluble CD40 or engagement of CD154 by CD40 (35). Alternatively, differences in IL-4 requirements may influence CD154 expression (36). In prolonged incubation experiments which favored IL-13 rather than IL-4 production, Levy et al. (28) showed that CD154 expression may be upregulated in the absence of IL-4. In the present study, CD154 expression was induced without a detectable secretion of IL-4.

In contrast to BP-induced CD154 expression on CD3⁺ lymphocytes that was dependent on atopic state but

unresponsive to LPS stimulation alone, the expression of CD11a and HLA-DR on all types of blood leukocytes was not influenced by BP sensitization (i.e., prick test positive and specific IgE). However, CD11a and HLA-DR expressions on monocytes were upregulated by LPS, as has also been demonstrated by others (37–39). Both CD11a and HLA-DR were also upregulated upon BP stimulation *in vitro*, although to a lesser extent than with LPS. LPS and BP costimulation did not induce levels of expression above those of LPS alone. Although presumed to be less essential than the CD154–CD40 interaction (40, 41), HLA-DR and CD11a/CD18-mediated interactions are involved in T- and B-cell activation interactions (see refs. 11, 13, 42 for review), in B-cell maturation (40, 41), in differentiation from native to memory cells (43), and in antigen-induced expression of CD154 (gp39) in immunity (44). Furthermore, the CD11a/CD18 ligand ICAM-1 provides an important costimulatory signal for T-cell receptor-mediated activation of resting T cells (45), and reagents that inhibit CD11a/ICAM-1 interactions also inhibit CD154 expression (46). Therefore, CD11a and CD154 seem to be coexpressed, since both are induced and upregulated in response to allergen stimulation.

LPS was chosen as the candidate to mimic bacterial infection. LPS, a component of the outer membrane of Gram-negative bacteria, is continuously shed into the environment. In man, acute inhalation of dust containing LPS induces a blood and lung inflammatory reaction (18, 19). Clinical symptoms, such as chest tightness, cough, dyspnea, sputum production, and fever (20, 47), and changes in lung function and bronchial responsiveness (20, 47, 48) emerge. In allergen-exposed atopic subjects, the no-response threshold level of LPS exposure is probably far less than in nonatopic individuals (49). Furthermore, mechanisms leading to bronchial involvement in the response to inhaled LPS seem to be independent of an IgE-mediated response (50), and induce nonspecific proliferative effects acting on both humoral and cellular immunity (17, 51, 52). This is in accordance with the results of the present study, in which no increase in IgE production could be detected after LPS stimulation. The monocytic secretion of several proinflammatory cytokines may be induced by LPS, an effect which is inhibited by the cytokine IL-10 (52), which may be a key factor in priming for development of allergy (22, 23). LPS may also induce various very potent costimulatory signals

influencing T-cell stimulation. For example, in a study by Noelle et al. (7), monoclonal antibodies against CD154 were able to block B-cell differentiation induced by activated T cells, but not by LPS.

The aim of this study was to examine a model whereby leukocytes in their natural habitat (blood) or in mononuclear cell cultures were exposed to the costimulatory effects of allergen challenge and mimicked bacterial infection in adults. The fraction of blood lymphocytes primed to respond to BP allergens, in both atopic and nonatopic individuals, is likely to be small, and peripheral blood may not be the optimal milieu for studying the allergic response. However, in this study and as also shown by others (53), blood leukocytes responded to appropriate stimuli and expressed CD154 molecules on their surface. The secretion of IL-5 in atopics after stimulation with BP allergens indicated that a Th2-mediated response to allergen stimulation had occurred. The induction of IgE synthesis also indicated an allergic response in atopics. However, this response was less obvious in nonatopics, as has also been demonstrated in other studies (28) where IgE was secreted from atopics, but not from nonatopics. In interpreting the results of this paper, it has to be taken into account that leukocytes were isolated from adult atopics sensitized to BP. It is likely that even after removal of specific allergens, allergic disease may continue, with other nonspecific factors triggering the symptoms. However, it is also well known that sensitization (i.e., specific IgE and prick test positive), allergic symptoms, and asthma can develop for the first time in adult life.

In conclusion, the results of this *in vitro* model of allergen challenge and subsequent bacterial infection in adults showed that bacterial products (i.e., LPS) did not qualitatively change the pattern of allergic, inflammatory responses, at least with respect to CD154 expression on CD3+ lymphocytes. Instead, bacterial infection seemed to introduce a lag phase for expression of CD154, and the allergic response even seemed to be potentiated by LPS. However, it has to be kept in mind that the LPS-modified response to allergens may be different in early life when the individual is exposed to the allergen for the first time. This study, therefore, did not support the hypothesis that persistent bacterial infection prevents the development of allergic disease.

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