

Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides

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In allergy and asthma, the fine balance between the T helper (Th) 1, Th2 and T regulatory cytokine responses appears to be shifted towards Th2. Here, we report that synthetic lipopeptides which contain the typical lipid part of the lipoprotein of gram-negative bacteria stimulate a distinct regulatory cytokine pattern and inhibit several Th2 cell-related phenomena. The most potent analogue of synthetic lipopeptides, lipopeptide CGP 40774 (LP40) was not active in MyD88-deficient mice and stimulated Toll-like receptor (TLR)-2, but not TLR-4. LP40 potentiated the production of IFN- γ and IL-10, but not IL-4 and IL-5 by human T cells. In addition, triggering of TLR-2 by lipopeptides promoted the *in vitro* differentiation of naive T cells towards IL-10- and IFN- γ -producing T cells and suppressed IL-4 production by Th2 cells. Accordingly, LP40 inhibited IgE production induced by allergen, anti-IgD antibody, *Nippostrongylus brasiliensis* or murine acquired immunodeficiency virus. Furthermore, ovalbumin-induced lung eosinophilic inflammation was abolished and *Schistosoma mansoni* egg-induced granuloma size and eosinophil counts were suppressed in mice by LP40. These results demonstrate that stimulation of TLR-2 by lipopeptides represents a novel way for possible treatment of allergy and asthma by regulating the disrupted cytokine balance.

Key words: Allergy / T cells / IgE / Lipopeptide / Toll-like receptor 2

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1 Introduction

Th2 cells and their cytokines, in particular interleukin (IL)-4, IL-13 and IL-5, are responsible for the production of IgE and generation of tissue eosinophilia in allergic diseases [1, 2]. Based on the recent knowledge of the pathogenesis of allergy and asthma, one mechanism to control the disease process is to control the over-expression of Th2 cytokine-secreting cells. Thus, it is expected that

selective inhibition of Th2 cytokines may control overexpression of IgE and eosinophils. IL-12 produced by macrophages, dendritic cells (DC) and B cells is a dominant factor in the differentiation of Th1 cells from naive T lymphocytes [3]. In contrast, the presence of IL-4 during the initial priming of naive T cells by antigen generates IL-4- and IL-5-producing Th2 cells while inhibiting Th1 development [2]. In addition to cytokines, several other substances such as histamine may influence the development of Th cell subsets [4].

IL-10 acts as a general inhibitor of proliferative and cytokine responses of both Th1 and Th2 cells *in vitro* and *in vivo* [5]. Recently, IL-10-derived regulatory CD4⁺ T cells, producing IL-10 and suppressing antigen-specific T cell responses, were identified in human and mice [6, 7]. In addition, IL-10 plays a key regulatory role in T cell tolerance to high dose of antigen exposure (bee sting of

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Abbreviations: LP40: Lipopeptide CGP 40774 PLA: Phospholipase A₂ TLR: Toll-like receptor JNK: c-Jun N-terminal kinase sBLP: Synthetic bacterial lipopeptide MAPK: Mitogen-activated protein kinases CsA: Cyclosporin A

healthy individuals) and during specific immunotherapy of allergy [5, 7]. IL-10 thus offers a means of dampening inflammatory responses altogether, without the risk of converting one deleterious response (e.g. Th2 response) to another (e.g. Th1 response).

The Toll-like receptor (TLR) family is a phylogenetically conserved mediator of innate immunity that is essential for microbial recognition [8]. So far, ten members have been reported [9]. TLR-2, TLR-4 and TLR-9 are responsible for immune responses to peptidoglycan, lipopolysaccharide (LPS) and unmethylated CpG dinucleotides of bacterial DNA, respectively [9]. Accumulating evidence has revealed therapeutic potential of TLR-9-triggering CpG DNA for cancer, allergy and infectious diseases [10, 11]. The present study demonstrates that stimulation of the innate immunity by lipopeptides via a TLR-dependent mechanism induces copious levels of the anti-inflammatory cytokine IL-10 and the Th1 cytokine IFN- γ , which regulate naive T cell differentiation, IgE and IgG antibody isotype regulation and lung eosinophilia in mice, as a novel approach for the treatment of allergy and asthma.

2 Results

2.1 Triggering of TLR-2 by lipopeptides

Signaling through TLR occurs through the sequential recruitment of the adapter molecule MyD88 and the serine/threonine kinase IRAK, and subsequently activates mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and the nuclear factor (NF)- κ B [12, 13]. We analyzed whether a synthetic lipopeptide, lipopeptide CGP 40774 (LP40), specifically activates NF- κ B via triggering of TLR-2 (Fig. 1A). For this purpose, we transfected human embryonic kidney (HEK293) cells that otherwise do not transduce signals by lipopeptides and LPS via TLR-2 [14]. Wild-type and TLR-2-transfected HEK293 cells were stimulated with biologically active synthetic bacterial lipopeptide (sBLP) and inactive Pam₃Cys in comparison to LP40. NF- κ B was activated by LP40 and sBLP as measured using an NF- κ B reporter luciferase assay demonstrating that LP40-mediated signal transduction is dependent on TLR-2.

We also analyzed activation of JNK1 in purified monocytes. Activation of JNK during signal transduction is demonstrated by dual phosphorylation of Thr 183 and Tyr 185 [15]. LP40 induced tyrosine phosphorylation of 46-kD JNK1 similar to LPS in purified monocytes (Fig. 1B). To further support that LP40 stimulates TLR-2, we analyzed IL-6 production by MyD88-deleted and TLR-4-deleted mice spleen cells (Fig. 1C). LP40 stimu-

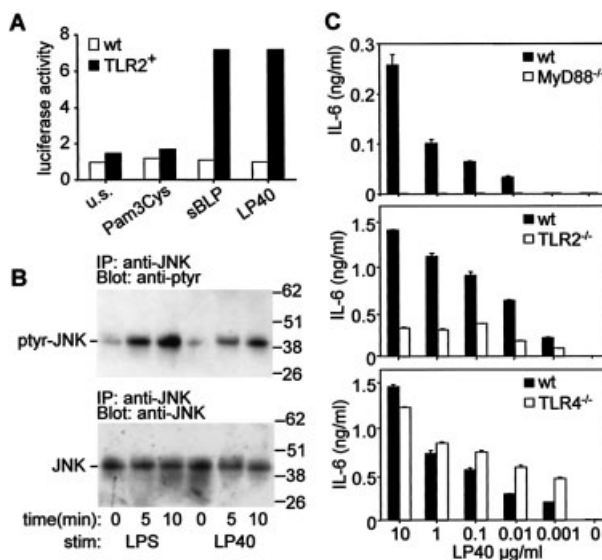


Fig. 1. LP40 triggers TLR-2. (A) NF- κ B activation triggered by LP40 and sBLP. Wild-type and TLR-2-transfected HEK293 cells were stimulated for 6 h with sBLP (1 μ g/ml), Pam3Cys (1 μ g/ml), and LP40 (1 μ M). NF- κ B activation was measured using an NF- κ B reporter plasmid containing the luciferase gene. Data normalized for transfection efficiency are expressed as relative luciferase activity, i.e. multiples of the results obtained in unstimulated wild-type HEK293 cells (by definition =1). Shown is one representative experiment out of three. (B) LP40 activates JNK1. Purified human monocytes were stimulated with 100 ng/ml LPS and 1 μ M LP40. JNK1 was immunoprecipitated and Western blots were stained with anti-phosphotyrosine and anti-JNK1 mAb. Both LPS and LP40 induced tyrosine phosphorylation of JNK1. Results are representative of three experiments. (C) LP40 signals through a TLR-2-dependent, MyD88-dependent, but TLR-4-independent pathway. Spleen cells from TLR-2^{-/-}, TLR-4^{-/-}, or MyD88^{-/-} mice and wild-type mice were cultured with the indicated concentrations of LP40 for 24 h and the culture supernatants assayed for IL-6 by ELISA. Representative of three experiments with three mice per group in each experiment is shown.

lated IL-6 production in TLR-4-deleted spleen cells dose-dependently without showing any effect in MyD88-deficient spleen cells. In the case of TLR-2-deficient mice, there was a marked diminution of LP40-induced response, although a significant residual response was observed. Same results were obtained for the induction of TNF- α (data not shown). Together these data demonstrate that LP40 signals through TLR-2 and through a non-TLR-2, MyD88-dependent receptor.

2.2 Induction of IFN- γ , IL-10 and IL-12 production

The immunomodulatory effects of synthetic lipopeptides were studied in human peripheral blood mononuclear cells (PBMC) and during naive T cell differentiation to Th2 and Th0 cells (Fig. 2). First, we determined the effect of LP40 on human antigen-specifically stimulated PBMC cultures. LP40 substantially enhanced the antigen-induced production of IL-10, IFN- γ and IL-12, but

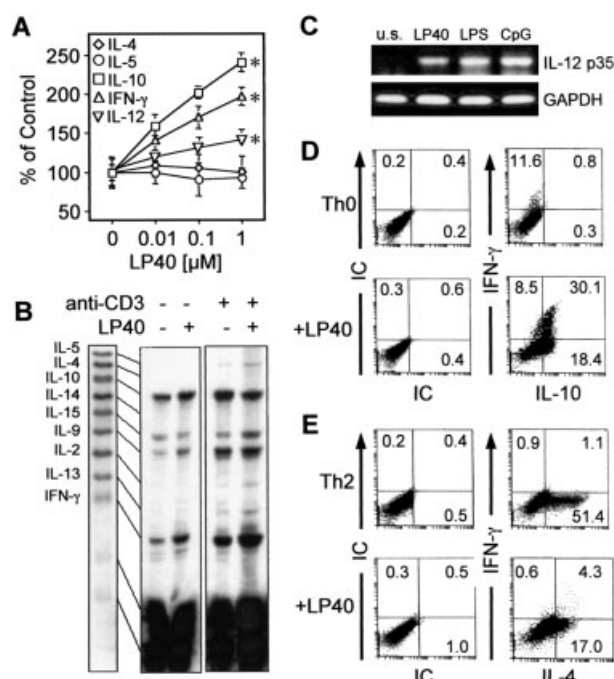


Fig. 2. LP40 enhances the production of IFN- γ , IL-10 and IL-12 but not Th2-type cytokines. (A) PBMC from bee venom-allergic individuals were stimulated with the antigen (PLA) in different doses of LP40 and cytokine levels were determined by ELISA. Results shown are representative of one out of four experiments. In the absence of LP40, IFN- γ production was 1.4 ± 0.4 ng/ml, IL-4 was 0.3 ± 0.1 ng/ml, IL-5 was 2.2 ± 0.5 ng/ml, IL-10 was 1.8 ± 0.4 ng/ml and IL-12 was 0.3 ± 0.2 pg/ml. (B) Human PBMC were exposed to 1 μ M LP40 in the absence or presence of anti-CD3 stimulation for 6 h. RNA was isolated and subjected to RNase protection assay. Same results were obtained in two other experiments. (C) IL-12 p35 mRNA was detected after 8 h stimulation of human PBMC with 1 μ M LP40, 100 ng/ml LPS and 3 μ M CpG. (D, E) Purified human cord blood CD45RA⁺ T cells were differentiated into Th0 cells (D) and Th2 cells (E) in the absence or presence of 1 μ M LP40 for 12 days. IC: isotype control antibody. Intracytoplasmic cytokines were determined 12 h after anti-CD2/anti-CD3/anti-CD28 mAb stimulation. One out of four experiments with similar results is shown; * $p < 0.001$.

exerted no effect on IL-4 and IL-5 in response to bee venom allergen phospholipase A₂ (PLA) (Fig. 2A). Similar results were obtained in other antigen systems such as tetanus toxoid and protein-purified derivative of *Mycobacterium bovis* as well as anti-CD3 stimulation (data not shown). We then analyzed effect of LP40 on cytokine mRNA of human PBMC. LP40 strongly enhanced IL-10 and IFN- γ mRNA and moderately enhanced IL-9 and IL-15 mRNA expression (Fig. 2B). In addition, LP40 induced IL-12 p35 mRNA expression in human PBMC similar to LPS and CpG (Fig. 2C).

Since triggering of TLR-2 by LP40 altered several cytokines that may influence Th cell subset differentiation, we determined its effect on cytokine modulation during the differentiation of naive Th cells to Th0 cells and Th2 cells. CD45RA⁺ cord blood T cells were stimulated with mAb against CD2, CD3 and CD28. In the presence of IL-2 for 12 days, LP40 induced a unique cytokine profile and enhanced IL-10 and IFN- γ production during *in vitro* differentiation of memory Th0 cells (Fig. 2D). LP40 induced a profound enhancement in the frequency of IL-10- and IFN- γ -producing T cells in Th0 cells. The percentage of IFN- γ -producing cells increased threefold and the percentage of IL-10-producing cells increased 41-fold. The percentage of both IL-10- and IFN- γ -producing cells increased 37-fold. Interestingly, LP40 inhibited the *in vitro* differentiation of naive T cells to Th2 cells (Fig. 2E). IL-4 production was inhibited by 2.4-fold during differentiation to Th2 cells. In contrast, the percentage of IFN- γ -producing cells increased by 2.5-fold in IL-4⁺ IFN- γ ⁺ T cells showing a switch from Th2 to Th0 cytokine profile.

We also attempted to modulate the cytokine profile of pure cloned T cells by LP40, which did not show any direct effect (data not shown). Accordingly, modulation of T cell cytokine profile by LP40 appears to be indirect rather than a direct effect on T cells. In summary, triggering of the innate immune response by lipopeptides efficiently modulates cytokine profile during Th cell differentiation by suppressing IL-4 and abundantly enhancing IL-10 and IFN- γ .

2.3 Inhibition of IgE production

Since LP40 stimulated the production of IL-10, IL-12 and IFN- γ , but not IL-4 and IL-5, the question was posed, whether IgE production could be regulated. In human PBMC, the lipopeptide significantly inhibited the allergen-induced production of bee venom PLA-specific IgE, while it simultaneously enhanced PLA-specific IgG4 levels ($p < 0.001$; Fig. 3A).

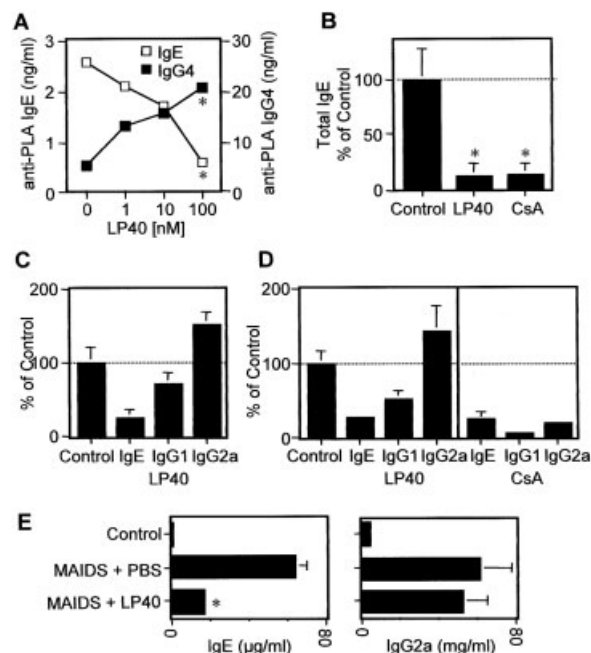


Fig. 3. LP40 selectively suppresses IgE production. (A) PBMC from a bee venom-allergic donor were stimulated with PLA and antigen-specific IgE and IgG4 levels were determined by ELISA. LP40 (100 nM) completely suppressed IgE, while enhancing IgG4. (B) Mice were injected s.c. with *N. brasiliensis* (700 larvae/mouse). LP40 and CsA were given i.p. daily except for day 5 and 12, and at day 10 serum samples were taken and IgE levels were determined by ELISA. At day 10, LP40 (3 mg/kg) and as control CsA (20 mg/kg) suppressed the IgE production by 88% and 87%, respectively. Animals treated with vehicle alone produced 30.3 µg/ml of IgE (100% value). (C) Groups of six mice were injected with goat anti-mouse IgD antibody (3 mg/mouse) at day 0, and treated orally with LP40 (10 and 30 mg/kg) at days –1, 0 and 2. At day 8, serum samples were taken and total isotype levels were determined by sandwich ELISA. Animals treated with vehicle alone produced 67 µg/ml IgE, 28 mg/ml IgG1 and 345 µg/ml IgG2a (100% values). Bars show the level of suppression caused by 30 mg/kg LP40. SE of the mean is given. LP40 (10 mg/kg) suppressed IgE by ~10%, IgG1 by 20%, and enhanced IgG2a by 151% (data not shown). (D) Mice were treated as in (C) but LP40 was administered i.p. (0.1 and 1 mg/kg) daily from day 0 to day 7 except day 6. Total isotype levels were determined by sandwich ELISA at day 8. Animals treated with vehicle alone produced 28 µg/ml IgE, 39 mg/ml IgG1 and 545 µg/ml IgG2a (100% values). Bars show the level of suppression caused by 1 mg/kg LP40. SE of the mean is given. LP40 (0.1 mg/kg) suppressed IgE by 38%, did not effect IgG1 level, and enhanced IgG2a by 172% (data not shown). CsA (20 mg/kg) significantly inhibited IgE, IgG1 and IgG2a as control. (E) Groups of ten female C57BL/6 mice were injected i.p. with the LP-BM5 viral stock and treated with 3 mg/ml LP40 i.p. once a week for 5 weeks. After 11 weeks serum samples were taken and IgE and IgG2a levels were determined; * $p < 0.001$. Results are representative of three (A) and two experiments (B–E).

The *in vivo* effects of LP40 on antibody isotype regulation were intensively analyzed in three mouse models. In *Nippostrongylus brasiliensis*-infected mice [16], LP40 inhibited the extremely high levels of IgE similar to cyclosporin A (CsA) (Fig. 3B). In the goat anti-mouse IgD antibody model given orally (Fig. 3C) or i.p. (Fig. 3D), LP40 strongly inhibited IgE ($p < 0.001$) and partially inhibited IgG1 ($p < 0.01$), reflecting total and partial IL-4 dependency of these isotypes. In contrast, the IFN- γ -dependent isotype IgG2a [17] was consistently enhanced ($p < 0.001$). In comparison to LP40, CsA inhibited all Th cell-dependent isotypes, IgG1, IgG2a and IgE ($p < 0.001$; Fig. 3D). We also examined whether LP40 could selectively inhibit IgE in a chronic virus model. It is known that, similar to humans, in the murine model of AIDS (MAIDS), virally-infected mice produce high levels of IgE, particularly in the later stages of infection [18]. Here, LP40 suppressed serum IgE levels without significantly affecting IgG2a levels (Fig. 3E).

2.4 Inhibition of allergen- and schistosoma-induced lung eosinophilia

The murine lung inflammation model is widely used to study asthma. Previous studies demonstrated that T cell or T cell cytokine inhibitors such as CsA, anti-CD4, anti-IL-4 and anti-IL-5 antibodies can effectively suppress lung eosinophil infiltration in mice [19]. In response to ovalbumin (OVA), which was applied three times i.p. before the antigen rechallenge, as little as 0.03 mg/kg of LP40 significantly reduced the number of eosinophils in bronchoalveolar lavage (BAL) fluid (Fig. 4A). Almost complete inhibition of eosinophil accumulation was obtained at 3 mg/kg, which was as potent as CsA. LP40 was also found to be orally active (Fig. 4B), albeit much higher doses were required. Interestingly, a single oral dose given before the antigen rechallenge inhibited BAL eosinophil counts by 82% (Fig. 4C).

Th2-type T cells and eosinophils are important players in immune defense against parasites. *Schistosoma mansoni* eggs are known to induce a Th2 response-mediated eosinophilic granuloma in mice [20]. Treatment of mice with LP40 significantly decreased granuloma size and the tissue eosinophilia. Moreover, soluble egg antigen-induced IL-4 and IL-5 were reduced by 30% and 93% after LP40 treatment, respectively (Table 1).

3 Discussion

The present study demonstrates that stimulation of the innate immune response by lipopeptides elicits a unique Th profile, characterized by abundant levels of IL-10 and

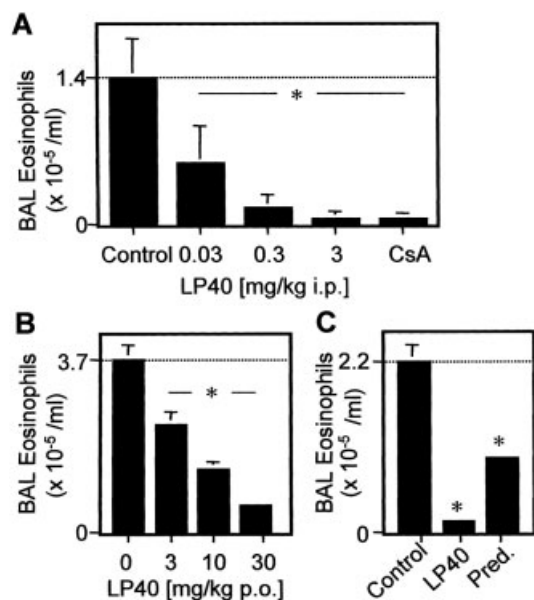


Fig. 4. Suppression of eosinophilic responses by LP40. (A) For the induction of lung eosinophilia by antigen, mice were sensitized with OVA and on day 21 challenged with OVA intranasally. One day later bronchoalveolar cells were counted. Various doses of LP40 were administered i.p. daily starting at day 13 until day 21. For LP40, ED₅₀ was 0.03 mg/kg. (B) LP40 was given orally three times at days 21, 22 and 23. ED₅₀ for LP40 for oral treatment was ~6 mg/kg. In the same experiment, ED₅₀ was <0.3 mg/kg when the compound was administered i.p. (data not shown). (C) Conditions were the same as in (B) except that the LP40 (30 mg/kg) was administered orally, once on day 21, 1 h before antigenic challenge. Prednisolone (1 mg/ml) caused 55% inhibition of lung eosinophilia under the same conditions. Results are representative of two separate experiments with groups of eight mice (A–C); **p*<0.001.

IFN- γ , and inhibits several important features of allergic inflammation including Th2 cells, IgE production and eosinophilia *in vivo*. In chronic allergic diseases a significant proportion of T cells display the Th2 pheno-

type, which is defined by the predominant release of IL-4, IL-5 and IL-13 [2]. Inhibition of Th2 cells or Th2 cytokines, Th2 cytokine receptors and Th2 cytokine signal transduction may provide a direct way to treat allergy and asthma. A more general mechanism of Th2 cell inactivation in allergic inflammation can be achieved by the induction of high levels of IL-10. Microbial lipopeptides are also potent stimulators of IL-12 production by TLR [14]. Accordingly, LP40 induces a potent IL-12 and IFN- γ response to counter-regulate Th2-mediated allergic inflammation. Stimulation of the immune response by synthetic lipopeptides appears to utilize both of these strategies. As shown in allergen-induced cytokine responses, LP40 does not inhibit IL-4 and IL-5 production directly. However, during differentiation of naive T cells to memory T cells, LP40 enhances the number of IL-10- and IFN- γ -producing T cells leading to a distinct cytokine pathway and thereby inhibiting IL-4-producing T cells.

The induction of high levels of IL-10 by LP40 is consistent with a recent study, which reports that certain TLR-2 ligands induce IL-10 and T regulatory cells [21]. It has been postulated that signaling through TLR always induces Th1 responses [22]. However, more recent studies indicate that signaling through TLR-2 may also induce Th2 responses [23, 24] or T regulatory responses [21]. Different forms of lipopeptides may activate DC subsets to produce different cytokines, and induce distinct types of adaptive immunity *in vivo*. For example, *Escherichia coli* LPS was shown to induce IL-12 p70 in the CD8 α^+ DC subset, while *Porphyromonas gingivalis* LPS, a TLR-2 ligand, did not [23]. In this context, our data suggest that LP40 induces a unique cytokine profile, characterized by induction of IL-10, IL-12 and IFN- γ . This unique cytokine profile cannot be characterized as a typical Th1 or a T regulatory response. Therefore, the present study highlights the complexity that is inherent in the cytokine profiles of Th responses, and the futility of trying to “fit” this complexity into the simple Th1-Th2 paradigm.

Table 1. LP40 suppresses *S. mansoni* egg-induced granuloma size, eosinophil content and IL-5 production^{a)}

	Granuloma size (mm ³ ×10 ³)	Eosinophils	IL-4 (U/ml)	IL-5 (ng/ml)	IFN- γ (ng/ml)
Control	3.6±0.9	50.0±9.4	6.72	38.99	1.07
LP40	1.9±0.4	6.0±1.3	4.68	2.77	1.55
(% inhibition)	(46%)	(88%)	(30.4%)	(93.0%)	(0.0%)

^{a)} Mice (*n*=5) were treated with LP40 at the time of i.v. challenge with *S. mansoni* eggs. Single-cell suspensions of lung-associated lymph node cells were stimulated with soluble egg antigen. Cytokines were determined after 72 h. Percent inhibition by LP40 is given in parenthesis.

IL-4 and IL-13 are known to induce IgE switch in B cells [25, 26]. Allergen-specific IgE antibodies mediate type 1 hypersensitivity, whereas specific IgG4 antibodies afford normal protective immunity to the respective allergen [27]. Several studies have demonstrated the IgE inhibitory effects of IL-10, IL-12 and IFN- γ [7, 28]. In addition, IFN- γ was successfully used to treat hyper-IgE patients [29]. Consistent with this, the present study demonstrates that LP40 inhibits IgE in several mice models.

The dramatic effect of a single dose of LP40 to inhibit lung eosinophilia prior to airway antigen challenge suggests a strong effect on the recruitment phase of this response. Inhibition of IL-4 may down-regulate vascular cell adhesion molecule (VCAM)-1 expression on the endothelium [30] and IL-5 is an important cytokine for increased eosinophil life span [31]. In addition, IL-10 down-regulates eosinophil function and activity in several ways [32, 33] and IL-12 treatment of asthmatic patients decreases blood and sputum eosinophils [34].

It has been demonstrated that immunostimulatory DNA sequences containing CpG motifs prevent the development of allergic airway responses in murine models of disease [35]. Recently, it has been demonstrated that allergen-DNA conjugates can reverse established Th2-driven allergic airway responses [36]. In experiments with TLR-2^{-/-}, TLR-4^{-/-} and MyD88^{-/-} mice, we have demonstrated that LP40 utilizes an MyD88-dependent, TLR-2-dependent and TLR-4-independent signaling pathway. However, the significant residual response observed in TLR-2^{-/-} mice suggests that an additional receptor is important for the LP40 signal. Recently, several new components of the TLR signaling cascade have been identified. Most pathways involved in the induction of NF- κ B and activator protein (AP)-1 were demonstrated to be activated by triggering of TLR [37]. The present study demonstrates that NF- κ B is activated specifically in TLR-2-transfected cells and 46-kDa JNK1 is activated in human monocytes by LP40. These results are supported by the finding that a close analog of LP40 was reported to cause phosphorylation of MAPK in macrophages [38]. Similar to our findings, synthetic lipopeptides were shown to activate extracellular signal-regulated kinases (ERK)1/2 and MAPK/ERK kinases (MEK)1/2 independent from TLR-4 pathway [39]. In addition, TLR-2 can heterodimerize with either TLR-1 or TLR-6, and it is possible that the different combinations promote different responses [40].

The possibility that TLR triggering by bacterial or viral components may protect against allergic disease is supported by several epidemiological studies. Positive tuberculin response predicted a lower incidence of asthma and atopy and correlated with increased Th1 and

reduced Th2 cytokines in serum [41]. Currently, there is convincing evidence suggesting that growing up in a farm protects against the development of childhood allergic diseases [42]. Dramatic increase in the frequency of atopic disorders particularly in the western world has been linked in part to reduced Th1-type immunity because of reduced childhood infections [43].

Therapy with cytokines and growth factors represents a novel approach for the treatment of several diseases. The present study demonstrates that induction and regulation of cytokines by low-molecular-weight innate immunity-triggering molecules could represent a practical approach in the treatment and prevention of allergic diseases.

4 Materials and methods

4.1 Chemical synthesis of LP40

We have synthesized several lipopeptides, and after acquiring information on the structural requirements for specific biological activities, we synthesized the most effective lipoprotein designated LP40 of 1.2 kDa [44]. Briefly, L-(R)-cysteine was converted in several steps to N-palmitoyl-S-[2(R),3-dilauroyloxypropyl]-(R)-cysteine, which was then coupled to S-glutamic acid-bis-taurine amid resulting in LP40.

4.2 Lymphocyte cultures and measurement of cytokine production

PBMC from bee venom-allergic individuals were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood and stimulated by PLA as described [7]. Cytokines (IL-4 and IL-12 at 24 h, IL-5, IL-10 and IFN- γ at day 5) were determined by sandwich ELISA [7]. IL-12 ELISA was from PharMingen, San Diego, CA (clones 20C2 and C8.6). Purification of T cell subsets was performed by magnet-activated cell sorting (Miltenyi Biotec AG, Marburg) [31]. Purified cells were stimulated with a mixture of soluble anti-CD3 and anti-CD28 mAb (PharMingen), both 10 μ g/ml, for 72 h. Keyhole limpet hemocyanin (KLH; Calbiochem)-specific responses in mice were induced as described [45, 46]. Briefly, regional lymph node cells of BALB/c mice (10⁵ cells/0.2 ml/microtiter well) were cultured with the compounds (tested at 0.1–10 μ M range) and/or KLH (3 μ g/ml). Spleen cells of TLR-2^{-/-}, TLR-4^{-/-}, MyD88^{-/-} and wild-type mice (generous gift of Dr. Shizuo Akira, Osaka, Japan) were cultured with different doses of LP40.

Cytokines were determined by chemiluminescent immune assay or by ELISA [47]. For cytokine determination by RNase protection assay, PBMC were cultured at 4 × 10⁶ cells/ml in 2-ml wells and stimulated with 1 μ g/ml

anti-CD3 in the presence or absence of 1 μ M LP40. After 12 h, cells were lysed with guanidinium thiocyanate solution. RNA was precipitated and cytokine mRNA levels were analyzed by RNase protection assay using the Riboquant multiprobe set (PharMingen). IL-12 p35 mRNA expression was detected as described [48]. LPS was from Sigma Chemicals Co. and CpG oligonucleotide (5'-TCGTCGTTTTGTCGTTT-TGCTGTT-3') was from Microsynth, Balgach, Switzerland.

4.3 *In vitro* differentiation of Th2 and Th0 cells

Human cord blood CD4⁺ CD45RA⁺ T cells were purified by negative selection. Purity was more than 90%; less than 1% CD14⁺ monocyte contamination was observed; main contaminating cells were CD16⁺, CD14⁺, CD19⁺, CD3⁺, CD4⁺, CD8⁺ cells. Cells were stimulated with anti-CD2 (two mAb, 4B2 and 6G4, each 0.5 mg/ml), anti-CD3 (1 mg/ml), and anti-CD28 (1 mg/ml) and IL-2 (25 U/ml). For Th2 differentiation, human IL-4 (25 ng/ml) and neutralizing anti-IL-12 (10 μ g/ml) were used. Th0 cells were grown in the presence of IL-2 only. LP40 (1 μ M) was added to cultures from the start.

Intracellular staining of cytokines in T cells after priming was performed by fixing and permeabilizing the cells after stimulation with anti-CD2, anti-CD3, and anti-CD28 mAb mixture for 12 h. Monensin (2 mM, Sigma) was added during the last 10 h. Cells were fixed and permeabilized with a paraformaldehyde and saponin solution (Ortho PermeaFix, Ortho Diagnostic Systems, Raritan, NJ). After washing with PBS containing 5% FCS, 1.5% BSA (Sigma) and 0.0055% EDTA (Fluka Chemie) cells were stained with 0.5 mg/ml FITC- or PE-labeled anti-IL-4, anti-IL-10 or anti-IFN- γ mAb and FITC- or PE-labeled rat IgG1 and rat IgG2a control antibodies (all from PharMingen) for 30 min at 4°C. The flow cytometric analysis was performed with an Epics XL (Coulter, Hialeah, FL).

4.4 Immunoprecipitation and immunoblotting analysis

Cells were lysed in 0.5% Triton X-100 containing leupeptin, pepstatin A and aprotinin (each at 10 μ g/ml), sodium orthovanadate (100 μ M), EDTA (5 mM) and iodoacetamide (50 mM) (all from Sigma). Lysates were incubated for 45 min on ice, centrifuged for 10 min and supernatants were collected. Immunoprecipitations were performed in supernatants for 2 h at 4°C with anti-JNK1 mAb (Sigma). Sepharose-protein G was added for 2 h at 4°C (Sigma). Immunoprecipitates were washed with cold lysis buffer, boiled in Laemmli sample buffer and then subjected to electrophoresis on Tris-glycine gels (Novex AG, Frankfurt, Germany). Immunoblotting was performed on nitrocellulose membranes (Amersham Life Science, UK) by anti-phosphotyrosine, anti-JNK1 mAb and visualized by chemiluminescence detection system (Pharmacia Biotech Limited, UK).

4.5 TLR-2 transfection and NF- κ B reporter assay

HEK293 cells were purchased (ATCC-No. CRL-1573, TIB-202, CCL-185). HEK293 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% low endotoxin FBS (Hyclone Labs., Inc.) and 1% antibiotic-antimycotic solution (Life Technologies, Basel, Switzerland). The TLR-2 expression plasmid is based on a pFLAG-CMV-1TM vector (Sigma) and was a kind gift from Dr. C. Janeway, New Haven, CT. sBLP (Pam3CysSerLys4) was purchased from Boehringer Mannheim Biochemica and prepared in endotoxin-free water with 0.05% human albumin. The biologically inactive Pam3Cys serving as negative control was obtained from Novabiochem, Switzerland.

HEK293 cells (2×10^5 per well) were seeded in 6-well plates. After 24 h the cells were transfected with the TLR-2 expression plasmid (2 μ g plasmid per ml media) by calcium phosphate precipitation [49]. For the NF- κ B reporter assay 1.25 μ g/ml pNF- κ B-Luc reporter plasmid (Clontech) and 0.6 μ g/ml pCMV-lacZ were added to the transfection mix. The cells were lysed and luciferase activity was measured with a tri-carb liquid scintillation analyzerTM (Packard, Meriden, CT) using reagents from the luciferase assay system of Promega Inc. The luciferase activities normalized for transfection efficiency are presented as multiples of the normalized luciferase activity of the untreated wild-type sample, which served as calibrator.

4.6 Induction of human and mouse IgE responses

PBMC (2.5×10^6) from bee venom-allergic subjects were cultured in 5 ml medium in 6-well plates and stimulated with IL-4, sCD40L, anti-CD2 mAb and the PLA antigen for 12 days with different doses of LP40 added to culture from the start. Anti-PLA IgE and anti-PLA IgG4 were measured in cultures by ELISA as described [7].

For parasite-induced IgE response, female BALB/c mice were injected s.c. with the nematode *N. brasiliensis* as described [16]. Compounds were given daily, and at day 10, serum samples were taken and antibody levels were determined by sandwich ELISA using IgE isotype-specific antibodies [50]. For anti-IgD-induced IgE response, BALB/c mice were injected i.p. with purified goat anti-mouse IgD antibody (produced in house, given 3 mg/animal) as described [17]. These mice evoked profound IgE (20–80 μ g/ml) and IgG1 (20–30 mg/ml) antibodies, which were shown to depend on T cell activation and IL-4 production. Antibody levels were determined by ELISA [50]. For MAIDS-induced IgE production, female C57BL/6 mice were injected i.p. with the LP-BM5 viral stock inducing a disease referred to as MAIDS as previously described [18]. LP40 was given 3 mg/ml i.p. weekly for 5 weeks. Serum Ig levels were measured after 11 weeks.

4.7 Induction of lung eosinophilia

Briefly, groups of male BALB/c mice were sensitized by two i.p. injections of 10 µg OVA in alum on days 0 and 14 [46]. Six days after the second sensitization (day 20), mice were challenged by intranasal administration of 25 µg OVA in 50 µl PBS. At 24 h after the challenge, bronchoalveolar cells were harvested and differential cell counts were performed (Baxter, Düringen, Switzerland).

4.8 Induction of parasite egg-induced granulomas

S. mansoni eggs were isolated from the livers of infected mice (Biomedical Research Institute, Rockville, MD) and 5,000 eggs/animal were injected i.v. [20]. Unsensitized animals were sacrificed at day 14 after i.v. egg challenge. For measurement of granulomas, the left lung was inflated with Bouin-Hollande fixative. The size and cell composition of the pulmonary granulomas were determined in Giemsa-stained histological sections. For cytokine measurements, lung-associated lymph node cells (3×10^6 cells/ml) were stimulated with soluble egg antigen. Supernatants were harvested at 72 h and assayed for cytokine activity by ELISA.

4.9 Statistical interpretation

Results are shown as mean $\pm$ standard deviation. Paired and non-paired Student's *t*-test was used for statistical analysis.

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