

ORIGINAL PAPER

Maternal allergy influences p38-mitogen-activated protein kinase activity upon microbial challenge in CD14⁺ monocytes from 2-year-old children

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Clinical and Experimental Allergy

Summary

Background The development of allergic diseases is dependent on genetic and environmental factors. It has been shown previously that cord blood mononuclear cells (CBMCs) from infants with parental allergy have altered cytokine profiles upon bacterial encounter; it might be possible that such impairment persists during the early years of childhood.

Objective The aim of this study was to investigate anti-microbial responses with regard to p38-mitogen-activated protein kinase (MAPK) activity in CD14⁺ monocytes and IL-6 release from mononuclear cells in the same group of children at birth and at 2 years of age.

Methods Paired samples of CBMCs and peripheral blood mononuclear cells (PBMCs) were stimulated with either lipopolysaccharide (LPS) or peptidoglycan *in vitro*. CD14⁺ monocytes were analysed for p38-MAPK activity by flow cytometry, and soluble IL-6 receptor, soluble glycoprotein 130 and IL-6 release from PBMC cultures were quantified by ELISA.

Results CBMCs from newborns with allergic mothers tended to have a lower IL-6 response following an LPS ($P = 0.09$) challenge compared with the group without maternal allergy while p38-MAPK activation levels did not differ between the groups. PBMCs from 2-year-olds with allergic mothers released significantly less ($P < 0.05$) IL-6 upon peptidoglycan stimuli compared with age-matched infants with non-allergic mothers. Infants with allergic mothers displayed markedly reduced CD14⁺ monocyte p38-MAPK phosphorylation after LPS ($P < 0.05$) and peptidoglycan ($P < 0.01$) challenge. This altered anti-microbial response was attributed to maternal allergy rather than to being IgE-sensitized at 2 years of age.

Conclusion Monocytes from children with allergic mothers are less responsive to bacterial challenge than monocytes from children with non-allergic mothers, and this impairment persists during the first 2 years of infancy.

Keywords CD14⁺ monocytes, IL-6, maternal allergy, microbial stimuli, p38-MAPK

Submitted 16 May 2007; revised 2 November 2007; accepted 16 November 2007

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Introduction

A positive family history of allergy is an important risk factor for the development of allergic disease [1]. Nonetheless, the interplay between genetic factors and early encountered environmental agents has been suggested to play a role in the development of allergy [2]. The escalation of allergic disease in the developed countries has recurrently been explained by a decreased encounter of infectious agents during childhood, a theory referred to as the hygiene hypothesis. At present, the immunological basis of the hygiene hypothesis is controversial [3, 4].

Pathogen-associated molecular patterns (PAMPs) are evolutionarily conserved motifs that are expressed by microbes. PAMPs are recognized by pattern recognition receptors (PRRs), like the toll-like receptors (TLRs), expressed on several immune cells such as monocytes. A signalling pathway that is common to all TLRs mediates the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs), resulting in the production of inflammatory cytokines [5, 6]. The p38 group of protein kinases belongs to the MAPK family and has been subjected to intense research regarding inflammatory diseases [7]. Both lipopolysaccharide (LPS) and

Table 1. Subject demographics

	Children with allergic mothers	Children with non-allergic mothers	P-value
Group size, <i>n</i>	14	14	
Female/male, <i>n</i>	4/10	6/8	NS
Obstetric information*			
Delivery mode, vaginal (caesarean)	13 (0)	12 (2)	NS
Maternal age at delivery, years, median (range)	31 (23–37)	32 (24–35)	NS
Birth weight, g, median (range)	3680 (2495–4400)	3700 (3200–4570)	NS
Gestation, weeks, median (range)	41 (38–42)	41 (39–42)	NS
Environmental exposure*			
Exclusive breastfeeding, months, median (range)	4.5 (3–6)	4.3 (0–6)	NS
Attending day care, <i>n</i> , yes (no)	12 (1)	12 (2)	NS
Age at which day care started, months, median (range)	17 (13–24)	17 (12–26)	NS
Pets at home, <i>n</i> , Cat (dog)	1 (1)	3 (0)	NS
Older siblings at home, <i>n</i>			
1 sibling	4	6	NS
≥ 2 siblings	1	1	NS
Maternal smoking, <i>n</i> , yes (no)	0 (13)	1 (13)	NS
IgE sensitized at 2 years of age, <i>n</i> , yes (no)	9 (4)	4 (9)	
Total IgE at 2 years of age, kU/L, Median (Range) [†]	34 (17–105)	14 (9–80)	0.0003

*Data based on 13 subjects with allergic mothers and all subjects with non-allergic mothers.

[†]Data based on 11 subjects with allergic mothers and all subjects with non-allergic mothers. NS, not significant.

peptidoglycan [8] can signal through TLRs and have been found to initiate p38-MAPK-mediated and/or IL-6 release from cells of monocytic origin [9, 10]. IL-6 is a multi-functional cytokine that is believed to have a central role in innate immune responses in addition to having an important role in clearing the innate response and directing a shift to the subsequent acquired immunity [11, 12]. Furthermore, IL-6 has been shown to bypass the suppressive action of regulatory T cells upon microbial challenge of TLR4 [13]. Alterations in IL-6 levels have been associated with pathology in several inflammatory diseases [14, 15]. Notably, IL-6 has been shown to play a significant role in the generation of IL-17-producing T helper (TH) cells, which are believed to play an important role in autoimmune as well as allergic diseases [16, 17].

IL-6 exerts its biological functions through signalling via at least one subunit of glycoprotein (gp) 130, together with either its membrane-bound receptor (IL-6R) or its soluble receptor (sIL-6R). Whereas gp130 is ubiquitously expressed, IL-6R expression is limited to certain cell types. However, IL-6-unresponsive cells may be rendered IL-6 responsive via sIL-6R, a mechanism termed IL-6-trans-signalling [18]. Further, the soluble form of gp130 (sgp130) functions as an antagonist to IL-6-trans-signalling [19].

We and others have shown that cord blood mononuclear cells (CBMCs) from newborns destined to be allergic later in life have altered cytokine profiles after stimulation with allergens or mitogens [20, 21]. In addition, our group has reported a reduced IL-6 release from peptidoglycan-stimulated CBMCs from newborns with allergic mothers compared with newborn babies with non-allergic mothers, in spite of normal TLR2 and TLR4 expression levels on the CB

monocyte cell surface before and after stimulation [22]. This suggests that an event downstream the TLRs could be responsible for the reduced IL-6 levels. The aim of this study was to investigate the influence of maternal allergy on microbially stimulated IL-6 production during the early years of childhood – at birth and in the same infants at 2 years of age. By applying a novel flow cytometry-based technique [23], we have analysed p38-MAPK phosphorylation levels in CD14⁺ monocytes and found it to be a plausible mechanism behind the altered IL-6 concentrations in children with allergic mothers.

Materials and methods

Subjects

For this study, a total of 28 children were randomly selected from a cohort of infants participating in a larger prospective study [24]. The parents provided a blood sample and underwent a skin prick test (SPT). Only those parents whose SPT results confirmed their positive or negative history of respiratory allergy to pollen and/or furred pets were invited to continue. In the group with maternal allergy, 12 children had two allergic parents and two children had an allergic mother. In the group with no maternal allergy, none of the 14 children had a parental history of allergy. There was no significant difference between the two heredity groups regarding environmental exposures such as attending day care, having pets at home, number of older siblings, the duration of exclusive breastfeeding or maternal smoking (Table 1). The Human

Ethics Committee in Stockholm approved this study and the parents provided their informed consent.

Skin prick testing

At age 2 years, the children underwent SPT against food and inhalant allergens, which were performed according to the manufacturer's recommendation (ALK, Copenhagen, Denmark). The SPT included food allergens: egg white (Soluprick weight to volume ratio 1/100, ALK); cod (Soluprick 1/20); peanut (Soluprick 1/20); cow's milk (3% fat, standard milk); and soy bean protein (Soja Semp[®], Semper AB, Stockholm, Sweden). SPT was also performed for inhalant allergens: cat; dog; *Dermatophagoides farinae*; birch; and timothy (Soluprick 10 HEP). All parents were skin prick tested against the same inhalant allergens as the children but also against horse, rabbit and mugwort. The SPT was considered to be positive if the weal diameter was ≥ 3 mm after 15 min. Histamine chloride (10 mg/mL) and the allergen diluent served as positive and negative controls, respectively.

Determination of specific immunoglobulin E

The children were serologically tested for the same allergens as for SPT, all performed with Cap-FEIA[™] (Phadia AB, Uppsala, Sweden). The test was considered to be positive if allergen-specific IgE levels were ≥ 0.35 kU_A/L.

Classification of the children

The children were followed from birth until 2 years of age and were clinically examined by the same paediatrician (C. N.) at 6, 12, 18 and 24 months of age. In accordance with Johansson et al. [25], the child was classified as IgE sensitized if at least one or more SPT was positive (≥ 3 mm) and/or if specific IgE against at least one or more of the selected allergens was ≥ 0.35 kU_A/L. In order to optimize the classification in IgE-sensitized and IgE-non-sensitized children, the results of the *in vivo* and *in vitro* analysis were combined. Nine out of 14 children with allergic mothers and five out of 14 children with non-allergic mothers were classified as IgE-sensitized (Table 1). Eczema was found in 11 out of 14 children with allergic mothers and in one out of 14 children with non-allergic mothers. With regard to IgE sensitization, 10 out of 14 of the IgE-sensitized and two out of 14 of the non-sensitized children had eczema.

Blood sample collection and separation of mononuclear cells

For ethical reasons, blood samples with sufficient volume for cell separation were only drawn at birth (cord blood) and at 2 years of age. Cord blood samples were collected

from the umbilical cord vein right after delivery with the aspiration technique and transferred into heparinized tubes. Further, blood was collected from the same children at 2 years of age. Mononuclear cells were separated by Ficoll-Paque (Pharmacia-Upjohn, Stockholm, Sweden) gradient centrifugation and thereafter stored in liquid nitrogen until analysis.

Stimulation of mononuclear cells in vitro

Cells were thawed and thereafter washed three times, followed by trypan blue exclusion of the non-viable cells. For analysis of released cytokines, 10^6 cells/mL were resuspended in tissue culture medium and were stimulated during 24 h (at 37 °C with 5% CO₂) with either 1 ng/mL LPS (*Escherichia coli* B, Sigma Aldrich, Stockholm, Sweden), 1 µg/mL peptidoglycan (*Staphylococcus aureus*, Sigma Aldrich) or remained unstimulated in medium alone. The supernatants were collected by centrifugation and stored in – 85 °C until analysis. To study phosphorylation of p38-MAPK in monocytes, 10^6 cells/mL were resuspended in tissue culture medium and incubated (at 37 °C with 5% CO₂) for 2 h before being stimulated for 30 min with either 10 ng/mL LPS, 10 µg/mL peptidoglycan or in medium alone. Phorbol 12-myristate 13-acetate (PMA) and ionomycin (both from Sigma Aldrich) served as positive controls.

Surface and intracellular target detection by flow cytometry

In order to detect phosphorylated p38-MAPK in monocytes, BD[™] Phosflow Phosphorylation State Analysis (BD Biosciences Pharmingen, San Diego, CA, USA) was performed according to the manufacturer's instructions for human peripheral blood mononuclear cell (PBMC) protocol 1. In brief, immediately after 30 min of stimulation, the cells were fixed (Fix Buffer I, BD Biosciences Pharmingen) for 10 min at 37 °C with 5% CO₂, then pelleted by centrifugation, after which the supernatant was discarded. Cells were resuspended and washed three times with permeabilization/washing buffer (Perm/Wash buffer I, BD Biosciences Pharmingen) before being labelled with titrated amounts of monoclonal mouse anti-human CD14 conjugated to phycoerythrin (PE, IgG2b, clone MφP, BD Biosciences, San Jose, CA, USA) and monoclonal mouse anti-phospho-p38-MAPK T180/Y182 conjugated to Alexa Fluor[®] 488 (AF488, IgG1, clone 36, BD Biosciences Pharmingen) for 30 min at room temperature in the dark. PE-conjugated monoclonal mouse IgG2b (anti-dansyl, BD Biosciences Pharmingen), AF488-conjugated monoclonal mouse IgG1 (clone MOPC-21, BD Biosciences) or unstained cells served as negative controls. The isotype control for anti-phospho-p38-MAPK did not display unspecific binding in unstimulated, LPS- or peptidoglycan-stimulated cells. However, low levels of phosphorylated

p38-MAPK were detected with anti-phospho-p38-MAPK in the unstimulated CD14⁺ cells, suggesting that unstimulated cells serve as an appropriate control. Gating was performed on live CD14⁺ monocytes that were analysed for phosphorylated p38-MAPK. Figure 1 illustrates representative plots of p38-MAPK phosphorylation in children with allergic and non-allergic mothers following peptidoglycan stimuli. The results are based on analysed geometrical mean fluorescence intensity (GeoMFI) values and are presented as a stimulation index (GeoMFI of phosphorylated p38-MAPK in stimulated CD14⁺ monocytes/GeoMFI of phosphorylated p38-MAPK in unstimulated CD14⁺ monocytes). All flow cytometric data were acquired by a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and further analysed with the software BD CellQuest™ Pro version 5.2.1.

Interleukin-6, soluble interleukin-6 receptor and soluble glycoprotein 130 enzyme-linked immunoassay

IL-6, sIL-6R and sgp130 in the cell-culture supernatants were quantified by the sandwich ELISA technique according to the manufacturer's instructions (RnD Systems, Abingdon, UK). All supernatants were centrifuged to remove particulate matter and kept frozen at -85°C until

analysis. The optical density (OD) was determined using a microplate reader (Molecular Devices, Sunny Vale, CA, USA) set at 450 nm. The detection limits for IL-6, sIL-6R and sgp130 were 0.70, 6.5 (mean value) and 0.05 ng/mL, respectively.

Statistical evaluation

The Mann-Whitney *U*-test was used to test for differences between the two groups of children. All box-plots display the following information: the central square within the boxes represents the median, the box covers 50% of the values lying between the 25th and 75th of the percentiles and the whiskers span to the non-outlier maximum and minimum values. Extreme outliers are not shown in the figures. Correlations between variables (IL-6 concentration and p38-MAPK stimulation index) were determined by Spearman's rank correlation test. Two-factor ANOVAS were performed to deduce which of the following factors, maternal allergy or IgE sensitization, exerted the largest effect on p38-MAPK phosphorylation after LPS and peptidoglycan stimulation. Data were log-transformed in order to better fulfil the ANOVA assumptions (normal distributed data with equal variance). All statistical analyses were performed with Statistica 7.1 software package

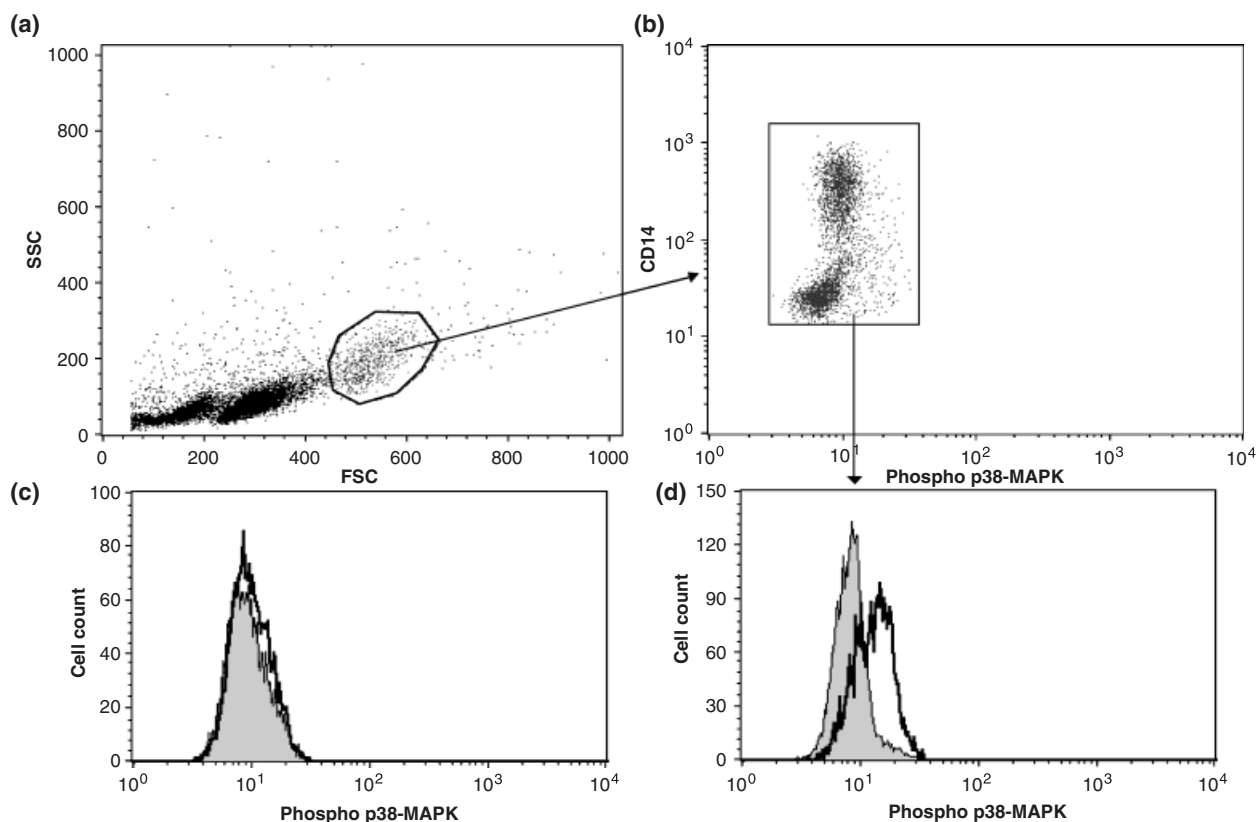


Fig. 1. Gating performed in order to define CD14⁺ monocytes for phospho-p38-MAPK analysis (a and b). Representative histogram plots displaying phosphorylated p38-MAPK upon peptidoglycan challenge (unshaded lines) in 2-year-olds with allergic mothers (c) and with non-allergic mothers (d). Unstimulated controls are shaded. MAPK, mitogen-activated protein kinase.

(StatSoft Scandinavia AB, Uppsala, Sweden), except two-factor ANOVAS, which were performed with Stata 8 software (StataCorp LP, College Station, TX, USA). *P*-values below 0.05 were considered to be statistically significant.

Results

Interleukin-6 release after peptidoglycan stimulation of peripheral blood mononuclear cells is reduced in 2-year-old children with allergic mothers

To investigate how maternal allergy affects microbially stimulated IL-6 production in children, we stimulated mononuclear cells from paired samples of children at birth and at 2 years of age with LPS and peptidoglycan for 24 h and determined the IL-6, sIL-6R and sgp130 concentrations with ELISA. There were no significant differences between the groups of infants concerning basal levels (unstimulated cells) of IL-6, neither at birth nor at the age of 2 (Fig. 2a). There was a trend towards lower IL-6 release in stimulated CBMC cultures in the group with maternal allergy compared with the group without maternal allergy. This trend was stronger for LPS than for peptidoglycan. At 2 years of age, PBMCs from the group of children with allergic mothers released signifi-

cantly lower ($P < 0.05$) amounts of IL-6 upon peptidoglycan challenge compared with PBMCs from the group with non-allergic mothers. There was also a persistent trend towards a reduced IL-6 release in PBMCs from children with allergic mothers following LPS stimulation. Increasing the sample size of the 2-year-old groups confirmed these differences and trends (Fig. 2b). No significant changes in IL-6 response were noted from birth to 2 years of age in the paired samples. There was no correlation in IL-6 responses from birth to the age of 2. Only low amounts of sIL-6R and sgp130 could be detected in the culture supernatants after LPS and peptidoglycan stimulation of mononuclear cells, both at birth and at 2 years of age, and there were no significant differences between the groups regarding these soluble factors (data not shown).

Two-year-old children with allergic mothers have reduced p38-mitogen-activated protein kinase phosphorylation in their CD14⁺ monocytes following lipopolysaccharide or peptidoglycan challenge

To investigate whether the decreased release of IL-6 after LPS and peptidoglycan stimulation could be a consequence of reduced activity of p38-MAPK in monocytes, we analysed p38-MAPK phosphorylation ability in stimulated CD14⁺ monocytes by flow cytometry. Throughout the study, we used an unstimulated control within each individual sample in order to determine the basal levels of phosphorylated p38-MAPK in the CD14⁺ monocytes. In the paired samples (Fig. 3a), there was no difference between the groups of newborns regarding the stimulation index of p38-MAPK phosphorylation after 30 min of challenge with LPS or peptidoglycan. At 2 years of age, however, peptidoglycan induced a significantly lower degree of p38-MAPK phosphorylation in CD14⁺ monocytes from children with allergic mothers than in the CD14⁺ monocytes from the group of infants with non-allergic mothers ($P < 0.05$). Regarding p38-MAPK response from birth to the age of 2, we noticed decreased p38-MAPK phosphorylation levels in 2-year-old children with allergic mothers ($P = 0.07$ for LPS and $P = 0.03$ for peptidoglycan). This was not noted in the group without maternal allergy. On comparing responses from birth to the age of 2, we observed no correlation in p38-MAPK response within the two groups. Notably, on increasing the number of 2-year-old individuals, we observed a larger significant difference regarding peptidoglycan challenge ($P < 0.01$), and there was also a significant difference between the groups following LPS stimuli ($P < 0.05$, Fig. 3b). Also, p38-MAPK phosphorylation levels in stimulated CD14⁺ monocytes strongly correlated with the quantified IL-6 levels in the corresponding PBMC culture supernatants ($r_s = 0.72$, $P < 0.001$ after LPS stimuli and $r_s = 0.81$, $P < 0.001$ after peptidoglycan stimuli).

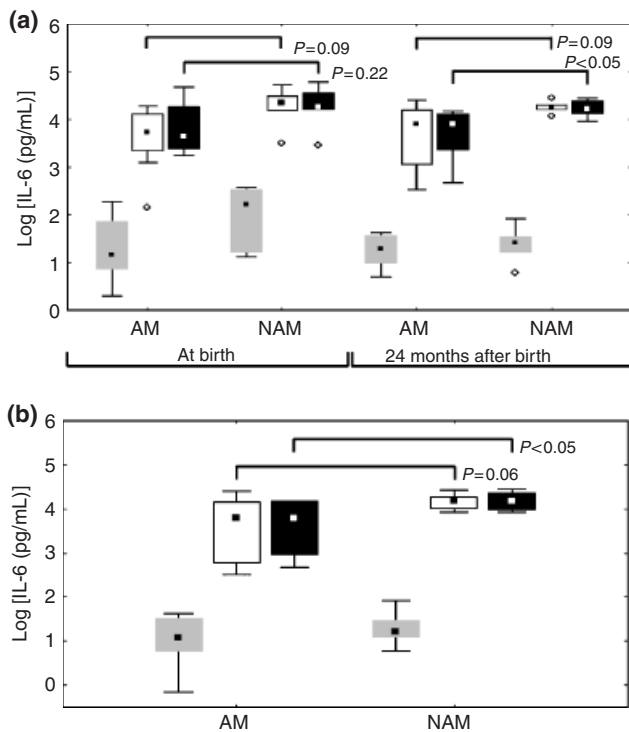


Fig. 2. IL-6 release from lipopolysaccharide- (□) or peptidoglycan (■)-stimulated mononuclear cells from children with allergic mothers (AM) and children with non-allergic mothers (NAM). (a) Paired samples from birth and at 2 years of age. (AM $n = 8$, NAM $n = 5$). (b) Extended group of 2-year-old samples (AM $n = 11$, NAM $n = 8$). ■, unstimulated mononuclear cells; ○, outliers.

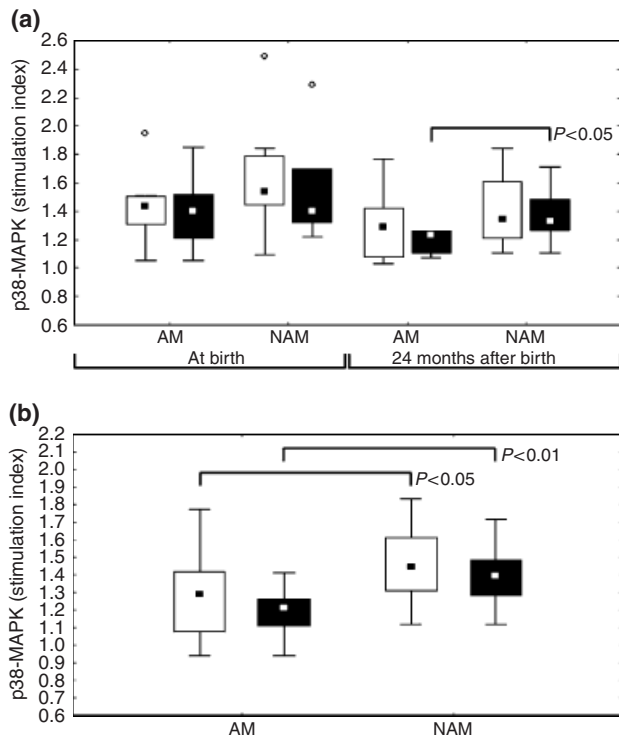


Fig. 3. p38-MAPK phosphorylation in CD14⁺ monocytes from children with allergic mothers (AM) and children with non-allergic mothers (NAM). (a) Paired samples from birth and at 2 years of age (AM $n = 10$, NAM $n = 9$). (b) Extended group of 2-year-old samples (AM $n = 14$, NAM $n = 14$). Stimulation index: GeoMFI of phosphorylated p38-MAPK in lipopolysaccharide- (□) or peptidoglycan (■)-stimulated CD14⁺ monocytes/GeoMFI of phosphorylated p38-MAPK in unstimulated CD14⁺ monocytes after 30 min of stimuli. ○, outliers. MAPK, mitogen-activated protein kinase; GeoMFI, geometrical mean fluorescence intensity.

Maternal allergy, but not immunoglobulin E sensitization, has a significant influence on p38-mitogen-activated protein kinase activation capacity in CD14⁺ monocytes of 2-year-old children

Because both groups of 2-year-old infants included IgE-sensitized and IgE-non-sensitized subjects, we wanted to address the contributory effect of maternal allergy vs. IgE sensitization on the observed differences in p38-MAPK phosphorylation. Therefore, we subdivided these two groups of children (Table 2) and evaluated this aspect statistically with a two-factor ANOVA for each stimulation (LPS and peptidoglycan) separately. Table 2 shows the back-transformed ANOVA estimates of the average stimulation index for p38-MAPK phosphorylation in each subgroup. After peptidoglycan challenge, there was a significantly higher p38-MAPK phosphorylation in the group of children with non-allergic mothers compared with children with allergic mothers (estimated ratio 112%, $P = 0.01$). The effect of IgE-sensitization status on p38-MAPK activity was not significant (estimated ratio 104%, $P = 0.85$). The results were less clear regarding LPS stimu-

lation (estimated ratio 115%, $P = 0.11$ regarding the factor maternal allergy, and estimated ratio 101%, $P = 0.58$, for the factor IgE sensitization), although they followed a trend similar to that for peptidoglycan challenge. There were no significant interactions between IgE sensitization and maternal allergy ($P = 0.75$ for LPS and $P = 0.73$ for peptidoglycan).

Discussion

By applying a novel phospho-specific flow cytometry-based method [23], we have studied p38-MAPK activity within CD14⁺ monocytes on a single-cell basis. To our knowledge, we are the first to demonstrate an association between maternal allergy and reduced p38-MAPK phosphorylation following microbial stimulation in CD14⁺ monocytes in 2-year-old children. The decreased p38-MAPK phosphorylation levels of CD14⁺ monocytes correlated strongly with reduced IL-6 levels quantified in the PBMC culture supernatants.

Pregnancy is an immunologically complex phenomenon that skews newborns towards a TH2-biased immunity during their neonatal period. By encountering pathogenic and non-pathogenic microbes during early childhood, there will be a progressive shift towards a TH1-type-immunity initiated by a proper regulatory T cell activity and secretion of TH1-polarizing cytokines [26]. In the wake of the hygiene hypothesis, PRR-mediated signalling and its potential to link innate and adaptive immunity has been in focus in allergy research. The associations between polymorphisms in TLRs and anti-microbial responses as well as the development of allergic disease have been investigated [27, 28]. Earlier work from our group has shown that CBMCs from newborns with allergic mothers released less IL-6 following peptidoglycan challenge than did CBMCs from babies with non-allergic mothers [22]. Our previous findings suggested that maternal allergy might modify the intrauterine environment, an alteration that could have an impact on immunity already at birth and possibly also on immune maturation during early years. This could have consequences for anti-microbial responses and subsequently also for allergy development. This is further supported by another study reporting that 2-year-old children with atopic disease displayed reduced basal human leucocyte antigen (HLA)-DR levels on their cord blood monocytes compared with 2-year-olds without atopic disease [29].

In the present study, we investigated whether the reduced IL-6 response to microbial stimuli at birth in children with allergic mothers persisted through childhood. Further, we aimed to elucidate a potential mechanism behind the reduced IL-6 response. Because we observed previously that it was not altered basal or stimulated TLR2 or TLR4 expression on the monocyte surface that caused this impaired IL-6 response, this time

Table 2. Estimated averages of the p38-MAPK stimulation index after LPS and peptidoglycan challenge in each subgroup of 2-year-old children ($n = 28$)

	Maternal allergy		Stimuli
	Yes	No	
IgE sensitized	1.23*	1.41 [†]	LPS
IgE non-sensitized	1.31 [‡]	1.44 [§]	
IgE sensitized	1.19*	1.39 [†]	Peptidoglycan
IgE non-sensitized	1.22 [‡]	1.38 [§]	

On comparing these estimates, the P -values were 0.58 and 0.85 between the IgE-sensitization status after LPS and peptidoglycan stimuli, respectively. The P -values were 0.11 and 0.01 between the allergic status of the mothers after LPS and peptidoglycan stimuli, respectively.

* $n = 9$, [†] $n = 5$, [‡] $n = 5$, [§] $n = 9$.

MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide.

we searched for an explanation within the monocyte. We considered p38-MAPK to be a suitable intracellular target for this study because it is involved in both LPS- and peptidoglycan-mediated signalling [9, 10], and it has also been implicated as a potential target for therapeutics [7]. Members of the NF- κ B signalling pathway such as I κ B α would also have been highly relevant to study in the context of anti-microbial responses [5, 6]; yet, the difficulty of detecting phosphorylated I κ B α before its rapid ubiquitination and degradation [30] encouraged us to select p38-MAPK in this particular study. Moreover, p38-MAPK has been shown to play a crucial role in the maturation of dendritic cells after LPS challenge [31]. Whether peptidoglycan is recognized by TLR2 has been questioned as the intracellular nuclear oligomerization domain 2 (NOD2) has been proven recently to be a ligand for peptidoglycan [32]. However, novel studies confirm the role of both TLR2 and NOD2 as ligands for peptidoglycan and show that p38-MAPK can be activated via both pathways [reviewed in [33] and [34].

Here we found that at birth, CBMCs from children with allergic mothers tended to have reduced IL-6 levels following microbial stimulation, although the difference did not reach statistical significance. This does not entirely agree with our previous findings, where IL-6 levels differed significantly between the two groups at birth, but can be explained by a relatively small (paired) sample size and the fact that CBMCs from our previous study originated from neonates who were delivered with elective caesarean section in order to limit the 'background effect' of labour on pro-inflammatory cytokine production [35]. In this prospective study, all the CBMCs from which IL-6 release was quantified originated from babies delivered vaginally, which makes it possible that the baby's actual IL-6 levels upon microbial challenge are partly 'masked' by the delivery process.

Interestingly, PBMCs from the same children at 2 years differed in their IL-6 response following stimulation with peptidoglycan, where PBMCs from children with allergic mothers released significantly lower amounts of IL-6 ($P < 0.05$). Notably, the increased sample size of the 2-year-old groups confirmed the observation regarding peptidoglycan challenge and, in fact, pointed towards a similar tendency upon LPS challenge as well ($P = 0.06$). It has been demonstrated that IL-6 bypasses the suppressive effect of regulatory T cells after bacterial challenge in mice [13], which could be important for the generation of proper anti-microbial (TH1-dominated) immune responses and could skew the immune system away from a profile governing for example allergic responses. However, IL-6 is a multi-faceted cytokine that also promotes the generation of TH17 cells [16], which in turn have been implicated as cells driving several diseases, including allergy.

IL-6 trans-signalling, which is antagonized by the presence of sgp130 [19], has been shown to be a critical point in the maintenance of several inflammatory diseases [18]. It has also been shown that adult patients suffering from allergic airway inflammation display higher amounts of sIL-6R in their bronchoalveolar lavage fluid both at basal levels and upon allergen challenge [36]. Considering the complexity of IL-6 signalling, we were also interested in quantifying sIL-6R and gp130 in culture supernatants upon LPS and peptidoglycan challenge. We found low detectable amounts of both these soluble factors, with no significant difference between the two groups of children, either at birth or at 2 years of age, indicating that no altered levels of these soluble factors could be detected systemically.

In this study, we can report that following LPS and peptidoglycan challenge, 2-year-old infants with allergic mothers phosphorylated p38-MAPK in their CD14⁺ monocytes to a significantly lower degree than their age-matched counterparts with non-allergic mothers. There were no significant differences between the groups at birth, which could be explained in a manner similar to the results for IL-6, discussed above. We have been able to study intracellular phosphorylation in a distinctly phenotyped cell population by applying a recently developed flow cytometric method in which the phosphorylated p38-MAPK levels detected have been shown to correlate highly to the levels detected by Western blot analysis [37]. The strong correlation between p38-MAPK phosphorylation and IL-6 release in this study following both LPS and peptidoglycan stimulation suggests that an altered monocyte function may play an important role in the reduced anti-microbial IL-6 response in PBMCs from children with allergic mothers. Our findings are supported by other studies showing that peptidoglycan-stimulated mouse macrophages produce IL-6 through pathways involving p38-MAPK [9] and that p38-MAPK-mediated signalling is involved in LPS-induced monocyte inflammatory

responses [38]. However, we are not excluding that the altered activity of other signalling intermediates could contribute to the reduced IL-6 levels. Further, as the measurement of total p38-MAPK levels was not possible with the phospho-specific cytometric technique, we cannot rule out the possibility that the total p38-MAPK protein levels in the subjects might vary. However, because basal phosphorylation levels of p38-MAPK were taken into account before activation of the cells we do not consider variations of total p38-MAPK levels as a major contributing factor to the observed differences, which are expressed as ratios between stimulated and unstimulated CD14⁺ cells.

Soluble CD14 (sCD14) has been shown to bind to both LPS and peptidoglycan and influence their signalling, suggesting that it could affect these microbial responses. sCD14 levels are highly influenced by genetics, and low levels of sCD14 have been associated with allergic disease in some studies [39]. We did not address this question in our study, but it is possible that the CD14 genotype and/or sCD14 levels contribute to the decreased LPS- and peptidoglycan-induced monocyte responses in children with allergic mothers.

Because both groups of 2-year-old children included infants with mixed IgE sensitization (i.e. being or not being sensitized at the age of 2 years) it was of interest to evaluate the role of maternal allergy vs. IgE sensitization with respect to CD14⁺ monocyte p38-MAPK activation capacity. When comparing 2-year-old children with or without allergic mothers, we show that children with a parental history of allergy have a significantly lower capacity to activate their CD14⁺ monocyte p38-MAPK upon challenge with peptidoglycan irrespective of the IgE-sensitization status. A similar trend was observed after LPS challenge although not statistically significant. This clearly suggests that the degree of p38-MAPK phosphorylation following microbial challenge is related to maternal allergy and not to IgE sensitization at 2 years of age. It should be emphasized that there were no statistically significant differences between the two groups of children regarding environmental exposure factors such as number of siblings, maternal smoking, duration of breastfeeding or having pets at home (Table 1), which could have influenced monocyte activity at 2 years of age. The utilization of antibiotics would also have been interesting to take into consideration; however, this was not possible in this study. How p38-MAPK activity relates to a developed allergic disease later in life remains to be elucidated. An active allergic disease is accompanied by an inflammatory response, which could very well be associated with elevated p38-MAPK levels.

Our results are interesting in relation to the hygiene hypothesis, which postulates a relationship between microbial exposure and the development of allergic disease. The data presented here, together with our previous

study, indicate that maternal allergy renders the monocyte population less responsive to microbial challenge and that this impairment persists during the first 2 years of life.

Acknowledgements

We thank Monica Nordlund, Anna Stina Ander and Ann Sjölund for their valuable assistance, and Jan-Olov Persson for providing excellent statistical support. Supported by grants from the Swedish Asthma and Allergy Association's Research foundation, the Swedish Research Council (Medicine) grant no 74X-15160, the Swedish Medical Society, the Golden Jubilee Memorial Foundation, the Crown-princess Lovisa & Axel Tielman, Vardal, Golje, Hesselman and Åhlén foundations. Saghafian-Hedengren S. was partly supported by The BioMalPar European Network of Excellence (LSMP_CT-2004-503578).

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