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ORIGINAL ARTICLE

Exposure to dogs is associated with a decreased tumour necrosis factor-α-producing capacity in early life

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Summary

Background It appears that contacts with furred animals early in life and already during gestation contribute to the immunological development in humans, but the mechanisms and relevant exposures are not clear.

Objective To investigate whether exposure to animals during pregnancy and the first year of life is associated with early immune development, determined as stimulated cytokine responses of children at birth and at age 1 year.

Methods Cord blood ($n = 228$) and peripheral venous blood ($n = 200$) samples 1 year after birth were collected and stimulated with Gram-positive superantigen Staphylococcal enterotoxin B, Gram-negative bacterial lipopolysaccharide (LPS) and the combination of mitogenic phorbol 12-myristate 13-acetate and calcium ionophore ionomycin (P/I) for 24 and 48 h. TNF-α, IFN-γ, IL-5, IL-8 and IL-10 responses were measured by ELISA. For each cytokine, the time-point with the highest response was chosen for further analyses. Animal contacts were surveyed by self-administered questionnaires.

Results Dog ownership was associated with decreased TNF-α-producing capacity at birth (P/I: median 841 vs. 881 pg/10^6 WBC, $P = 0.05$) and 1 year after birth (P/I: 1290 vs. 1530, $P = 0.01$; LPS: 425 vs. 508, $P = 0.02$). Associations remained significant after adjustment for potential confounders. Cat ownership was not associated with cytokine production.

Conclusion Having a dog in the household in infancy and already during pregnancy may be associated with reduced innate immune responses in early childhood. The observed attenuation of cytokine production may help in preventing exaggerated immune responses against harmless antigens later in life. Thus, intensive exposure to dogs in early life may be beneficial during normal immune maturation.

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Introduction

The immune system goes through a major maturation process in early life, starting already before birth [1]. At the same time, the most prevalent childhood diseases, atopy and allergic disorders, begin to develop [1, 2]. There is a growing body of evidence to suggest that exposure to microbial compounds [3, 4] as well as pet-keeping [5, 6] make a strong contribution to these complex immunological processes. Moreover, the timing of animal contact during the first years of life appears to play a crucial role [7]. It is also believed that maternal exposure to stables and an increasing number of different farm animals already during pregnancy affect the expression of the receptors in the innate immunity of the child [8]. The effect of early life exposures to pets on the development of allergies and immune system is of special interest, not least because pet-keeping is very common, but also due to the previous conflicting findings suggesting that pets could either promote [9–11] or prevent [11–16] the development of allergic diseases. In addition, complicated interactions between environmental exposures and the genetic constitution of the child make it difficult to assess the influence of one particular environmental stimuli,

because the same exposure may either increase or decrease (or have no influence on) the risk of asthma or allergic sensitization, depending on the genetic susceptibility of the child [17].

The underlying immunological mechanisms to explain how microbial exposures can influence the development of immune functions and allergic disorders are still poorly understood but initially it was postulated that diminished exposure to microbes and their components in early life could favour the maintenance of postnatally default T-helper type 2 (Th2)-responses (missing immune deviation) [18]. An increased understanding of the many components of the immune system including Toll-like receptors (TLRs), T-regulatory cells and dendritic cells indicate that this theory may be an oversimplification. Moreover, long-term intensive exposure to house dust endotoxin appears to be associated with down-regulated cytokine responses among exposed children [3]. Nonetheless, the immunological pathways activated following animal exposures are still not clear, although it has been shown that exposure to cat allergens in childhood may result in elevated IgG and IgG4 production without the risk of asthma or sensitization (modified Th2 response) [19, 20]. Both of the postulated alternative mechanisms could be related to the development of tolerance against environmental antigens among exposed children.

Our previous study detected an association between elevated house dust endotoxin and IFN-γ-producing capacity from birth to 3 months of age. Also farming, poor cleanliness and having a dog or a cat appeared to increase the subjects levels of IFN-γ [21]. However, due to low number of samples studied we could not assure which of these exposure variables was the best predictor for IFN-γ. Because there are few other studies that have investigated the effect of pet ownership on the maturation of the immune system during infancy [5, 6], in this paper we have focused on animal exposures. Earlier, we have examined the general maturation of the immune system by comparing the stimulated production of pro-inflammatory (TNF-α), Th1 (IFN-γ), Th2 (IL-5) and anti-inflammatory (IL-10) cytokines as well as chemokine (IL-8) in whole blood samples of children at birth and at the age of 1 year [22]. In order to investigate further if immunological development is affected by exposures to animals during pregnancy and in early life, stimulated cytokine responses were compared with animal contact data gathered from a written questionnaire filled in by the mothers during pregnancy.

Methods

Study population

The study subjects ($n = 228$, mother–child pairs from Eastern Finland) belong to a prospective birth cohort study called LUKAS2. More detailed information about the participants, recruitment process and inclusion criteria have been published previously [22, 23]. Briefly, an invitation letter was sent to 1763 pregnant women of which 886 fulfilled inclusion criteria (maternal age of 18 years or older, singleton pregnancy, native language Finnish, no plans of moving from the study area and not living in an apartment). A total of 458 were willing to participate and 228 of them fulfilled further inclusion criteria (parturition at term (≥ 37 weeks of gestation), no neonatal congenital abnormalities, successful cord blood sampling, delivery between Sunday afternoon and Thursday morning). All the children were born between May 2004 and May 2005 at Kuopio University Hospital. Written questionnaires were sent to mothers during the last trimester of pregnancy and again at 2 and 12 months after delivery. All mothers signed an informed consent form. Ethical permission was granted by the Research Ethics Committee, Hospital District of Northern Savo.

LUKAS2 is an extension to LUKAS1 Study. LUKAS1 is the Finnish part of the European birth cohort study called Protection against Allergy – Study in Rural Environments (PASTURE) which includes other mothers and their children from Austria, Switzerland, France and Germany [24]. LUKAS2 follows a similar protocol as the LUKAS1, but only 11 out of 228 mothers were from actively operating farms whereas the focus of LUKAS1 is on farming families [23]. This is due to the fact that initially the LUKAS2 was established to serve a larger study population (without a farm perspective) for additional studies started in LUKAS1, such as a more detailed assessment of dietary habits and a broader determination of moisture problems and the microbial environment. Participants of LUKAS2 were not selected according to either occupation or area of living. However, in order to make housing conditions comparable between LUKAS2 and LUKAS1, only mothers from non-apartment households were enrolled into the study.

Blood sampling and immunoglobulin E analysis

Cord blood samples ($n = 228$) at birth and peripheral venous blood samples at the age of 1 year ($n = 200$) were collected in lithium-heparin and in EDTA tubes by an aspiration technique (Vacutainer, Becton&Dickinson, Plymouth, UK). Temporary storage and the transportation of blood samples were carried out at 4 °C. Samples were processed for further analysis within 27 h. The atopic status of the mothers and children (at the age 1 year) was assessed by measuring specific IgE levels for six common food and 13 inhalant allergens from serum [22] in the PASTURE Central Laboratory (Marburg, Germany). Atopy was defined as an IgE value $\geqslant 0.35$ kU/L for any of the measured allergens.

Stimulation of whole blood cell culture and analysis of cytokine production

Preparation and stimulation of whole blood culture and the measurement of TNF-α, IFN-γ, IL-5, IL-8 and IL-10 responses are described previously [22]. Briefly, to stimulate the production of cytokines, whole blood culture was incubated for 24 and 48 h with Staphylococcal enterotoxin B (SEB) (final concentration 100 ng/mL), lipopolysaccharide (LPS) (100 ng/mL) and the combination of phorbol 12-myristate 13-acetate (PMA) (5 ng/mL) and ionomycin (1 μg/mL) (all from Sigma Chemicals, St Louis, MO, USA, except LPS from Research Center Borstel, Borstel, Germany). The production of cytokines was analysed with sandwich ELISA (OptEIA^{TM} Human ELISA set, BD Biosciences, San Diego, CA, USA). The manufacturer's protocol was followed in cytokine analysis except that the optimal antibody concentrations were determined using in-house titration experiments and the number of washes was increased. The ranges of cytokine measurements were set according to the validation tests: IFN-γ (12.5–800 pg/mL), IL-5 (4.7–300 pg/mL), IL-8 (15.6–1000 pg/mL), IL-10 (6.3–400 pg/mL) and TNF-α (15.6–1000 pg/mL). Cytokine concentrations below the detection limit were given a value of 0 pg/mL. If the concentration of measured cytokine exceeded the upper limit of the standard curve, the sample was diluted to obtain a concentration within the range. The individual white blood cell (WBC) count was measured from EDTA–blood using a Sysmex KX-21N blood cell analyzer (Sysmex Corporation, Kobe, Japan).

Although for most of the cytokines, the highest concentrations were detected with the 48 h stimulation, the productions of IL-10 (P/I and LPS stimulation) and TNF-α (LPS stimulation) were higher at the 24 h stimulation. The results for each cytokine are presented from the timepoint when the measured concentrations were at their highest. IFN-γ and IL-5 responses following LPS stimulation both at birth and at 1 year of age and SEB-stimulated IL-5 in cord blood are not presented, because $>60\%$ of values were below the detection limits.

Determination of animal exposures

The animal exposure data were acquired from written questionnaires collected at the third trimester of pregnancy. Mothers in questionnaires also filled 2 and 12 months after birth, but because pet-keeping remained nearly constant during the 1-year period, pet ownership was determined from the pregnancy questionnaire. The current farming status of the family was asked: 'Are you living in an actively operating farm?' (Yes/No). Maternal contact to different farm animals was determined for each species separately: 'How often were you in contact with the following animals during this pregnancy: horse, cow, pig, sheep/goat, hare/rabbit, chicken/poultry?' (Never or rarely/Several times each month/Several times each week/Daily). Mothers were regarded as exposed if they had animal contact several times each month or more. Dog ownership was enquired as follows: 'Do you have a dog' (Yes/No), 'Is/are the dog(s) kept indoors (Never/Sometimes/Often/Always)?' Cat owner status was asked with similarly formulated questions.

Statistical analysis

To avoid the possible confounding effect of individual variation in baseline cytokine production, cytokine data are expressed as Δ values (stimulated cytokine values minus unstimulated values). Negative Δ values (stimulated value smaller than the unstimulated value) were given a value of 0 pg/mL. In addition, cytokine Δ values were standardized according to the number of leucocytes (picograms/one million WBC, pg/10^6 WBC). Each of the animal exposure variables was dichotomized. The effects of animal exposures on cytokine-producing capacity were analysed using the non-parametric Mann–Whitney test. In addition, statistically significant ($P \leqslant 0.05$) associations in non-parametric tests were analysed further: the cytokine data were ln-transformed to achieve a more normal distribution and linear regression models were used to adjust for potential confounding variables. Residuals of linear regression models were examined to reveal possible outliers (absolute value of standardized residual above three) and influential observations. These examinations lead to the exclusion of a few observations from the analyses, namely 22 observations from the analyses of TNF-α (P/I, cord blood $n = 5$, 1 year, $n = 2$; SEB, cord blood $n = 0$, 1 year $n = 7$; LPS, cord blood $n = 5$, 1 year, $n = 3$). Based on biological plausibility and preliminary results from multivariate linear regression tests including a wide variety of different variables, the following potential confounders were selected for further analyses: mode of birth, number of earlier deliveries (four groups: 0/1/2/$\geqslant$3), use of antibiotics during pregnancy, maternal atopy (specific IgE$\geqslant$0.35 kU/L), maternal doctor-diagnosed asthma, maternal education (comprehensive school, vocational school, upper secondary school or academic) number of 0–12-year-old siblings in the household (three groups: 0/1/$\geqslant$2), maternal smoking (four groups: never smoked over five packets of cigarettes in their life/given up smoking/given up smoking during pregnancy/smoked during pregnancy) and gender of the child. Only those confounders, which changed the effect estimate of the exposure of interest by $>10\%$, were included in the final multivariate models.

Only a few households had pets other than cats or dogs, thus other domestic animals were not taken into account in the analysis. Because of the small number of mothers reporting contact with different farm animal species (cow, pig, sheep/goat, hare/rabbit and chicken/poultry),

associations between cytokines and these exposures were also not considered. Because the number of farming homes was only 11 out of 228, the farming status was not included into models as a potential confounding factor. In non-parametric tests, children from farming families expressed generally lower cytokine responses in comparison with their peers from non-farming households at birth and at the age of 1. Thus, significant associations between cytokine responses and dog ownership were tested also by excluding these farming families from the analyses. When evaluating associations between maternal horse exposure and cytokine-producing capacity in multiple regression models, cat or dog ownership was also included among the confounding variables. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software version 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P \leqslant 0.05$. *P*-values were not adjusted for multiple testing [25].

Results

Animal exposures

During the last trimester of pregnancy, it was more common to have a dog (36%) than a cat (17%) in the household. Pets were typically kept indoors: only 7% of the dogs and 10% of the cats were only housed outdoors. Dog ownership among dog-sensitized mothers (38%) was similar to those of non-sensitized (35%). In addition, the prevalence of a cat in the household among cat-sensitized mothers (19%) was close to that of non-sensitized (16%). Dog and cat ownership changed only marginally during the 1-year period: 10 of 82 households had given up keeping dogs and two new families became dog owners, whereas two of 38 families gave up their cats and three households took a cat into their home. Therefore, in the statistical analysis, animal exposure data from pregnancy questionnaire were used.

Mothers also were exposed to some extent to horses (11%), cows (8%) and hare/rabbits (4%) whereas exposure to other animals (pig, sheep/goat and chicken/poultry, separately) was $< 1\%$ among the study population. Many of those who were exposed to horses ($n = 24$) had also cat/s (33%) or dog/s (54%) in the household. As expected, animal contacts among farming families ($n = 11$) were more frequent than among non-farming ($n = 217$) households: Dog-keeping, 82% of farming vs. 34% of non-farming families; cat-keeping 64% vs. 14%; horse exposure 36% vs. 9% and cow exposure 73% vs. 4%.

Associations between animal exposures and cytokine-producing capacity in infancy

Dog and cat. The presence of a dog in the home during pregnancy was significantly associated with a decreased capacity to produce TNF-α at birth (Table 1: P/I, $P = 0.05$) and at the age of 1 year (Table 2: P/I $P = 0.01$; LPS, $P = 0.02$). Associations remained statistically significant following adjustment for potential confounders (Table 3). Because farm exposure may affect cytokine responses [21, 26], the relationship between dog ownership and TNF-α production was further studied by excluding the farming families from the multiple linear regression tests;

Table 1. *Cord blood*: Stimulated cytokine-producing capacity (median of individual Δ values, IL-8 as ng/10^6 WBC, other cytokines as pg/10^6 WBC) according to animal exposure variables

		IL-5	IL-10			TNF-α			IFN-γ		IL-8		
	*n**	P/I	P/I	SEB	LPS	P/I	SEB	LPS	P/I	SEB	P/I	SEB	LPS
Dog													
Yes	82	76.6	14.8	9.61	483	841	19.4	81.7	3130	111	189	18.7	110
No	145	74.9	13.8	8.63	522	881	24.2	83.7	3840	103	236	19.5	126
P-value		0.62	0.65	0.85	0.14	**0.05**	0.24	0.22	0.42	0.72	0.09	0.78	0.35
Cat													
Yes	38	68.6	13.7	7.43	454	722	22.8	74.8	3340	96.5	211	16.1	102
No	189	78.7	14.5	9.34	520	873	23.4	85.9	3480	107	224	19.3	129
P-value		0.38	0.84	0.80	0.25	0.50	0.68	0.79	0.57	0.44	0.66	0.94	0.37
Horse													
Yes	24	79.4	13.7	9.41	477	841	9.11	69.0	3590	125	254	18.2	115
No	204	75.9	14.4	8.66	514	861	24.2	83.5	3440	100	211	19.3	125
P-value		0.71	0.56	0.84	0.29	0.81	**0.03**	**0.05**	0.96	0.95	0.89	0.96	0.14

*Total number of subjects; Because of priority order in stimulations, the number of individual cytokine results varied between 228 and 225 for 24 h stimulation (IL-10 P/I, IL-10 LPS, TNF-α LPS) and between 172 and 169 for 48 h stimulation.

Statistically significant differences ($P \leqslant 0.05$) in non-parametric tests (Mann–Whitney) are indicated with a bold font.

P/I, phorbol 12-myristate 13-acetate and ionomycin; SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide; WBC, white blood cell.

Table 2. *Child, 1 year*: Stimulated cytokine-producing capacity (median of individual Δ values, IL-8 as ng/10^6 WBC, other cytokines as pg/10^6 WBC) according to animal exposure variables

		IL-5		IL-10			TNF-α			IFN-γ		IL-8		
	*n**	P/I	SEB	P/I	SEB	LPS	P/I	SEB	LPS	P/I	SEB	P/I	SEB	LPS
Dog														
Yes	67	110	50.4	81.5	79.8	600	1290	310	425	14500	836	73.3	66.4	91.9
No	127	130	45.6	84.3	77.7	593	1530	274	508	16800	680	89.7	46.3	107
P-value		0.60	0.89	0.86	0.25	0.97	**0.01**	0.53	**0.02**	0.16	0.71	0.11	0.43	0.08
Cat														
Yes	32	139	47.4	80.7	71.9	569	1340	262	428	16300	684	71.2	42.3	95.2
No	162	116	48.5	84.2	78.0	598	1460	298	484	16300	744	80.3	53.0	100
P-value		0.98	0.84	0.35	0.58	0.74	0.76	0.28	0.15	0.54	0.43	0.22	0.07	0.44
Horse														
Yes	18	66	36.5	80.6	73.2	555	1410	311	451	19900	1040	74.8	56.6	73.4
No	177	130	52.0	83.8	78.8	600	1430	289	473	16000	704	78.4	52.2	103
P-value		0.17	0.18	0.71	0.95	0.96	0.91	0.62	0.60	0.42	0.75	0.50	0.67	0.31

*Total number of subjects; Because of priority order in stimulations, the number of individual cytokine results varied between 195 and 192 for 24 h stimulation (IL-10 P/I, IL-10 LPS, TNF-α LPS) and between 193 and 188 for 48 h stimulation.
Statistically significant differences ($P \leqslant 0.05$) in non-parametric tests (Mann–Whitney) are indicated with a bold font.
P/I, phorbol 12-myristate 13-acetate and ionomycin; SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide; WBC, white blood cell.

Table 3. The effect of multivariable adjustment for assessing associations between dog and horse exposures and stimulated TNF-α responses at birth and at the age of 1 year

			Difference in TNF-α producing capacity†			
			Cord blood		1 year of age	
Animal exposure	Stimulant	Model*	%	Significant	%	Significant
Dog	P/I	Unadjusted	−21	**0.03**	−16	**0.01**
		Adjusted	−25	**<0.01**	−16	**0.01**
	SEB	Unadjusted	−33	0.18	19	0.25
		Adjusted	−19	0.54	17	0.29
	LPS	Unadjusted	−17	0.11	−18	**0.02**
		Adjusted	−15	0.16	−16	**0.04**
Horse	P/I	Unadjusted	−11	0.53	8.1	0.51
		Adjusted	28	0.24	13	0.31
	SEB	Unadjusted	−68	**0.04**	39	0.22
		Adjusted	−41	0.39	34	0.30
	LPS	Unadjusted	−34	**0.02**	−8.3	0.54
		Adjusted	−24	0.12	−1.5	0.92

*Confounders, which changed the exposure effect estimate (= B value) > 10%, were included in the adjusted models.
†Describes the fraction ($(e^B)-1\times100\%$) by which the TNF-α-producing capacity of the exposed group differs from non-exposed.
Statistical significance ($P \leqslant 0.05$) indicated with a bold font.
P/I, phorbol 12-myristate 13-acetate and ionomycin; SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide.

associations remained significant for P/I-stimulation at birth (Fig. 1) and at 1 year of age (Fig. 1), and almost for LPS-stimulation at the age of 1 year ($P = 0.07$). The production of other measured cytokines (IL-5, IL-10 and IFN-γ) at birth or at the age of 1 year was not affected by the exposure to dogs. Cat ownership did not have an influence on measured cytokine responses.

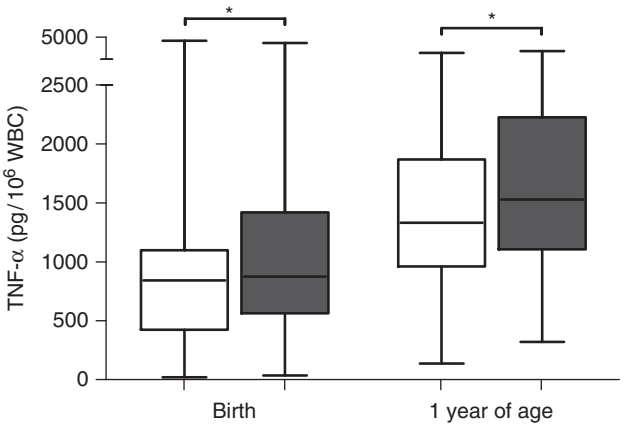


Fig. 1. Associations between dog ownership and TNF-α-responses after exclusion of farmers' children. TNF-α-producing capacity (pg/10^6 WBC) following 48 h P/I-stimulation at birth and 1 year after birth in children from non-farming households with (empty boxes) or without (filled boxes) a dog in the home. Box plots represent the inter-quartile range, median (horizontal line) and extremes (whiskers) of individual cytokine Δ values. *$P \leqslant 0.05$ after multiple linear regression analysis of ln-transformed data. WBC, white blood cell.

Horse. Maternal horse-exposure during pregnancy was associated with decreased cord blood TNF-α responses (Table 1: SEB, $P = 0.03$; LPS, $P = 0.05$). However, after adjustment for potential confounders, the findings were no longer statistically significant (Table 3). At the age of 1 year, no associations were detected between maternal horse exposure and cytokine-producing capacity.

Discussion

The focus of this paper was to examine whether exposure to animals during the pregnancy and the first year of life can affect the development of immune functions in infancy. Our study supports the hypothesis that animal exposure may influence immune responses during early life. Furthermore, it offers new insights into the potential causal immunological mechanism to explain how exposure to animals may be linked to the maturation of immune system. The main finding from this prospective birth cohort study was that children living in households with a dog/s had reduced TNF-α-producing capacity in comparison with their peers with no dog in the home. The effect of dog exposure was seen both in cord blood (P/I stimulation) and in peripheral blood 1 year after birth (P/I and LPS). Associations between dog exposure and TNF-α responses also remained significant after multivariable adjustment for potential confounders. Although cytokine production was also down-regulated among children living in a farming environment, dog exposure was found to be independently associated with lower TNF-α responses. Previously, only a few studies have investigated the effect of early life animal exposures on immune development. Gern et al. [6] demonstrated that dog exposure was associated with increased IL-10 and IL-13 responses at the age of 1 year in children with a parental history of atopy or asthma, but the production of TNF-α was not measured. In our previous paper, we noted that the IFN-γ-producing capacity following mitogen stimulation at the age 3 months was associated with cat and/or dog ownership [21]. No such association was found in the present study. This may be due to methodological differences between these two cohorts: in the earlier study e.g. stimuli, duration of stimulation and classification of pet ownership were all different. However, as far as we are aware, this is the first publication reporting associations between animal exposures and the production of pro-inflammatory cytokine TNF-α in infancy.

Although cytokines themselves cannot be regarded as a clinical outcome, they are sensitive markers providing important information about the complex network between immunological cells and different parts of the immune system. Early life immune responsiveness against different types of stimuli were examined by incubating whole blood samples with Gram-positive bacterial SEB (a potent activator of cells in both innate and adaptive immunity), Gram-negative bacterial LPS (stimulation of innate immunity) and the combination of mitogenic PMA and calcium ionophore ionomycin (P/I; inducer of adaptive T cell responses). It is not clear why associations between dog exposure and TNF-α responses were observed both with LPS and P/I stimulations at age 1 year but only in P/I at birth. One explanation could be that children are exposed to certain microbes/microbial groups or other currently unknown components due to direct dog contact during their first year of life, which may reduce their immunological capability to respond to LPS stimulation at the age of 1 year. In addition, in this study population, P/I had the highest potency to induce the production of TNF-α in infancy [22], which may explain why differences in children's immune responsiveness can be seen more clearly following this stimulation. One should also keep in mind that, in order to mimic the *in vivo* setting, cytokine responses were assessed using whole blood samples. Whole blood culture may contain factors, which either inhibit or intensify the effects of specific stimulants.

Because associations were seen not only with P/I but also with LPS-stimulation, the currently unknown immunological pathway to decreased TNF-α responses among dog-exposed individuals could be related to the exposure to innate immune stimuli, such as Gram-negative bacterial endotoxin. Previous studies have shown that the presence of dogs is associated with elevated endotoxin levels in house dust [27] and in indoor air [28]. An increase in the number of dogs also appeared to correlate with the endotoxin levels [29], but it is not clear whether having more than one dog at home offers better protection against atopy and wheezing [14] or not [30]. The study of Campo et al. [29] suggests that endotoxin may play a crucial role in mediating the effect of dog exposure, because the protection against wheezing was found only when there was a high endotoxin load together with several dogs. Neither endotoxin nor dog exposure were independently associated with the reduced risk of wheezing [29]. This may be partly explained by gene–environment interactions: specific polymorphism in the gene encoding the endotoxin receptor (CD14-159TT) among dog-exposed children has been shown to offer better protection against the development of atopic dermatitis [6]. On the other hand, many studies have failed to prove that the influence of dog exposure on health outcomes and immune responses is due to the increased exposure to endotoxin. Bufford et al. [5] found that the concentration of house dust dog allergen (Can f 1) was associated with increased levels of IL-10, IL-13 and IL-5 at the age of 1 year, but the findings were not explained by the concentrations of microbial markers (endotoxin, muramic acid and ergosterol) in house dust. Moreover, dog ownership has been linked to a reduced risk of childhood asthma [16], wheezing [12] as well as allergic outcomes such as hayfever [16] and inhalant sensitization [31], independently of house dust endotoxin levels. Thus, it is also possible that the effect of dog exposure is mediated via a mechanism other than the endotoxin-dependent immune response and could be related to allergen or other types of microbial exposures for which dog exposure only serves as a surrogate. Dog-derived allergen and microbial exposures may activate different cytokine pathways

(allergen → Th2 vs. endotoxin → Th1) and have a bidirectional effect on allergic outcomes, as suggested by Wright [32]. In addition, the lifestyle of dog owners may include other unidentified immunomodulatory factors. More and extensive sophisticated indoor microbial and allergen characterization may be needed to clarify which exposure agents account for the putative dog -related attenuation of innate immune responses in infancy.

Although endotoxin is commonly regarded as an inducer of immune responses, the levels of house dust endotoxin have been associated with decreased cytokine responses (including TNF-α) in children [3], indicating that continuous exposure to a high microbial load could lead to the development of tolerance against microbial antigens. The theory of immunological adaptation against external non-pathogenic antigens is supported also by our recent publication showing that 3-month-old children, with increasing concentrations of Gram-negative bacterial marker (3-hydroxy fatty acids, $C_{10:0}$–$C_{14:0}$) in their bed dust, had reduced capability to produce TNF-α following mitogen stimulation [4]. Because LPS-stimulated TNF-α responses were also lowered among dog owners' children, the present findings indicate that dog exposure may have similar down-regulating effects as Gram-negative bacterial exposure on the development of immune responses. Thus, despite the missing knowledge concerning causal components, possible protective effects of dog exposure against the manifestation of allergic diseases may be related to the establishment of tolerance. The link between early life exposure and the development of atopy, however, could not have been demonstrated in our study, because dog-keeping was not associated with children's inhalation atopy or food atopy at the age of 1 year (data not shown). In addition, inhalation atopy or food atopy were not associated with TNF-α responses at birth or at the age of 1 year [22]. The intended 6-year follow-up of these children will allow us to investigate if the development of atopy can be linked to either dog exposure or to TNF-α responses later in childhood.

No associations were found between cytokine-producing capacity and cat-keeping. In line with our results, neither levels of cat allergen (Fel d 1) in house dust [5] nor cat ownership [6] have been associated with cytokine responses of children at the age of 1 and 3 years. In addition, the effect of cat exposure on health outcomes appears to be controversial: different studies have suggested that contact with cats reduces [16], increases [9] or has no effect [30] on the risk of wheezing and atopy. The lack of association between cytokines and cat exposure may be explained, at least partly, by behavioural aspects. The size of the animal and the frequency of outdoor visits may influence the exposure factors: the larger the animal, the more it carries and spreads microbial particles and allergens indoors. Cats and dogs also differ in their social behaviours, i.e., direct contact with cats may be less intense than that with dogs. Indeed, a recent study found contrasting effects for the early-life exposure to different pets i.e. an increase in the risk of asthma was seen among children exposed to cats and a protective effect in children exposed to dogs [11].

As far as we are aware, this is the first study to assess the associations between pre-natal horse exposure and the maturation of cytokine responses in infancy. Maternal horse exposure during pregnancy was associated with decreased cord blood TNF-α responses (SEB and LPS stimulation) in univariate testing, but not after adjustment for confounders. The true contribution of horse exposure on infant's immune responses may be difficult to assess due to a great confounding effect of dog ownership among the horse-exposed mothers (54% of the horse exposed had also dog/s in this study). Nevertheless, pre-natal farm animal exposures appear to have an influence on neonatal immune responses as demonstrated by the study of Ege et al. [33] suggesting that maternal exposure to animal sheds during pregnancy are inversely associated with cord blood IgE levels to inhaled allergens. Ege and associates have also shown that maternal exposure to stables and to an increasing number of different farm animal species during pregnancy increases the expression of TLR-2 and -4 in school-aged children [8].

Avoidance of pet-keeping among allergic families can bias the observed associations between pets and measures of immune response. In our study, 25% of the households reported that they had avoided acquiring pets or had given up pets at some phase of life (before the birth of the study child) because of allergies. However, observed associations between dog exposure and cytokine responses remained, even if avoidance of pets was included into regression models as a confounder (data not shown). An atopic phenotype of the mother did not appear to be a major reason to avoid pets, because dog dander-sensitized mothers had more often dog/s than non-sensitized mothers. Similarly, cat-keeping among cat-sensitized mothers was somewhat more common compared with non-sensitized mothers. However, in order to minimize the possible confounding effect of the atopic heredity, maternal atopy and asthma were included in the regression analyses. Similar to maternal atopy, children's atopy (atopy against any inhaled or food allergen) at 1 year of age did not appear to influence dog ownership in the family (sensitized 33% vs. non-sensitized 35%). Changes in pet ownership during the study period did not confound the results because pet-keeping remained virtually constant from birth to 1 year.

When performing numerous statistical tests from the data set with many variables and outcomes, there is always a possibility that observed associations are due to chance (in this study, 5% of the significant associations). Thus, in some study designs, correction for multiple comparison (e.g. Bonferroni) is reasonable. The use of

multiple testing is based on the classical test theory, where statistical inference is made from randomly drawn samples to the universe and the samples and hypotheses tested are completely independent. In contrast, leading epidemiologists have argued strongly against adjustment for multiple comparisons [25]. In the present study, animal ownership was selected *a priori* as the exposure of interest and very few variables were included in the regression analyses. In addition, our findings on dog ownership are supported by biological plausibility, similar patterns following different stimuli and at different age (birth vs. 1 year). Thus, no correction was carried out for multiple testing.

In conclusion, factors affecting immunological development in children are a complex mixture of genetic factors and type, timing, duration and intensity of multiple pre- and postnatal environmental exposures. Although the underlying immunologic mechanisms and potential causal components by which animal exposures could possibly affect the development of immune system require further investigation, our findings suggest that exposure to dogs may promote the development of tolerance against external antigens, as reflected in the decreased capacity of leucocytes to produce TNF-α following antigen stimulation among dog-exposed individuals. This attenuation of cytokine production may help the immune system to prevent exaggerated responses to non-pathogenic antigens later in life. Thus, it appears likely that exposure to furred animals, at least to dogs, during pregnancy and during early postnatal life offers immunological challenges and thus could be advantageous for immune development. In the future, new strategies to prevent allergic disorders may include supporting the development of immunological adaptation against environmental antigens with factors known to induce tolerance.

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