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Development of asthma in inner-city children: possible roles of MAIT cells and variation in the home environment

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Abstract

Humans have populations of innate-like T lymphocytes with an invariant T cell antigen receptor (TCR) α chain that recognize non-peptide antigens, including invariant natural killer T (iNKT) cells and mucosal associated invariant T (MAIT) cells. iNKT cell involvement in human asthma is controversial, while there has been little analysis of MAIT cells. Using peripheral blood cells from 110 participants from Urban Environment and Childhood Asthma (URECA) birth cohort study, these cells were analyzed for number and function. We determined if iNKT cell or MAIT cell frequency at one year is correlated with the cytokine polarization of mainstream $CD4^+$ T cells and/or the development of asthma by age seven. Dust samples from 300 houses were tested for iNKT cell antigenic activity. Our results shows that a higher MAIT cell frequency at one year of age was associated with a decreased risk of asthma by age seven years. The frequency of MAIT cells was associated with increased production of IFNγ by activated $CD4^+$ T cells from URECA cohort. iNKT cell antigenic activity in bedroom dust samples was associated with higher endotoxin concentration and also with reduced risk of asthma. In conclusion, MAIT cell frequency at one year may reflect the tendency of the immune system toward Th1 responses and is associated with protection from asthma. Additionally, iNKT cell antigenic activity may be a marker of houses with increased microbial exposures and therefore also with protection from asthma.

Keywords

allergy; asthma; house dust; iNKT and MAIT

Introduction

Asthma is a significant health problem in industrialized countries, among children as well as adults. Some studies suggest asthma may be more prevalent in low-income inner-city populations, accompanied by higher morbidity and mortality rates (1-3). Different lymphocyte populations may contribute to asthma. Here we have focused on the two prevalent subsets of innate-like T cells that recognize nonpeptide antigens found in microbes. One of these populations is Invariant Natural Killer T cells (iNKT cells), which respond to glycolipids, and the other is Mucosal Associated Invariant T cells (MAIT cells), which respond to certain riboflavin metabolites (4). The cognate antigens for these cells are presented by nonclassical or nonpolymorphic class I antigen-presenting molecules: CD1d for iNKT cells and MR1 for MAIT cells (5). iNKT cells express surface proteins in common with Natural Killer (NK) cells, often including NK1.1 in mice and CD161 in humans, and surface proteins typical of T lymphocytes. The great majority of iNKT cells express an invariant TCRα chain formed by rearranged Vα24 and Jα18 (*TRAV10–TRAJ18*) gene segments in humans; (4,6). MAIT cells are characterized by the expression of a different conserved and invariant α chain: a Vα7.2-Jα33 (*TRAV1-2–TRAJ33*) α chain rearrangement in humans (7,8). These innate-like T cells share a number of properties including rapid cytokine responses, recognition of non peptide antigens, and preferential localization to tissues, such as the lung (4).

iNKT cells have been implicated in several mouse models of asthma. These include asthma induced either by allergens or inflammatory stimuli (9,10). In humans, it has been shown that Th2 cytokine secreting iNKT cells often were the dominant $CD4^+$ T cell subset in the airways of both allergic and non-allergic subjects with severe asthma, but they were almost undetectable in the airways of healthy controls (11). These results are highly controversial, however, with smaller or no iNKT cell increase observed in some other studies (12-15). These discordant results may be a reflection of the variability in iNKT cell numbers in human peripheral blood (16) or disease severity.

Sterile house dust extracts (HDE) have stimulatory compounds for innate immune cells (17) and adjuvant activity in a widely used model of airway inflammation, in which mice were sensitized to the antigen chicken ovalbumin (cOVA). Additionally, our laboratories have shown that the majority of HDEs also had antigenic activity for mouse and human iNKT cells (9). Furthermore, the adjuvant activity of the HDEs was partially iNKT cell-dependent; therefore airway inflammation in mice sensitized with HDEs and cOVA was significantly reduced in mice that did not have iNKT cells (9). The amount of antigenic activity in HDEs was highly variable when obtained from different houses, however, leading us to ask if this activity correlates with the number of iNKT cells in the blood, which is also quite variable (16), or the development of asthma. Much less is known about the role of MAIT cells in allergy and asthma. A recent study, however, found a reduction of MAIT cells in blood, sputum, and biopsy specimens from asthmatic patients, which was related to disease severity (18).

The Inner-City Asthma Consortium initiated the Urban Environment and Childhood Asthma (URECA) study in 2005. This birth cohort study was designed to assess the effect of environmental factors found in urban areas with a high poverty rate on the immune system and the development of allergy and asthma. In this study, pregnant women were enrolled from central urban areas of Baltimore, Boston, New York City, and St. Louis and their offspring, who have at least one parent with allergy or asthma (19), are being followed from birth through age 14-16 years. In this report, we focused on the frequency of iNKT cells and MAIT cells in blood samples obtained at age one year from children in the URECA study and determined their correlations with cytokine production by $CD4^+$ T lymphocytes, the presence of iNKT cell antigens in house dust, and clinical outcomes of recurrent wheeze and atopy at age 3 years and asthma at age 7 years.

Materials and Methods

URECA study and sample collection

The URECA birth cohort study is an observational study with a total of 560 infants, born at term in low-income, inner-city urban neighborhoods from Baltimore, Boston, New York City, and St. Louis, four cities at high risk for allergic disease. Prenatal entry criteria included a history of allergic disease or asthma in at least one parent. Subjects are predominantly African-American, and some are Latino or mixed race. Subjects underwent serial clinical evaluations and house dust samples were collected from their homes as previously described. Informed consent was obtained from all subjects. Detailed design of the study has been previously published (19). Briefly, the clinical outcomes that were used

for this study are recurrent wheezing (age 3 years), aeroallergen sensitization (age 3 years) and asthma (age 7 years). Sensitization to aeroallergens was defined as either a positive skin test or positive serum specific-IgE. For skin testing, children underwent prick skin testing with a collection of 14 common indoor and outdoor allergens such *Alternia tenuis*, Timothy grass and others as described (19) (Multi-Test II, Lincoln Diagnostics, Decatur, IL). Total and allergen-specific IgE were measured by fluoroenzyme immunoassay (UniCAP, Pharmacia & Upjohn, Diagnostics, Uppsala, Sweden). Recurrent wheezing is defined as at least two episodes of wheezing during the first three years of life, with at least one episode during the third year. Asthma up to age 7 years was based on several criteria as described (19). Briefly, children were classified as having asthma at age 7 years if at least one of three conditions was met: 1) a parent-reported physician diagnosis of asthma between age 4 and 7 years, combined with asthma symptoms or the use of asthma controller medication for 6 of the past 12 months; 2) methacholine PC20 ≤ 4 mg/ml or albuterol reversibility of FEV1 ≥ 10%, combined with asthma symptoms or use of asthma controller medication for 6 of the past 12 months; or 3) a report in the past 12 months of ≥2 wheezing episodes, ≥2 doctor visits for asthma/wheeze, ≥1 hospitalization for asthma/wheeze, or the use of controller medications for 6 of the past 12 months. Endotoxin levels in the bedroom dust samples were measured as described (20) at the age of 3 months.

Blood cell analysis

A total of 162 URECA PBMC samples were obtained from 110 unique subjects, plus 52 duplicate samples. All the blood samples were collected at the age of one year. Samples of frozen PMBCs were thawed at 37°C, washed twice with pre-warmed (37°C) RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) Pen-Strep-Glutamine containing 10,000 U/ml penicillin, 10,000 μ/ml streptomycin, 29.2 mg/ml L-glutamine (Invitrogen), distributed in plates and rested overnight (14-16h). To detect iNKT cells, we utilized the 6B11 mAb specific for the CDR3 region of the invariant TCR α chain (16). As the availability of antigen-loaded MR1 tetramers was highly limited, for MAIT cells we used surrogate markers including the combination of Vα7.2 and CD161, which provide a reasonably accurate detection of this population (21). 90% of the cells were then stimulated with PMA (200 ng/ml) and ionomycin (1μg/ml) (both Sigma-Aldrich) in the presence of 0.8 μl/ml Golgi-Plug and 0.55μl/ml Golgi-Stop (both from BD Bioscience) for 4h at 37°C. As a staining control, PBMCs were prepared from buffy coats pooled from two healthy donors from the San Diego Blood Bank. Control lymphocytes were purified with Ficoll, aliquoted and frozen.

One aliquot of these cells was thawed with every experimental set, *i.e.* 1 control and 5 URECA samples. Cells were surface stained for 30 min on ice, washed, fixed with CytoFix/Perm (BD Bioscience) for 15 min at 37°C, permeabilized and intracellular cytokine staining (ICS) was done for 30 min on ice. Flow cytometry was performed as previously described (22), cells were analyzed with an LSR II Fortessa (BD Bioscience) and data were processed with CellQuest Pro (BD Bioscience) and Flow Jo (Tree Star Inc.) software. The antibodies used in this study are described in Supplemental Fig. 1a. Gating strategy for various populations is described in Supplemental Fig. 1b. Details of the protocol and of the surface staining utilized have been published elsewhere (23). The study received IRB approval at the

clinical sites and the administrative center. An independent Data Safety and Monitoring Board (DSMB) run by the National Institutes of Health monitors the study. Approval for this study also was obtained from the Institutional Review Board at the La Jolla Institute for Allergy and Immunology.

Analysis of the antigenic activity in HDEs

300 house dust samples collected at the age of the 3 months from URECA subjects, including all unique subjects that donated blood. The samples were suspended in sterile PBS at 100 mg/ml and then were placed on a rotor at room temperature for 24h before filtration and storage at −80°C. House dust samples were diluted in PBS to a final concentration of 1 mg/ml for the experiment. To test for antigenic activity, stimulation of an iNKT cell hybridoma on plates coated with soluble, recombinant mouse CD1d was performed as described (24). Briefly, 96-well flat-bottom microwell plates were coated with mouse CD1d by incubation of 100 μl CD1d solution (1 μg/ml in PBS) for 1h at 37°C. Wells were then washed 5x times with PBS and blocked by incubation for 1h at 37°C with 10% (v/v) FBS (Mediatech) in PBS. After washing, HDE samples, and as a positive control the potent antigen α-galactosyl ceramide (αGalCer), were added to duplicate wells and incubated for 24h at 37°C. After being washed with medium (RPMI 1640 (Invitrogen) supplemented with 5% (v/v) FBS, 1% (v/v) Pen-Strep-Glutamine containing 10.000 U/ml penicillin, 10.000 μ/ml streptomycin, 29.2 mg/ml L-glutamine (Invitrogen), and 50 μM β-mercaptoethanol (Sigma), 1×10^5 DN3A4-1.2 Vα14i NKT hybridoma cells (25) were added to the plates and incubated for 16-20h at 37°C. Supernatants were collected and IL-2 was determined using a sandwich ELISA (BD PharMingen).

Statistical analysis

Matrices containing cell population statistics for different samples were created. For the 52 duplicate blood samples an average value was used. The ‘cor’ and ‘cor.test’ functions in the R ‘stats’ package were used to calculate R and p-values for Pearson correlations among the log-transformed frequencies of the various cell populations. The ‘t.test’ function was used to calculate p-values for equivalence of clinical parameter groups. Adjusted p-values were calculated using the Benjamini-Hochberg algorithm in the ‘p.adjust’ function of R, where appropriate. All statistical calculations were performed with R version 3.1.0.

Results

Subject characteristics

The baseline characteristics of the URECA consortium birth cohort have previously been described (19). Summary of demographics of 303 children related to this study are described in Table I and demographics for children who provided blood is described in Table II. Detailed demographic information on individual children is listed in Supplemental Table. From these 303 children, we received 300 house dust samples. Blood samples were available from 110 of these individuals, 107 that also provided house dust, plus the three additional donors (Table I and II). Both genders were well represented; almost 50% were female. The blood samples were collected from the subjects at the age of one year, whereas house dust was collected at 3 months of age. These ages were chosen to assess early influences on

asthma, but also reflect the availability of material that is widely shared among a consortium of investigators. Further, we analyzed the clinical data available for these individuals. One hundred and thirty-nine were found to be sensitized to aeroallergens and 102 had recurrent wheezing by the age of 3 years (Table III). By the age of seven years, some children dropped out of the study and at that time data were available for 270 children, of whom 77 (29%) developed asthma. Similarly, if we only consider the children who provided blood, by age 3, 42% were sensitized to aeroallergens and 25% developed recurrent wheezing. By the age of 7, 18.9% of these children developed asthma (Table IV).

Frequencies of iNKT and MAIT cells

The gating strategy for detecting T cell populations is shown in Supplemental Figure 1b and primary data on cell frequencies are in Supplemental Figure 1c. The mean frequency of MAIT cells in the population was 0.097% (median 0.07%) of total $CD3^+$ T cells. The proportion of iNKT cells was lower, at 0.038% (median 0.029%). The MAIT cell frequency of URECA participants is lower than what has been reported for adults, consistent with literature indicating that this cell population expands steadily after birth (21,26,27).

Correlation of MAIT cell frequency with IFNγ producing $CD4^+$ T cells

After a brief in vitro activation we gated on two sub-populations of $CD3^+CD4^+$ T lymphocytes: memory and effector. We observed a strong tendency for the activated $CD4^+$ T cells to produce IL-4 compared to IFNγ, although production of both cytokines was detectable. This Th2 pattern of cytokine skewing is consistent with other work indicating that T cells before birth and early in life produce stronger Th2 responses (28-30). We found a significant positive correlation between the frequency of MAIT cells and the cytokine secretion by $CD4^+$ T cells, prominently with their capability of producing IFNγ (Fig 1) and a negative correlation was observed in case of $CD4^+$ T cells producing IL-4. By contrast, there generally were no significant correlations between iNKT cell frequency and the $CD4^+$ T cell subpopulations capable of producing IFNγ or IL-4, (Supplemental Figure 2).

Association of innate-like cell populations with clinical outcomes

To test the relationship between the frequencies of iNKT and MAIT cells in the blood and clinical outcomes related with allergy and asthma, we compared the frequencies of the two innate-like T cell populations to subsequent aeroallergen sensitivity and recurrent wheezing at age 3 years, and asthma at age 7 years. iNKT cell population frequency distributions at age one were not related to the later development of allergic sensitization or asthma at ages 3-7 years (Fig 2a–2c). While MAIT cell frequency was not associated with risk of aeroallergen sensitization or wheezing at age 3 years (Fig 2d, 2e), the frequency of MAIT cells was significantly lower ($p=0.003$) in one year-old children that later developed asthma at age 7 years (Fig 2f). Benjamini-Hochberg adjustment indicated a low likelihood of false discovery ($q=0.015$).

It is possible that the type of immune responses mounted by iNKT cells and/or MAIT cells would reflect disease status more accurately than their frequencies. Therefore, we activated peripheral blood cells and measured cytokine production by iNKT and MAIT cells by intracellular cytokine staining. The cytokine production profiles of these two populations

were very different. iNKT cells from the one-year-old children were generally less responsive with relatively few cells that produced either IL-4 (Fig 3a) (mean 7.0%, median 9.2%) or IFNγ (mean 16.7%, median 12.6%). There was not a significant difference, however, in the percentage of iNKT cells producing one of these cytokines in children that subsequently became sensitized or that developed asthma (Supplemental Figure 3a-f). By comparison, MAIT cells from the children exhibited a vigorous immune response potential (Fig 3b), which closely resembled that reported for MAIT cells from mature adults. In the URECA cohort children, nearly all MAIT cells could be activated to produce IFNγ (mean 79.3%, median 82.8%) and very few produced IL-4 (Fig 3b), despite a strong Th2 or IL-4 skewing of the bulk $CD4^+$ T cell response. This Th1 cytokine bias of MAIT cells from the children is similar to their cytokine output in adults (27,31-33), but there were no significant associations with MAIT cell cytokine production and allergic sensitization, recurrent wheezing or asthma (Supplemental Figure 3g-i Fig).

Variation in iNKT cell antigen in house dust

In a previous publication, we demonstrated that households from Southern California, varied greatly in the antigenic activity for iNKT cells in their HDEs (9). To determine if iNKT cell antigenic activity in the environment is related to asthma, we analyzed house dust extracts from URECA cohort households using an antigen-presenting cell free-assay. Fig 4 depicts the amount of stimulation of IL-2 secretion by the HDE samples. In agreement with our previous results, the antigenic content in URECA cohort households varied greatly. Interestingly, although there are outliers with high iNKT cell antigen and little endotoxin, and *vice versa*, antigenic content was correlated with the amount of endotoxin in the bedrooms when analyzed at 3 months of age (Fig 5).

It was reported that the frequency of iNKT cells in peripheral blood also shows a large individual variation, with this frequency being stable over time (34). To identify a possible environmental influence on the iNKT cell frequency, we analyzed if there was a relationship between iNKT cell frequency and HDE antigenic activity. These measures were not correlated (Fig 6).

We also analyzed the association of iNKT cell antigenic activity in the HDEs collected at the age of 3 months with clinical outcomes. Dust-induced IL-2 secretion was similar in children grouped according allergic sensitization (Fig 7a), or recurrent wheezing (Fig 7b) at age 3 years. But interestingly, iNKT cell antigenic activity was increased in the households of the children that did not develop asthma at age 7 years (Fig 7c).

Discussion

MAIT cells and iNKT cells are two expanded populations of T cells that express αβ antigen receptors with invariant α chains that recognize non-peptide antigens, and that make rapid effector responses. In a cohort of children from asthma-prone families and environments, we tested whether the frequency and function of iNKT cells and MAIT cells early in life correlated with the subsequent development of clinical outcomes, including aeroallergen sensitization and recurrent wheeze at 3 years, and asthma at age 7 years. We also determined if variation in antigenic activity for iNKT cells in the living environment was associated with

allergic sensitization or asthma. We found that the average frequencies of iNKT cells and MAIT cells in the young children were not highly divergent, with MAIT cells only slightly more than two-fold more frequent than iNKT cells. By contrast, in adults MAIT cells have been found to be much more frequent than iNKT cells (35,36). Two lines of evidence indicate that we identified MAIT cells using surrogate markers such as Vα7.2 antibody, CD8α and CD161, rather than antigen loaded MR1 tetramers, which were not available when the analysis was done. First, the population identified as MAIT cells had a uniquely Th1-skewed capability of cytokine production compared to the other populations from the children, but similar to adult MAIT cells. Second, when we performed a subsequent staining of cells from young children, the great majority of cells positive for the surrogate markers stains with MR1 tetramers loaded with 5-OP-RU (data not shown), in agreement with work from a previous study of samples from young children (37). Despite their relatively low frequency compared to adults, our main findings are that a higher MAIT cell frequency in the blood of one-year-old children is associated with reduced risk of asthma. Additionally, although iNKT cell frequency in blood and cytokine production were not associated with asthma, increased iNKT cell antigenic activity in HDEs was associated with a lower probability of developing asthma.

Several factors distinguish this report from several previously published studies (11-15,18) that addressed similar questions for iNKT cells. These include the larger size of our study population, the very young age of the subjects, the close match of subjects and controls for their age, environment and background, the monitoring of the environment that was undertaken in parallel, the inclusion of an analysis of MAIT cell frequency and function, and the prospective design in which the subjects were followed for seven years.

We measured the production of IFNγ and IL-4 by mainstream $CD4^+$ T cells to determine the extent to which their immune response is related to the frequency or activity of innate-like T cells in peripheral blood. A higher percentage of the $CD4^+$ T cells from the children were capable of producing IL-4 than IFNγ, but there was a strong positive correlation between increased IFNγ production by $CD4^+$ T cells and an increased MAIT cell frequency. Compared to iNKT cells, MAIT cells are more dedicated to producing Th1 cytokines (21) and therefore our data suggest that the frequency of MAIT cells might reflect the presence of factors in the children's immune system driving a Th1-skewed response generally.

We also analyzed the association of iNKT cell and MAIT cell frequency, and cytokine production by these cells, with sensitization and wheezing up to age 3 years and asthma by age 7 years. Our data did not provide strong evidence for a protective or pathogenic role for iNKT cells, in agreement with several investigations that did not find differences in the frequency of iNKT cells in peripheral blood in asthma patients (38-42). The caveat for these studies is that events in the blood might not reflect the state of the tissue. We note that, unlike in our results, an increased Th2 cytokine response by iNKT cells was correlated with asthma in two previous studies (42,43); this correlation is more consistent with the majority of data from mouse asthma models (11,44). The discordant outcomes regarding iNKT cell frequencies and asthma could reflect study design, as nearly all of the previous reports analyzed adult patients with long-term disease, rather than pediatric patients, and none looked at the earliest stages of disease or had the prospective design and highly matched

control group that was part of our study. We note, however, that in a pediatric asthma study using somewhat older children, between ages 6-12 years, iNKT cells produced less IFNγ and more IL-4 in children with asthma (43).

When we analyzed MAIT cell populations, we also could not find an association with allergen sensitization or wheezing up to age 3 years. Despite this, MAIT cell frequency was increased in the blood of those children at age one year who did not develop asthma by age 7 years. This result is consistent with the notion that the immune system can be programmed early in life (45). While it is uncertain why there was not a correlation between MAIT cell frequency and wheezing at age 3, the data suggest that the early in life MAIT cell frequency may be one marker for imprinting of the immune system in young children that is relevant to subsequent risk for asthma. Blood samples from older children were not available for this study, but we note that in agreement with our results, a recent study found a decreased MAIT cell frequency in blood and lung of adult asthma patients (18).

One factor that might drive iNKT cell and MAIT cell frequencies could be exposure to certain microbes or their products, both the species that are present in the environment and the magnitude of the exposure. To analyze this parameter for iNKT cells, we measured the antigen content of HDEs in URECA cohort households. We found that there was a correlation in children that were protected from asthma and HDEs that contained a higher antigenic activity for iNKT cells, even though this was measured years earlier. We also found a positive correlation of antigenic content in HDEs and endotoxin in the bedrooms of children. These data suggest that environments with a higher load of microbial products may also contain a higher concentration of substances that stimulate iNKT cells. Overall, the association of NKT cell antigen in house dust with endotoxin and protection from asthma is consistent with the idea that increased microbial exposure in the first years of life is protective for asthma (the “hygiene hypothesis”). The iNKT cell antigenic content in house dust was not correlated, however, with the percentage of iNKT cells in peripheral blood. This result is perhaps not too surprising, considering that there likely are other routes of iNKT cell microbial antigen exposure (46), and that alterations in the population size and phenotype of iNKT cells were fairly subtle in germ free mice (47).

In conclusion, our study demonstrates that in one-year-old children from asthma-prone households in inner-city environments a higher MAIT cell frequency in blood was correlated with increased $CD4^+$ T cells producing IFNγ and protection from asthma at age 7 years. These findings suggest that an early in life tendency to increased MAIT cells could be asthma protective in many children as they grow older, and could reflect early immune programming or imprinting phenomena promoting Th1 responses. MAIT cells become highly abundant in adult humans, and although MAIT cell frequency may only be a biomarker of a broader Th1 cytokine skewing early in life that suppresses Th2 responses, the strongly Th1-skewed cytokine profile of MAIT cells might actually provide protective function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HDE	House dust extract

iNKT cells	Invariant natural killer T cells
IFNγ	Interferon gamma
MAIT cells	Mucosal associated invariant T cells
TCR	T cell antigen receptor
URECA	Urban environment and childhood asthma

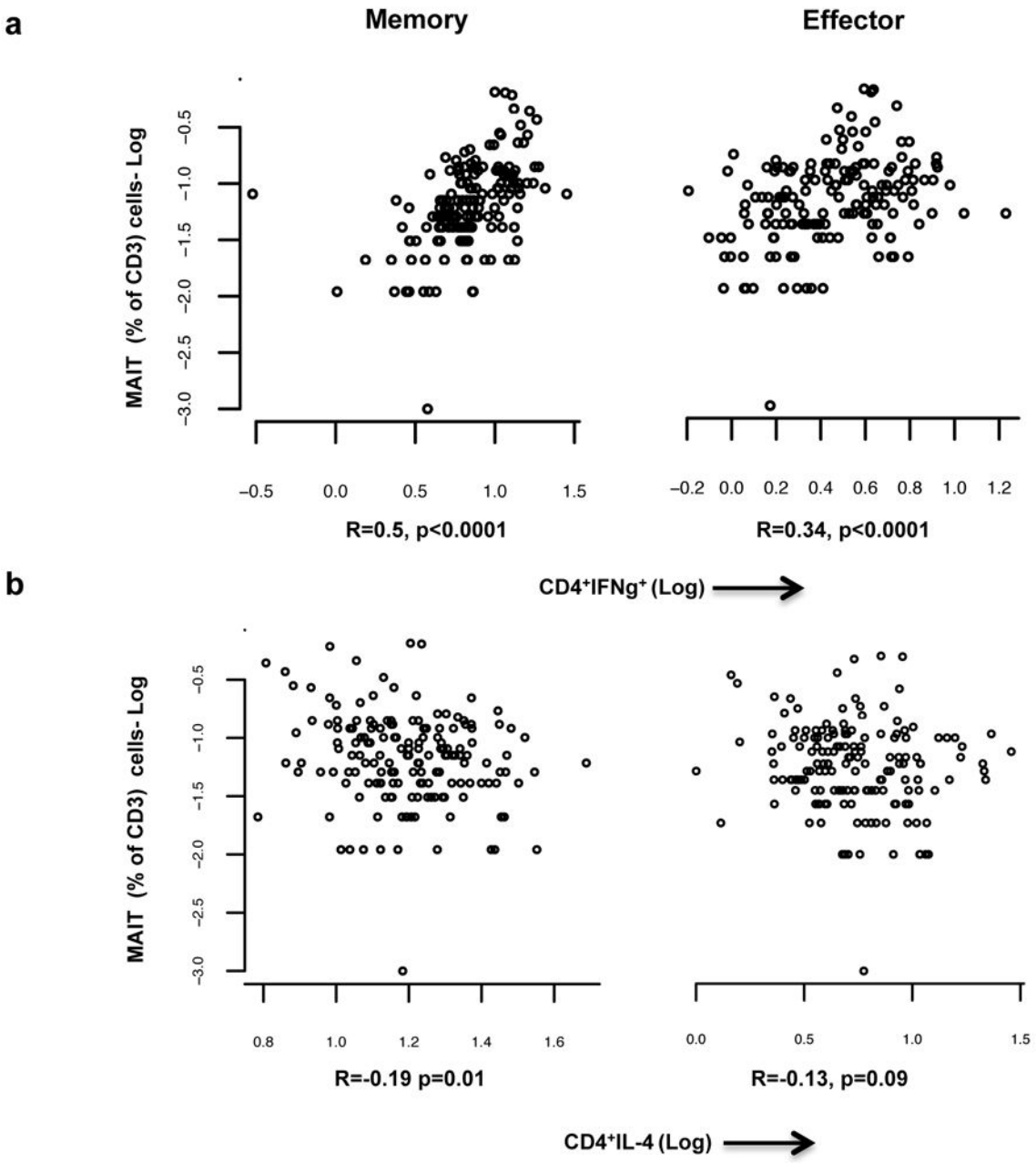

Fig 1. Correlation of MAIT cells with CD4$^+$ T cells producing IFNγ or IL-4

Pearson correlations were determined between MAIT cells as a percentage of total CD3$^+$ cells and cytokine production in the indicated CD4$^+$ T cell populations. Correlation coefficients and p-values are indicated below the plot (n = 110).

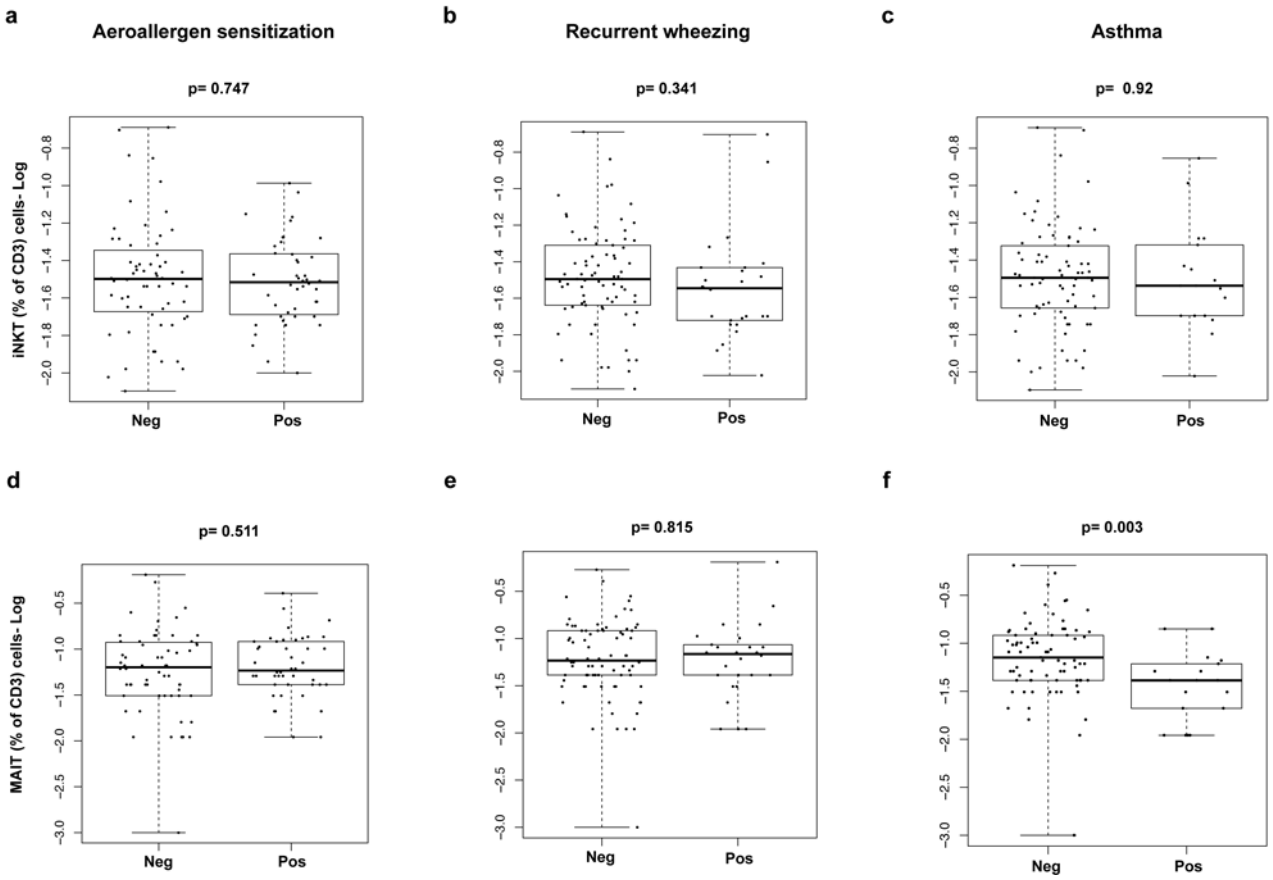

Fig 2. Association of iNKT and MAIT cell frequency with clinical outcomes

A comparison of the percentage of iNKT cells, of total $CD3^+$ cells, in one year-old children that did or did not show aeroallergen sensitization at age 3 years (Neg, n = 60, Pos, n = 44) (a), recurrent wheezing at age 3 years (Neg, n = 78, Pos, n = 26) (b), and asthma (c) at age 7 years (Neg, n = 77, Pos, n = 18). MAIT cell percentage in one year-olds were compared to subsequent development of (d) aeroallergen sensitization at age 3 years (Neg n = 60, Pos n = 44), (e) recurrent wheezing at age 3 years (Neg, n = 78, Pos, n = 26), and (f) asthma at age 7 years (Neg, n = 77, Pos, n = 18). p-values were calculated using a Student's t-test.

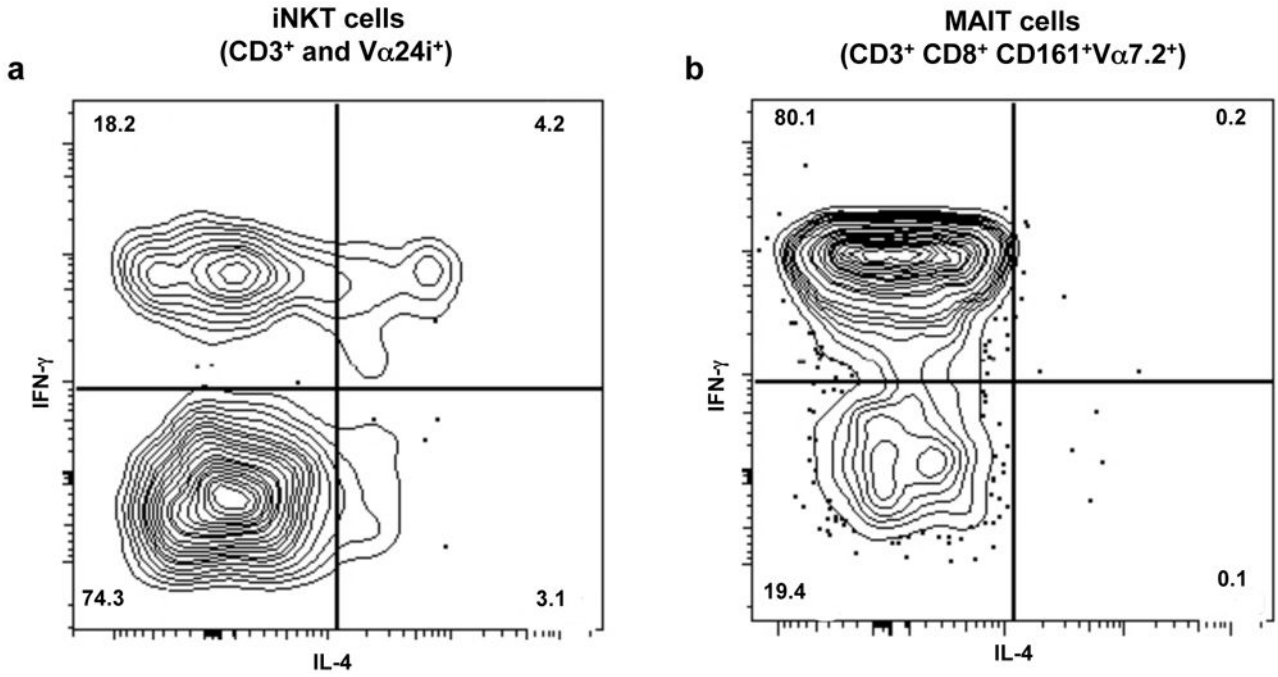

Fig 3. Cytokine production by iNKT and MAIT cells

Representative flow cytometry data for gated iNKT cells (a) and MAIT cells (b) activated with PMA and ionomycin and then analyzed by intracellular cytokine staining. Data from one of many similar analyses shown, see also Supplemental Figure 3 for compiled results for iNKT and MAIT cells.

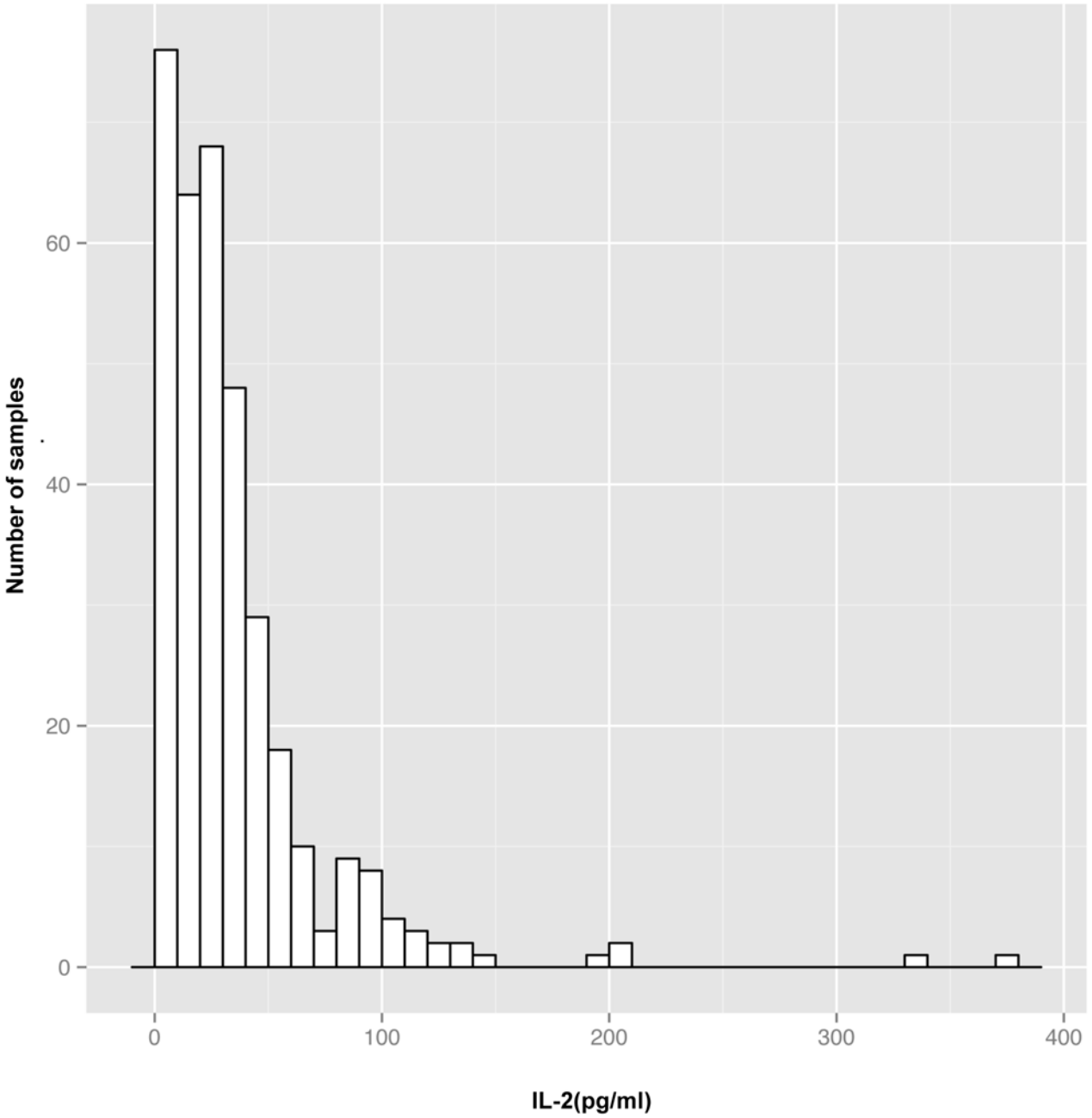

Fig 4. HDEs from different houses vary in their antigenic activity

iNKT cell antigenic activity (IL-2 release) of URECA HDEs (n = 300) grouped by the magnitude of cytokine release in 10 pg/ml intervals (0-10, 11-20, etc.).

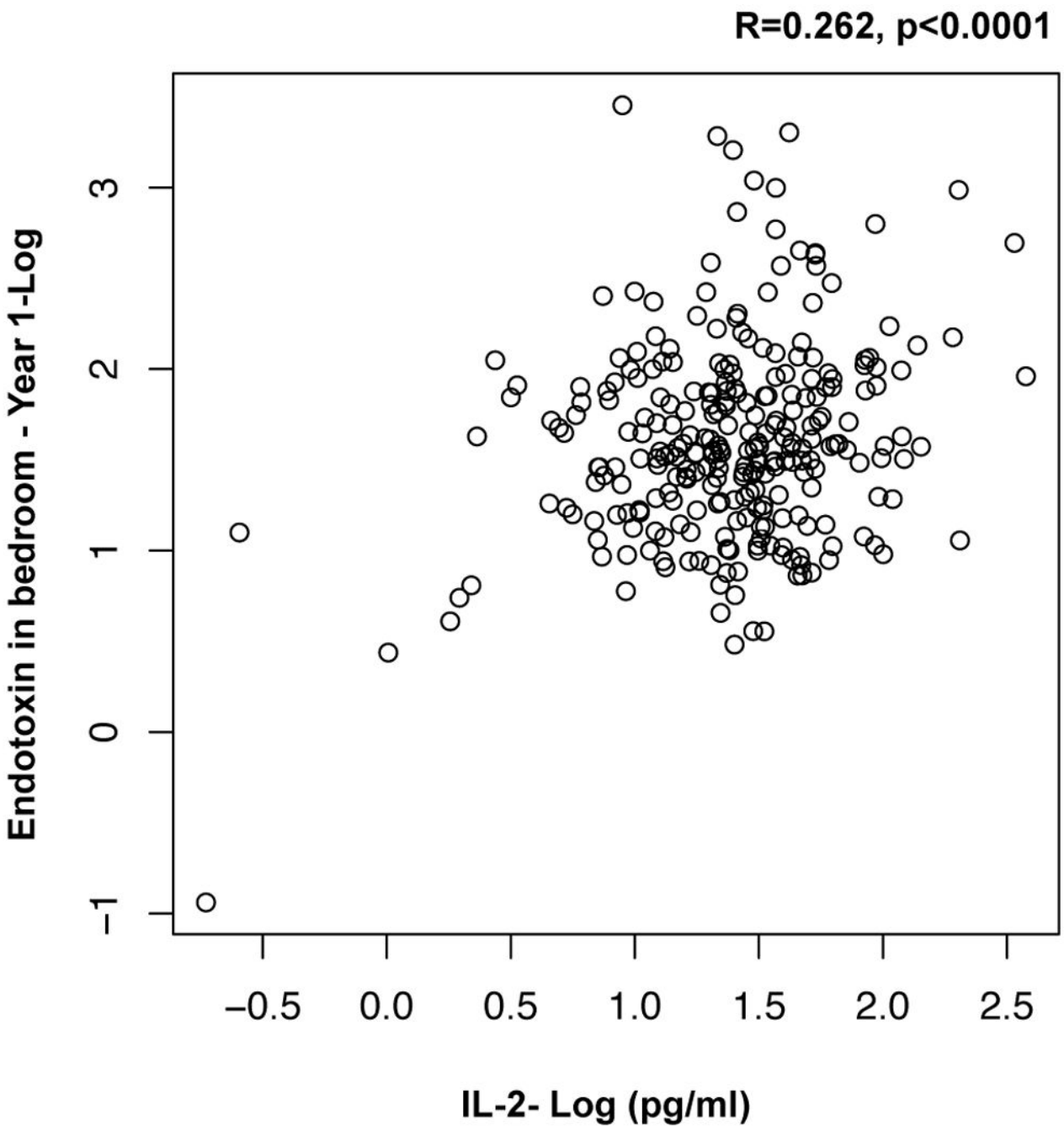

Fig 5. Endotoxin in bedroom is correlated with the amount of iNKT cell antigenic content in HDEs

Pearson correlation was determined between endotoxin from the bedrooms of children at age 3 months and the iNKT cell antigenic content in HDEs.

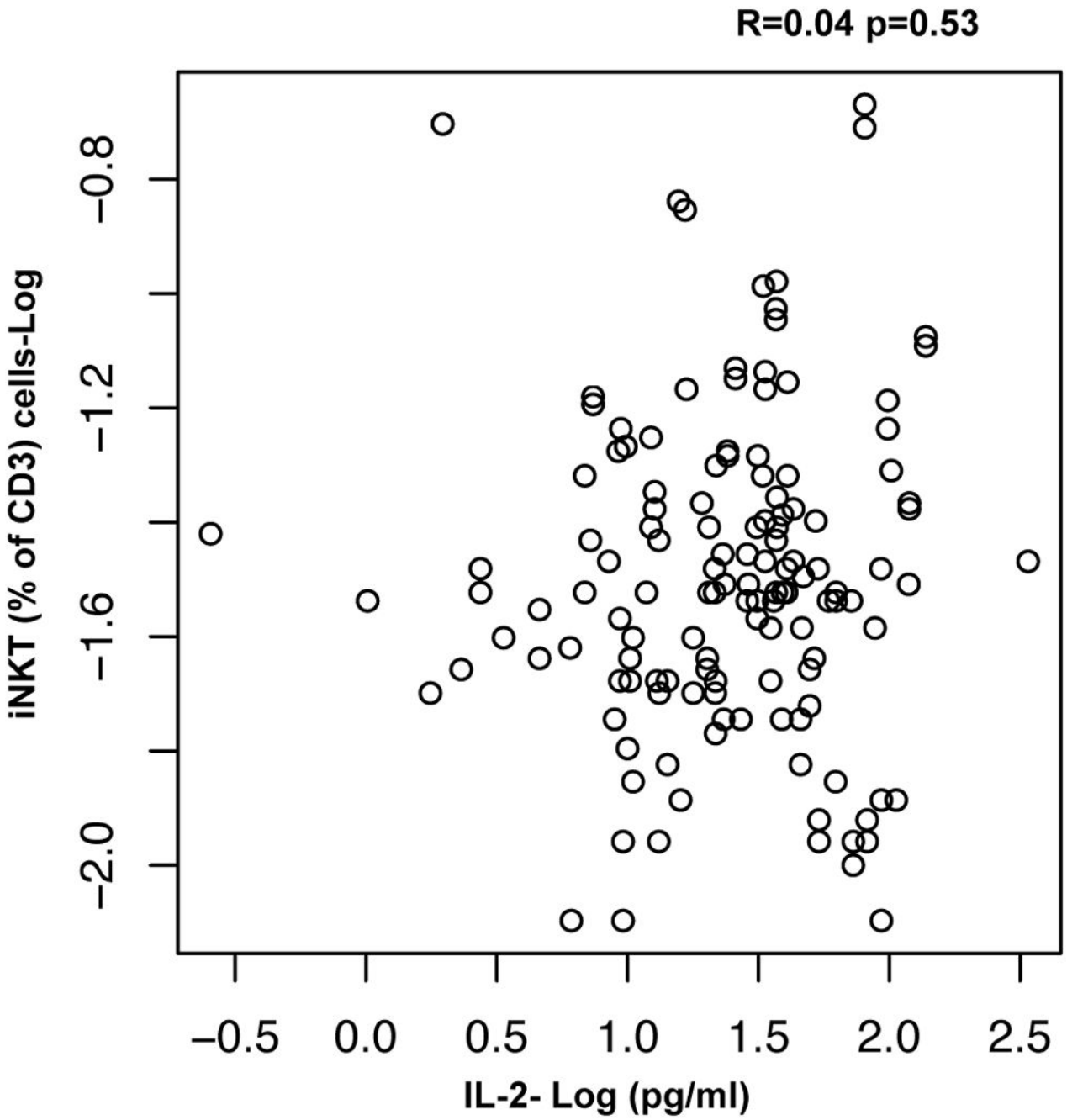

Fig 6. The frequency of iNKT cells is not correlated with the amount of iNKT cell antigen in HDEs

Pearson correlation was determined between the frequency of iNKT cells in peripheral blood, measured as a percentage of total $CD3^+$ cells, and the antigenic content in HDEs.

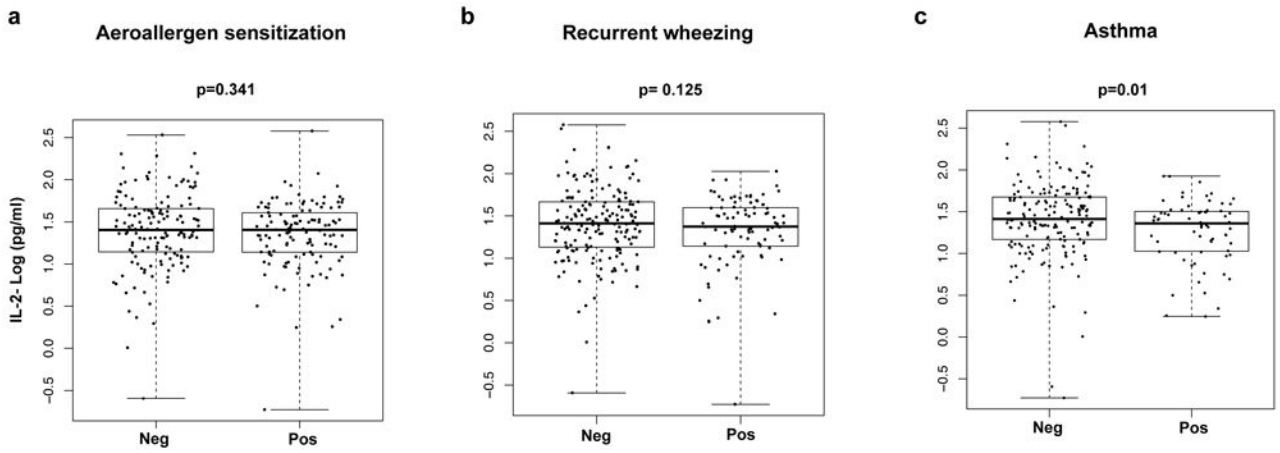

Fig 7. Association of HDE antigenic activity with clinical outcomes

iNKT cell antigenic activity (IL-2 release) in URECA HDEs at 3 months of age was compared to aeroallergen sensitization at age 3 (Neg, n = 152, Pos, n = 125) (a), recurrent wheezing at age 3 (Neg, n = 184, Pos, n = 93) (b) and asthma (c) at age 7 (Neg, n = 181, Pos, n = 67). p-values were calculated using Student's t-test.

Table I

Demographics of URECA cohort- Total Samples

Annual Household Income < $15K	C-Section	Mother Ever Breastfed	Child's Gender	Child's Race
207 (Y)	94 (Y)	170 (Y)	144 (Female)	221 (Black)
96 (N)	209 (N)	128 (N)	159 (Male)	56 (Hispanic)
				20 (Mixed)
				4 (Other)
				2 (White)

Y- Yes
N-No

Table represents the demographics of children that provided house dust or blood.

Table II

Demographics of URECA cohort- Blood donors

Annual Household Income < $15K	C-Section	Mother Ever Breastfed	Child's Gender	Child's Race
68 (Y)	31 (Y)	48 (Y)	52 (Female)	89 (Black)
36 (N)	73 (N)	54 (N)	52 (Male)	8 (Hispanic)
				6 (Mixed)
				1 (Other)
				0 (White)

Y- Yes
N-No

Table represents the demographics of children that provided blood.

Table III

Clinical outcome in URECA cohort- Total samples

	Allergic to aeroallergens (Age 3)	Recurrent wheezing (Age 3)	Asthma (Age 7)
Negative	164	201	193
Positive	139	102	77

Table represents clinical outcomes for the children that provided house dust or blood.

Table IV

Clinical outcome in URECA cohort- Blood donors

	Allergic to aeroallergens (Age 3)	Recurrent wheezing (Age 3)	Asthma (Age 7)
Negative	60	78	77
Positive	44	26	18

Table represents clinical outcomes for the children that provided blood.