

Received Date : 12-Nov-2015

Revised Date : 10-Apr-2016

Accepted Date : 10-May-2016

Article type : Original

***IL-33* polymorphisms are associated with increased risk of hay fever and reduced Regulatory T cells in a birth cohort**

Paul Christoph Schröder, PhD ^{a‡}, Vera Isabel Casaca, PhD ^{a‡}, Sabina Illi, PhD ^a, Maximilian Schieck, PhD ^{b, c}, Sven Michel, PhD ^{b, c}, Andreas Böck, PhD ^a, Caroline Roduit, MD ^d, Remo Frei ^{e, m}, Anna Lluís, PhD ^f, Jon Genuneit, MD ^g, Petra Pfefferle, PhD ^h, Marjut Roponen, PhD ⁱ, Juliane Weber, MD ^a, Charlotte Braun-Fahrländer, MD ^{j, k}, Josef Riedler, MD ^l, Roger Lauener, MD ^{m, r}, Dominique Angèle Vuitton, PhD ⁿ, Jean-Charles Dalphin, MD PhD ⁿ, Juha Pekkanen, MD ^{i, o}, Erika von Mutius, MD ^a, Michael Kabesch, MD ^{b, c}, Bianca Schaub, MD ^a and the PASTURE study group.

‡These authors contributed equally to this work.

^a LMU Munich, University Children's Hospital, Munich, Germany, Member of German centre for Lung Research (DZL), ^b Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO), Regensburg, Germany, ^c Department of Pediatric Pneumology, Allergy and Neonatology, Hannover Medical School, Hannover, Germany, ^d University of Zurich, Children's Hospital, and Christine Kühne-Center for Allergy Research and Education, Zurich, Switzerland, ^e Swiss Institute of Allergy and

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pai.12597

This article is protected by copyright. All rights reserved.

Asthma Research (SIAF), University of Zurich, Davos, Switzerland, ^f National Jewish Health, Denver, USA, ^g Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany, ^h Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany, ⁱ Living Environment and Health Unit, National Institute for Health and Welfare, Kuopio, Finland, ^j Swiss Tropical and Public Health Institute, Basel, Switzerland, ^k University of Basel, Basel, Switzerland; ^l Children's Hospital Schwarzach, Schwarzach, Austria, ^m Christine Kühne-Center for Allergy research and Education, Hochgebirgsklinik Davos-Wolfgang, Switzerland, ⁿ University Hospital of Besançon, the Research Unit Health and Rural Environment, University of Franche-Comté, Besançon, France, ^o Department of Public health, University of Helsinki, Helsinki, Finland, ^p University Hospital of Besançon and Plateforme de Biomonitoring, CIC-BT506, EFS Bourgogne Franche-Comté, INSERM UMR1098, University of Franche-Comté Besançon, France, ^q Utrecht University, Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht, NL, ^r Children's Hospital of Eastern Switzerland, St. Gallen, Switzerland

PASTURE Study group: A. Hyvärinen ⁱ, Karvonen A ⁱ, Hirvonen MR ⁱ, Tiittanen P ⁱ, Remes S ^o, Kaulek V ^p, Dalphin ML ^p, Ege M ^a, Depner M ^a, Loss G ^a, Renz H ^h, Doekes G ^q.

Running Title: *IL-33*-SNPs, hay fever and reduced Tregs

Correspondence to: Bianca Schaub, LMU Munich, University Children's Hospital, Lindwurmstrasse 4, D 80337, Munich, Germany, Ph: 0049-89-4400-57856, Fax: 0049-89-4400-54764. e-mail: Bianca.Schaub@med.uni-muenchen.de

Tables

Table 1. PASTURE/EFRAIM population characteristics

Table 2. Characteristics of *IL-33* polymorphisms in PASTURE/EFRAIM

Table 3. Identification of polymorphisms in linkage disequilibrium with rs928413 and rs1342326 using HaploReg

Table 4: *IL-33* polymorphisms, phenotypes and allergic sensitization in PASTURE/EFRAIM

Figures

Figure 1. Association of *IL-33* SNPs and CD4⁺CD25^{high}FOXP3⁺ Tregs in PASTURE/EFRAIM

Figure 2. Association of *IL-33* SNPs and *SOCS3* mRNA-expression in PASTURE/EFRAIM

Online Repository

Methods

Table S1: *IL-33* polymorphisms in relation to *TLR4* and *IL1RL1* mRNA expression and sST2 protein expression in PASTURE/EFRAIM children

Schröder (PS), Casaca (VC), Illi (SI), Schieck (MS), Michel (SV), Böck (AB), Roduit (CR), Frei (RM), Lluís (AL), Genuneit (JG), Pfefferle (PP), Roponen (MR), Weber (JW), Braun-Fahrlander (CBF), Riedler (JR), Lauener (RL), Vuitton (DAV), Dalphin (JCD), Pekkanen (JP), von Mutius (EVM), Kabesch (MK), Schaub (BS)

***IL-33* polymorphisms are associated with increased risk of hay fever and reduced**

Regulatory T cells in a birth cohort

Title of journal: Pediatr Allergy Immunol.

Abstract

Background: *IL-33* polymorphisms influence the susceptibility to asthma. *IL-33* indirectly induces Th2-immune responses via dendritic cell activation, being important for development of atopic diseases. Furthermore, *IL-33* upregulates Regulatory T cells (Tregs), which are critical for healthy immune homeostasis. This study investigates associations between *IL-33* polymorphisms during development of childhood atopic diseases and underlying mechanisms including immune regulation of Tregs.

Methods: Genotyping of *IL-33*-polymorphisms (rs928413, rs1342326) was performed by MALDI-TOF-MS in 880 out of 1133 PASTURE/EFRAIM children. In 4.5 years old German PASTURE/EFRAIM children (n=99) CD4⁺CD25^{high}FOXP3⁺ Tregs were assessed by flow cytometry following 24-hour-incubation of PBMCs with PMA/Ionomycin, LPS or without stimuli (U). *SOCS3*, *IL1RL1*, *TLR4*-mRNA-expression and sST2-protein levels *ex vivo* were measured in PASTURE/EFRAIM children by Real-Time-PCR or ELISA, respectively. Health outcomes (hay fever, asthma) were assessed by questionnaires at age 6 years.

Results: rs928413 and rs1342326 were positively associated with hay fever (OR=1.77, 95%CI=1.02-3.08; OR=1.79, 95%CI=1.04-3.11) and CD4⁺CD25^{high}FOXP3⁺-Tregs (%) decreased in minor allele homozygotes/heterozygotes compared to major allele homozygotes (p(U)=0.004;p(LPS)=0.005;p(U)=0.001;p(LPS)=0.012). *SOCS3*-mRNA-expression was increased in minor allele homozygotes and heterozygotes compared to major allele homozygotes for both *IL-33*-polymorphisms (p(rs928413)=0.032, p(rs1342326)=0.019) and negatively correlated to Tregs.

Conclusions: *IL-33*-polymorphisms rs928413 and rs1342326 may account for an increased risk for hay fever with age 6 years. Lower Tregs and increased *SOCS3* in combined heterozygotes and minor allele homozygotes may be relevant for hay fever development, pointing towards dysbalanced immune regulation and insufficient control of allergic inflammation.

Key words: childhood, hay fever, *IL-33*, polymorphisms, Tregs

Requests for offprints: Bianca Schaub, LMU Munich, University Children's Hospital, Lindwurmstrasse 4, D 80337, Munich, Germany, Ph: 0049-89-4400-57856, Fax: 0049-89-4400-54764. e-mail: Bianca.Schaub@med.uni-muenchen.de

ABBREVIATIONS

AD = Atopic dermatitis

bp = Base pairs

CI = Confidence interval

ct = Cycle threshold

DC = Dendritic cell

ERalpha = Estrogen receptor alpha

FOXP3 = Forkhead-Box-Protein P3

GMR = Geometric mean ratio

GWAS = Genome-wide association study

HNF1 = Hepatocyte Nuclear Factor 1

HP1 = Heterochromatin protein 1

HWE = Hardy-Weinberg equilibrium

IL-33 = Interleukin 33

LD = Linkage disequilibrium

LPS = Lipopolysaccharide

MAF = Minor allele frequency

OR = Odds ratio

PBMC = peripheral blood mononuclear cells

PI = Phorbol 12-Myristate 13-Acetate/Ionomycin (PMA)/ Ionomycin

RAR = Retinoid acid receptor

rs = Reference SNP

RXRA = Retinoid X acid receptor

SIC = Specific IgE class

SNP = Single nucleotide polymorphism

SOCS3 = Suppressor of cytokine signalling 3

IL1RL1 = Interleukin 1 receptor-like 1

sST2 = soluble Interleukin 1 receptor-like 1

TLR4 = Toll-like receptor 4

Tregs = Regulatory T cells

INTRODUCTION

Genome-wide association studies (GWAS) have identified several important candidate genes for the development of asthma, including *IL (Interleukin)-33* gene and its receptor, *IL1RL1* (1). Different studies have reported that IL-33 is also involved in additional immune-mediated disorders such as autoimmunity and other allergic diseases (2,3). IL-33 is a pro-inflammatory cytokine which also shares features of a nuclear factor. It localizes to the nucleus and acts as a suppressor of transcription (4,5). It is released during tissue injury by different cells such as keratinocytes, endothelial cells, fibroblasts and smooth muscle cells (6)

acting as a mediator or alarmin (3,7). It exists in a membrane-bound (IL1RL1) and soluble form (sST2), the latter acting as decoy receptor. IL-33 contributes to the development of allergic rhinitis by ragweed pollen (8) and two single-nucleotide-polymorphisms (SNP) of the *IL-33* gene, were associated to Japanese cedar pollinosis (9). Some studies have shown that IL-33 can influence Tregs, playing a crucial role in suppressing exuberant immune responses, by inducing and expanding them (10,11). In humans, suppressor of cytokine signalling (SOCS) proteins have been proposed as regulatory T cell regulators (12).

Given the importance of IL-33 in atopic and inflammatory diseases, we investigated whether the genetic variants rs928413 and rs1342326 near the *IL-33* gene locus, which were shown to be associated with childhood asthma in a genome-wide association study (GWAS) (1), were also associated with other childhood atopic phenotypes such as hay fever, and whether Treg-mediated immune modulation during childhood was relevant.

METHODS

Population

The PASTURE/EFRAIM study (Protection Against Allergy: Study in Rural Environments/Mechanisms of Early Protective Exposures on Allergy Development) is a prospective birth cohort including a total number of 1133 children from Austria, Finland, France, Germany and Switzerland (13).

All studies were approved by the local research ethics committee from each country and written informed consent was obtained from all parents for the participation in the study.

Questionnaires and atopic outcome definitions

Comprehensive questionnaires were completed by the parents and the prevalence of respiratory and clinical phenotypes with allergic symptoms and doctor's diagnoses were assessed at age 6 years, where a reliable phenotype can be established (14). Hay fever was defined as doctor's diagnosis of hay fever ever and/or nasal symptoms with itchy and/or watery eyes in the last 12 months without a cold. Asthma was defined as doctor's diagnosis of asthma ever and current wheeze at age 6 years. Atopic dermatitis was defined as doctor's diagnosis of atopic dermatitis ever at age 6 years. Other factors such as socioeconomic status, farming status and family history of atopy were reported (15).

Specific IgE to allergens was measured in the serum of 6 year old children by Allergy Screen test (Mediawiss Analytic, Moers, Germany) in a central laboratory in Marburg, Germany. Alder, birch, hazel and rye pollen, grass, ryegrass, alternaria, mugwort and plantain were categorized as seasonal and the aforementioned plus *D. pteronyssius*, *D. farinae*, cat, horse, dog as inhalant allergens. Three levels of specific IgE cut-off were used (specific IgE class [SIC] 3): ≥ 3.5 IU/ml (SIC3), ≥ 0.7 IU/ml (SIC2) and ≥ 0.35 IU/ml (SIC1).

Selection of polymorphisms and genotyping

Polymorphisms rs928413 and rs1342326 located upstream of *IL-33* were genotyped in 880 children from PASTURE/EFRAIM birth cohort with available material. The SNP selection was based on results from a GWAS on childhood onset asthma by Moffat *et al* (1). These SNPs showed a significant association in the two applied models (details Supporting Information).

Cell isolation and stimulation

At age 4.5 years blood samples in PASTURE/EFRAIM were collected, excluding children with signs of fever or infections. Peripheral blood mononuclear cells (PBMCs) were isolated within 24 hours from heparinized blood by density gradient centrifugation with Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA). 5×10^6 cells were then incubated with either mitogen PMA (5ng/ml) and Ionomycin (1μg/ml) (PI) or innate LPS (0.1 μg/ml) (Sigma-Aldrich Steinheim, Germany) or without addition of stimuli (unstimulated, U) for 24 hours (15).

Flow cytometry of Tregs

Tregs were assessed in 4.5 year old German PASTURE/EFRAIM children (n = 99; Table 1), where sufficient cells were available. Tregs defined as $CD4^+CD25^{high}FOXP3^+$ were measured by flow-cytometry (previously described in (15)) (details Supporting Information).

mRNA and protein expression

For the assessment of mRNA-expression of *SOCS3*, *IL1RL1* and *TLR4*, blood samples from PASTURE/EFRAIM children at the age of 4.5 years were collected in PAXgene® Blood RNA tubes containing a RNA-stabilizing solution (PreAnalytiX/Qiagen) and frozen to -80°C within 24 hours (details Supporting Information). For soluble ST2, concentrations were assessed in sera by ELISA (details Supporting Information).

Statistical analysis

Differences between population characteristics and genetic associations were calculated using the Chi-squared test. Associations between the different allergic outcomes and genotypes were calculated using the Chi-squared test. A stepwise variable selection model was used to

assess the relevance of individual allergens for hay fever. For Treg analysis only a limited number of minor allele homozygotes were available and a dominant minor allele effect was assumed by combining heterozygotes and minor allele homozygotes. Treg cell percentages were calculated in relation to the total number of gated lymphocytes. Differences in Treg cell percentages between heterozygotes and minor allele homozygotes compared to major allele homozygotes and the relation to hay fever were calculated with the Wilcoxon test. mRNA-expression and sST2 data were log-transformed and analyzed by using the comparative threshold method of Giulietti *et al* (16) and linear regression (details Supporting Information). The Pearson correlation coefficient was used. For all reported analyses, stratification for farming status was performed but not relevant. Differences were considered significant when $p < 0.05$. Because the study was based on previous knowledge and the variables were highly correlated, corrections for multiple testing were not performed (17). Statistical analyses were performed in SAS (version 9.3; SAS Institute, Cary, NC, USA) and R (<http://www.R-project.org/>).

RESULTS

Population and *IL-33* polymorphism characteristics

A total of 880 children from PASTURE/EFRAIM (Table 1) were genotyped for polymorphisms rs928413 and rs1342326, both located in the 5' upstream gene region of *IL-33*. The minor allele frequency was 0.26 for rs928413 and 0.18 for rs1342326 (Table 2). No deviation from Hardy-Weinberg equilibrium (HWE) was found ($p = 0.37$ for rs928413 and $p = 0.56$ for rs1342326). The overall genotyping rate was 0.995. Linkage disequilibrium (LD) between rs1342326 and rs928413 was $r^2 = 0.604$, which is almost identical to the observed LD in the 1000 Genomes CEU data set ($r^2 = 0.607$). Using the HaploReg database (18) further 19 SNPs (Table 3) in high LD with our target SNPs were detected ($r^2 \geq 0.8$; rs928413 $n = 8$ SNPs;

rs1342326 n=11 SNPs). Regulatory motifs HNF1 and HP1-site-factor for rs928413 and ERalpha-a, RAR, RXRA and Smad for rs1342326 as well as DNase hypersensitive sites (related to skin tissue for rs928413) and enhancer histone marks (in tissues like fat, bone-marrow derived mesenchymal stem cells, breast, muscle, skin, cervical carcinoma and bone) are potentially affected as described in Table 3.

No significant differences were found between the whole population, the genotyped and the genotyped Treg-subpopulation of the PASTURE/EFRAIM cohort in regards to maternal, paternal or parental atopy, number of siblings, sex, farming status, asthma and hay fever at age 6 years and sensitization (Table 1).

Association of *IL-33* polymorphisms rs928413 and rs1342326 with hay fever

In the total PASTURE/EFRAIM population both SNPs were positively associated with hay fever at age 6 years (rs928413 OR 1.77 (1.02-3.08), rs1342326 OR 1.79 (1.04-3.11)). The proportion of individuals affected of hay fever was highest in minor allele and heterozygotes of *IL-33* rs928413, compared to major allele homozygotes (AG/GG=9.6%, AA=5.7%, Table 4). A similar pattern was found for rs1342326 SNP (AC/CC=10.4%, AA=6.1%, Table 4).

When stratified for farming status, the magnitude of the effect was similar in farmers and non-farmers for both *IL-33* SNPs (data not shown). In regards to asthma and sensitization to inhalant and seasonal allergens (SIC 3), no differences were found for both SNPs (Table 4).

A further analysis showed that children with hay fever at age 6 years had significantly more asthma, atopic dermatitis, inhalant sensitization and food sensitization than children without hay fever. This was, however, independent of the genotype, i.e. the occurrence of other phenotypes in children with hay fever was not more common in children with the genotype as in children without the genotype (data not shown).

When allergens were considered independently a positive association of sensitization to

mugwort (specific IgE) was found for heterozygous/minor alleles for both SNPs (rs928413: %n/N(AA)=4.44, %n/N(GA/GG)=9.29, p=0.017; rs1342326: %n/N(AA)=4.85, %n/N(AC/CC)=10.26, p=0.012). However when a stepwise variable selection model was applied considering all seasonal allergens the SNP-associated mugwort specific IgE sensitization did not play a determinant role (data not shown).

Tregs decrease in heterozygotes and minor compared to major allele homozygotes of *IL-33* rs928413 and rs1342326

Tregs were consistently shown to be impaired in atopic diseases. Since *IL-33* can influence Tregs we assessed whether different *IL-33* genotypes were associated with the frequency of Tregs. Treg percentage in unstimulated and LPS-stimulated PBMCs showed a genotype-specific significant difference (Figure 1). In both conditions, Treg percentage was consistently higher in major allele homozygotes when compared to the heterozygotes and minor allele homozygotes of *IL-33* rs928413 and *IL-33* rs1342326 (p(U)=0.004, p(LPS)=0.005, Figure 1A/C; p(U)=0.001, p(LPS)=0.012, Figure 1D/F, respectively). Upon PI stimulation, Treg percentage did not differ significantly although a trend was observed for rs928413 showing lower percentages of Tregs in heterozygotes and minor allele homozygotes (Fig. 1 B/E). Further stratification of Treg data was not feasible due to the low amount of children with AG/GG and AC/CC genotypes, respectively, and positive outcome for hay fever.

***SOCS3* mRNA-expression is increased in heterozygotes and minor allele homozygotes of *IL-33* rs928413 and rs1342326**

Due to modulation of Tregs by suppressor of cytokine signalling 3 (*SOCS3*), we assessed mRNA-expression of *SOCS3* in relation to *IL-33* polymorphisms *ex vivo* in the whole PASTURE/EFRAIM population. For both *IL-33* polymorphisms rs928413 and rs1342326 heterozygotes and minor allele homozygotes showed higher mRNA-expression of *SOCS3* compared to major allele homozygotes ($p(\text{rs928413})=0.032$, $p(\text{rs1342326})=0.019$; Figure 2). Additionally, a decrease in unstimulated Treg percentage was negatively correlated with increased *SOCS3* mRNA-expression only in heterozygotes and minor allele homozygotes (rs928413: $r=-0.40$, $p=0.03$, $N=39$; rs1342326: $r=-0.38$, $p=0.06$, $N=33$) but not in major allele homozygotes (rs928413: $r=-0.09$, $p=0.45$, $N=74$; rs1342326: $r=-0.13$, $p=0.28$, $N=80$, not shown).

Modulation of Treg cells through LPS-receptor *TLR4*, *IL-33*-receptor *IL1RL1* and soluble sST2 has been described (19,20). We assessed mRNA-expression *TLR4* and *IL1RL1* as well as soluble sST2 protein levels *ex vivo* in relation to *IL-33* polymorphisms. However, expression of *TLR4* and *IL1RL1* and sST2 remained unchanged for both polymorphisms on mRNA and/or protein levels *ex vivo* in the whole PASTURE/EFRAIM cohort (Table S1).

DISCUSSION

In the international European PASTURE/EFRAIM birth cohort study, we suggest an association between *IL-33* polymorphisms and hay fever during childhood. *IL-33* rs928413 and rs1342326, previously shown relevant for childhood asthma, was associated with increased risk of hay fever at age 6 years. Both *IL-33* polymorphisms showed significant genotype-dependent effects on Regulatory T cells (Tregs) levels and *suppressor of cytokine signalling 3* (*SOCS3*) mRNA-expression at age 4.5 years.

IL-33, secreted by epithelial cells, has been shown to contribute to the development of allergic rhinitis (AR) by ragweed pollen (8). Only one study demonstrated that other polymorphisms of the *IL-33* gene, different from the ones in the present study, were positively associated to Japanese cedar pollinosis (a common form of hay fever in the Japanese population) (9). Another study found a positive association of *IL-33* polymorphism rs1342326 with skin prick test (21). Interestingly, a variant near *IL-33* (rs7032572), which reported an association with increased risk of hay fever (22), is in high LD with rs1342326 ($r^2 = 0.898$, $D' = 1.00$) and lower LD with rs928413 ($r^2 = 0.545$, $D' = 1.00$) according to the 1000 Genomes CEU data set (23). We provided evidence for an association of *IL-33* polymorphisms rs928413 and rs1342326 with an increased risk of hay fever in the PASTURE/EFRAIM (n=880) birth cohort. Noteworthy, it cannot be excluded that other polymorphisms in linkage with rs928413 or rs1342326 may be relevant in the association with hay fever. An *in silico* analysis of polymorphisms in high LD with rs928413 and rs1342326 showed some potential functional implications (Table 3) related to histone marks, DNase hypersensitive sites and DNA-protein binding as well as affected regulatory motifs which might be influenced by allele-specific effects. Future functional studies comprising large numbers of in depth clinically phenotyped children, ideally with detailed immunological and genetic data, are required to disentangle allele-specific causal changes for hay fever development until later childhood, when the phenotype is more frequent.

To further understand potential mechanisms of *IL-33* and an increased risk for the development of hay fever, we assessed immune regulation including mRNA-expression and Treg levels at age 4.5 years in PASTURE/EFRAIM children. Clinical outcomes were assessed at the age of 6 years to encompass a more reliable phenotype (14) as compared with age 4.5 years. We investigated whether modulated Tregs at 4.5 years may precede later

subsequent atopic disease at the age of 6 years. A link between IL-33 and Tregs has already been demonstrated by a few studies showing that IL-33 might be important for the induction and expansion of Tregs (11,24). Yet, to date there are no reports about genetic variants of the *IL-33* gene and modulation of Tregs. In this study, both *IL-33* polymorphisms were associated with reduced unstimulated and LPS-stimulated Tregs numbers in PASTURE/EFRAIM. Lower Tregs at the age of 4.5 years may be indicative of less counter-regulatory mechanisms via Tregs in the combined group of *IL-33* heterozygotes and minor allele homozygotes and potentially lead to subsequent development of hay fever at school-age. More detailed functional studies on the role of Tregs were not feasible in this large birth cohort studies due to insufficient number of Tregs. *SOCS3* mRNA-expression was increased in heterozygotes and minor allele homozygotes compared to major allele homozygotes in both rs928413 and rs1342326. Supporting our finding of a negative *SOCS3*-Treg correlation, Feng *et al* showed that *SOCS3* negatively regulates the Treg-associated marker FOXP3 (12). *TLR4*, *IL1RL1* expression and sST2 levels *ex vivo* showed no significant differences on mRNA level. The lack of differences for both *TLR4* and *ST2* *ex vivo* depending on the *IL-33* polymorphisms in our study – and considering a low number of children with polymorphism – may actually be due to the requirement of specific, most likely innate stimulation of cells, as indicated by studies of Sweet *et al* (20) and Brint *et al* (19). Thus, a *TLR4* or *IL1RL1*-dependency requires further functional studies including, e.g. blocking of the receptor.

Considering this study was performed in a large rural cohort (n=1133), further analysis are required in order to prove if these effects can be generalized to general populations.

Both polymorphisms, rs928413 and rs1342326, were associated with asthma onset in a GWAS (1). In the present study no associations were found between carrying the analyzed genetic variants and the development of asthma. Insufficient power due to low number of

children with asthma in the subgroups with polymorphisms (three children and one child homozygous for the minor allele for rs928413 and rs1342326, respectively) could explain these negative results. Thus, it is possible that effects for asthma may have been missed in this cohort. Another potential reason for the lack of association between *IL-33* polymorphisms and asthma in our study could be due to a difference in asthma definition. In the GWAS, doctor diagnosis of asthma was used, while in our study we used doctor diagnosis and current wheeze at the age 6 years, which we believe is specific for present asthma at the time of investigation. Also, the age range of children in the GWAS was larger while we focused on asthma at age 6 years.

Overall, the strength of this study relies on the investigation of important *IL-33* genetic variations in a longitudinal birth cohort assessing development of hay fever during immune maturation including assessment of biological data on Regulatory T cells, *SOCS3*, *IL1RL1*, *sST2* and *TLR4*.

In conclusion, our data suggest that *IL-33* polymorphisms may contribute to increased susceptibility to hay fever in European children at the age of 6 years. Impairment of Tregs regulated by *SOCS3* at the age of 4.5 years may be partly involved preceding hay fever development. Further investigations of the impact of *IL-33* genetic variants on immune mechanisms and longitudinal follow-up into later childhood are yet required to clarify these findings.

ACKNOWLEDGMENTS

We would like to thank all the families for participation in the study and all PASTURE/EFRAIM coworkers involved in recruitment and sample collection. We thank Isolde Schleich and Susanne Loeliger for technical assistance.

This work was supported by EFRAIM EU FP7- KBBE-2007-1, Bavarian Research Association (VC), SFB TR22 (BS, AL), DFG SCHA 997/3-1 (BS, PS), Else-Kröner Fresenius Stiftung (AB, BS), Marie Curie Grant (MEST-CT-2005-020524-GALTRAIN, AL), DZL (German Centre for Lung research, Comprehensive Pneumology Centre, BS).

CONFLICT OF INTEREST

PS, VC, SI, MS, SM, AB, CR, AL, RF, PP, MR, CBF, JR, DAV have nothing to disclose. JG reports grants from European Union during the conduct of the study. JW reports grants from European Commission, during the conduct of the study. RL reports grants from Kühne Foundation, grants from EU outside the submitted work. JCD reports grants from Novartis Pharma, personal fees from Novartis Pharma, Chiesi, Intermune, GSK, AstraZeneca, Boehringer Ingelheim, non-financial support from Novartis, GSK, AstraZeneca, Intermune, Chiesi, Boehringer Ingelheim, Stallergenes outside the submitted work. JP has nothing to disclose. EVM reports personal fees from Glaxo Smith Kline, Novartis, Astellas Pharma Europe Ltd., ALK Abelló, grants from Friesland Campina, personal fees from Journal of Allergy and Clinical Immunology, New England Journal of Medicine, outside the submitted work. MK is a consultant for Sanofi and speakers Bureau: Roxall, Glaxo Wellcome, Novartis, Allergopharma. BS reports funding from Comprehensive Pneumology Centre, and through grants from EU, grants from DFG outside the submitted work.

FIGURE LEGENDS

Figure 1. Association of *IL-33* SNPs and $CD4^+CD25^{high}FOXP3^+$ Tregs in PASTURE/EFRAIM

Corresponding box plots represent Treg percentages in relation to gated lymphocytes. Data are shown as medians, first and third quartile. Whiskers account for the lowest/highest value of 1.5*IQR. AA = major allele homozygotes for both SNPs, AG (rs928413), AC (rs1342326)

= heterozygotes, GG (rs928413), CC (rs1342326) = minor allele homozygotes, PI = PMA and Ionomycin, LPS = Lipopolysaccharide. Maximum number of children: rs928413: AA = 68, AG/GG = 31; rs1342326 AA = 73, AC/CC = 25; Wilcoxon test.

Figure 2. Association of *IL-33* SNPs and *SOCS3* mRNA-expression in PASTURE/EFRAIM

mRNA-expression of *Suppressor of cytokine signaling 3* (*SOCS3*) in the whole PASTURE/EFRAIM cohort for rs928413 and rs1342326 *IL-33* SNPs is shown. Data are shown as means, whiskers account for the 95% confidence interval. AA = major allele homozygotes for both SNPs, AG (rs928413), AC (rs1342326) = heterozygotes, GG (rs928413), CC (rs1342326) = minor allele homozygotes. Number of children: rs928413: AA = 301, AG/GG = 251; rs1342326: AA = 368, AC/CC = 184.

TABLES

Table 1. PASTURE/EFRAIM population characteristics

	PASTURE/EFRAIM Total population N=1133	PASTURE/EFRAIM rs928413/rs1342326 genotyped N=880	PASTURE/EFRAIM rs928413/rs1342326 genotyped + Treg 4.5y N=99	p-value (Chi ² -test) Total vs genotyped† Total vs genotyped+Treg 4.5y‡
Parental atopy (%) (n/total N)	53.78 (591/1099)	52.53 (456/868)	50.51 (50/99)	0.11† 0.49‡
Maternal atopy (%) (n/total N)	32.77 (371/1132)	32.99 (290/879)	33.33 (33/99)	0.77† 0.90‡
Paternal atopy (%) (n/total N)	31.61 (342/1082)	30.60 (265/866)	30.30 (30/99)	0.15† 0.76‡
Siblings (%) (n/total N)	33.45 (379/1133)	33.64 (296/880)	31.31 (31/99)	0.81† 0.64‡
Females (%) (n/total N)	48.62 (530/1090)	49.89 (437/876)	49.49 (49/99)	0.09† 0.86‡
Farming (%)	46.78	48.07	52.53	0.10†

(n/total N)	(530/1133)	(423/880)	(52/99)	0.23‡
Asthma 6 years	4.53	4.55	7.07	0.94†
(%)	(42/928)	(34/747)	(7/99)	0.19‡
(n/total N)				
Sensitization				
Inhalant 6 years	15.44	14.33	15.29	0.08†
(%)	(115/745)	(87/607)	(13/85)	0.97‡
(n/total N)				
Sensitization				
Seasonal 6 years	11.41	10.87	12.94	0.33†
(%)	(85/745)	(66/607)	(11/85)	0.64‡
(n/total N)				

Population characteristics at age 6 years are shown for: the whole PASTURE/EFRAIM, rs928413 and rs1342326 genotyped PASTURE/EFRAIM children as well as rs928413 and rs1342326 genotyped PASTURE/EFRAIM children where Treg data were available at age 4.5 years; y = years, n = number of children with positive clinical outcome, within the total number of children analyzed (N). † Total vs genotyped, ‡ Total vs genotyped + Treg 4.5y

Table 2. Characteristics of *IL-33* polymorphisms in PASTURE/EFRAIM

Gene	SNP	Distance to TSS*	Position in the Gene Structure	Major/Minor Allele	TOTAL PASTURE/EFRAIM MAF
<i>IL-33</i>	rs928413	-2,399bp	5' upstream	A/G	0.26
	rs1342326	-25,710bp	5' upstream	A/C	0.18

*SNP distance to transcription start site (TSS) is based on *IL-33* transcript variant 1 (NM_033439.3; GRCh38). bp = Base pairs, IL-33 = Interleukin 33, MAF = Minor allele frequency, rs = Reference SNP.

Table 3. Identification of polymorphisms in linkage disequilibrium with rs928413 and rs1342326 using HaploReg

pos (hg38)	LD (r ²)	variant	Ref	Alt	EUR freq	Promoter histone marks	Enhancer histone marks	DNase hypersensitive sites	DNA-Protein binding	Regulatory Motifs changed	GENCODE genes
chr9:6192796	0.93	rs2095044	T	C	0.76					Spdef	23kb 5' of IL33
chr9:6193455	0.96	rs2381416	C	A	0.76					Foxa,Foxp1,HDAC2,HNF6,Pou5f1,Sox	22kb 5' of IL33
chr9:6196645	0.80	rs7033058	T	C	0.72			PANC			19kb 5' of IL33
chr9:6197392	0.94	rs1888909	T	C	0.76			ESC,SKIN	CTCF	Gm397,Ik-2,Myc	18kb 5' of IL33
chr9:6209697	0.96	rs992969	A	G	0.76					Bbx,NF-Y,Nkx2,Prrx2	6.1kb 5' of IL33
chr9:6210099	0.96	rs3939286	T	C	0.76			SKIN		Foxp1,RREB-1,Zfp105	5.7kb 5' of IL33
chr9:6213148	0.99	rs928412	A	G	0.75		FAT, BMMSC, BRST, SKIN, CRVX, BONE	BRST,CRVX,MUS, BRN			2.7kb 5' of IL33
chr9:6213387	1.00	rs928413	G	A	0.75		FAT, BMMSC, BRST, MUS, SKIN, CRVX, BONE	SKIN		HNF1,HP1-site-factor	2.4kb 5' of IL33
chr9:6213468	0.99	rs7848215	C	T	0.25		FAT, BMMSC, BRST, SKIN, CRVX, BONE			FAC1,Gfi1,Pbx-1,RREB-1,TATA	2.3kb 5' of IL33
chr9:6172380	0.88	rs7032572	A	G	0.16		ESC, IPSC, SKIN	IPSC		PPAR	43kb 5' of IL33
chr9:6175855	0.91	rs72699186	A	T	0.15					EBF	40kb 5' of IL33
chr9:6176871	0.89	rs72699188	C	G	0.15					CTCF,ERalpha-a	39kb 5' of IL33
chr9:6177302	0.91	rs10975468	C	T	0.15					RXRA,YY1	39kb 5' of IL33
chr9:6177453	0.91	rs9775039	G	A	0.15					Foxa,GR,Pax-4,Pou2f2,RREB-1	38kb 5' of IL33
chr9:6178525	0.91	rs144511606	G	A	0.15					ATF3	37kb 5' of IL33
chr9:6185295	0.91	rs12349858	C	T	0.16	GI, BLD	GI, VAS	BLD	BATF,IRF4,MEF2A, MEF2C,PAX5N19,PU1,S P1,SRF,NFKB		31kb 5' of IL33
chr9:6190076	1.00	rs1342326	A	C	0.15					ERalpha-a,RAR,RXRA,Smad	26kb 5' of IL33
chr9:6197377	0.98	rs10975479	A	G	0.15			ESC,SKIN	CTCF	Pax-5	18kb 5' of IL33
chr9:6201163	0.94	rs1929995	T	C	0.16			BRN		HNF4	15kb 5' of IL33
chr9:6208030	0.96	rs144829310	G	T	0.15					Ascl2,Myf,TAL1,ZID	7.8kb 5' of IL33
chr9:6211813	0.93	rs72699191	T	C	0.16					GR,TATA,TEF	4kb 5' of IL33

Query for SNPs rs928413 (grey shaded) and rs1342326 (white shaded) with $r^2 \geq 0.8$ in HaploReg v3. LD: linkage disequilibrium, Ref: reference, Alt: alternative allele, freq: frequency, EUR: European. Tissues affected by promoter or enhancer histone marks and DNase hypersensitive sites are shown. GI: gastrointestinal (stomach smooth muscle), BLD: B-cell lymphoblastoid, BMMSC: bone marrow-derived mesenchymal stem cells, BRST: breast, CRVX: cervical carcinoma, MUS: muscle, ESC: embryonic stem cells, IPSC: induced pluripotent stem cells, VAS: vascular, PANC: pancreas, BRN: brain. DNA-Protein binding names and regulatory motif changes (gene names) are shown.

Table 4. *IL-33* polymorphisms, phenotypes and allergic sensitization in PASTURE/EFRAIM

<i>IL-33</i> rs928413	Genotypes				Dominant
	AA	AG	GG	AG/GG	p[†]
	% (n/N)				OR (95% CI)
Hay fever	5.7 (23/404)	8.0 (23/287)	18.2 (10/55)	9.6 (33/342)	0.041 1.77 (1.02-3.08)
Asthma	4.2 (17/405)	4.9 (14/286)	5.4 (3/56)	5.0 (17/342)	0.613 1.19 (0.60-2.38)
Inhalant class 3	14.5 (49/338)	12.4 (28/226)	23.3 (10/43)	14.1 (38/269)	0.897 0.97 (0.61-1.53)
Seasonal class 3	10.4 (35/338)	10.2 (23/226)	18.6 (8/43)	11.5 (31/269)	0.646 1.13 (0.68-1.88)
rs1342326	AA	AC	CC	AC/CC	
	% (n/N)				
Hay fever	6.1 (30/495)	10.5 (24/228)	8.7 (2/23)	10.4 (26/251)	0.035 1.79 (1.03-3.10)
Asthma	4.7 (23/495)	4.4 (10/228)	4.2 (1/24)	4.4 (11/252)	0.862 0.94 (0.45-1.95)
Inhalant class 3	14.8 (61/412)	13.1 (23/176)	15.8 (3/19)	13.3 (26/195)	0.629 0.89 (0.54-1.45)
Seasonal class 3	10.9 (45/412)	10.8 (19/176)	10.5 (2/19)	10.8 (21/195)	0.955 0.98 (0.57-1.70)

rs928413, AA = major allele homozygotes, AG = heterozygotes, GG = minor allele homozygotes. rs1342326, AA = major allele homozygotes, AC = heterozygotes, CC = minor allele homozygotes. n = number of children with positive clinical outcome, within the total number of children carrying a specific genotype, N. OR = Odds Ratio, 95% CI = 95% Confidence Intervals. † Chi square (χ^2).

REFERENCES

- 1 Moffatt MF, Gut IG, Demenais F, *et al.* A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med.* 2010; **363**: 1211-21.
- 2 Miller AM. Role of IL-33 in inflammation and disease. *J Inflamm (Lond).* 2011; **8**: 22.
- 3 Ohno T, Morita H, Arae K, Matsumoto K, Nakae S. Interleukin-33 in allergy. *Allergy.* 2012; **67**: 1203-14.
- 4 Carriere V, Roussel L, Ortega N, *et al.* IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A.* 2007; **104**: 282-7.
- 5 Roussel L, Erard M, Cayrol C, Girard JP. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep.* 2008; **9**: 1006-12.
- 6 Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol.* 2010; **10**: 103-10.
- 7 Fux M, Pecaric-Petkovic T, Odermatt A, *et al.* IL-33 is a mediator rather than a trigger of the acute allergic response in humans. *Allergy.* 2014; **69**: 216-22.
- 8 Haenuki Y, Matsushita K, Futatsugi-Yumikura S, *et al.* A critical role of IL-33 in experimental allergic rhinitis. *J Allergy Clin Immunol.* 2012; **130**: 184-94 e11.
- 9 Sakashita M, Yoshimoto T, Hirota T, *et al.* Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy.* 2008; **38**: 1875-81.
- 10 Schiering C, Krausgruber T, Chomka A, *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature.* 2014; **513**: 564-8.
- 11 Turnquist HR, Zhao Z, Rosborough BR, *et al.* IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *J Immunol.* 2011; **187**: 4598-610.
- 12 Lan F, Zhang N, Zhang J, *et al.* Forkhead box protein 3 in human nasal polyp regulatory T cells is regulated by the protein suppressor of cytokine signaling 3. *Journal of Allergy and Clinical Immunology.* 2013; **132**: 1314-21. e3.
- 13 von Mutius E, Schmid S, Group PS. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. *Allergy.* 2006; **61**: 407-13.
- 14 Depner M, Fuchs O, Genuneit J, *et al.* Clinical and epidemiologic phenotypes of childhood asthma. *Am J Respir Crit Care Med.* 2014; **189**: 129-38.
- 15 Lluís A, Depner M, Gaugler B, *et al.* Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol.* 2014; **133**: 551-59 e10.
- 16 Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods.* 2001; **25**: 386-401.
- 17 Rothman KJ. No Adjustments Are Needed for Multiple Comparisons. *Epidemiology.* 1990; **1**: 43-46.
- 18 Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic acids research.* 2012; **40**: D930-D34.
- 19 Brint EK, Xu D, Liu H, *et al.* ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol.* 2004; **5**: 373-9.

- 20 Sweet MJ, Leung BP, Kang D, *et al.* A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol.* 2001; **166**: 6633-9.
- 21 Tulah AS, Holloway JW, Sayers I. Defining the contribution of SNPs identified in asthma GWAS to clinical variables in asthmatic children. *BMC medical genetics.* 2013; **14**: 100.
- 22 Hinds DA, McMahon G, Kiefer AK, *et al.* A genome-wide association meta-analysis of self-reported allergy identifies shared and allergy-specific susceptibility loci. *Nat Genet.* 2013; **45**: 907-11.
- 23 Consortium TGP. An integrated map of genetic variation from 1,092 human genomes. *Nature.* 2012; **491**: 56-65.
- 24 Duan L, Chen J, Zhang H, *et al.* Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med.* 2012; **18**: 753-61.

Supporting Information

IL-33 polymorphisms are associated with increased risk of hay fever and reduced

Regulatory T cells in a birth cohort

Paul Christoph Schröder, PhD ^{a‡}, Vera Isabel Casaca, PhD ^{a‡}, Sabina Illi, PhD ^a, Maximilian Schieck, PhD ^{b, c}, Sven Michel, PhD ^{b, c}, Andreas Böck, PhD ^a, Caroline Roduit, MD ^d, Remo Frei ^{e, m}, Anna Lluís, PhD ^f, Jon Genuneit, MD ^g, Petra Pfefferle, PhD ^h, Marjut Roponen, PhD ⁱ, Juliane Weber, MD ^a, Charlotte Braun-Fahrländer, MD ^{j, k}, Josef Riedler, MD ^l, Roger Lauener, MD ^{m, r}, Dominique Angèle Vuitton, PhD ⁿ, Jean-Charles Dalphin, MD PhD ⁿ, Juha Pekkanen, MD ^{i, o}, Erika von Mutius, MD ^a, Michael Kabesch, MD ^{b, c}, Bianca Schaub, MD ^a and the PASTURE study group.

‡These authors contributed equally to this work.

^a LMU Munich, University Children's Hospital, Munich, Germany, Member of German centre for Lung Research (DZL), ^b Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO), Regensburg, Germany, ^c Department of Pediatric Pneumology, Allergy and Neonatology, Hannover Medical School, Hannover, Germany, ^d University of Zurich, Children's Hospital, and Christine Kühne-Center for Allergy Research and Education, Zurich, Switzerland, ^e Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland, ^f National Jewish Health, Denver, USA, ^g Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany, ^h Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany, ⁱ Living Environment and Health Unit, National Institute for Health and Welfare, Kuopio, Finland, ^j Swiss Tropical and Public Health Institute, Basel, Switzerland; ^k University of Basel, Basel, Switzerland; ^l Children's Hospital Schwarzach, Schwarzach, Austria; ^m Christine Kühne-

Center for Allergy research and Education, Hochgebirgsklinik Davos-Wolfgang, Switzerland, ⁿ
University Hospital of Besançon, the Research Unit Health and Rural Environment, University
of Franche-Comté, Besançon, France, ^o Department of Public health, University of Helsinki,
Helsinki, Finland, ^p University Hospital of Besançon and Plateforme de Biomonitoring, CIC-
BT506, EFS Bourgogne Franche-Comté, INSERM UMR1098, University of Franche-Comté
Besançon, France, ^q Utrecht University, Institute for Risk Assessment Sciences (IRAS), Division
of Environmental Epidemiology, Utrecht, NL, ^r Children's Hospital of Eastern Switzerland, St.
Gallen, Switzerland

PASTURE Study group: A. Hyvärinen ⁱ, Karvonen A ⁱ, Hirvonen MR ⁱ, Tiittanen P ⁱ, Remes S
ⁱ, Kaulek V ^p, Dalphin ML ^p, Ege M ^a, Depner M ^a, Loss G ^a, Renz H ^h, Doekes G ^q.

Correspondence to: Bianca Schaub, LMU Munich, University Children's Hospital,
Lindwurmstrasse 4, D 80337, Munich, Germany, Ph: 0049-89-4400-57856, Fax: 0049-89-4400-
54764. e-mail: Bianca.Schaub@med.uni-muenchen.de

METHODS

Selection of polymorphisms and genotyping

A genome-wide association study by Moffat *et al* identified multiple strong associations of polymorphisms with childhood-onset asthma in the *IL-33* gene region (1). Of these, the SNPs, rs928413 and rs1342326, displayed the strongest associations in the two applied statistical models in GABRIEL consortium. Genotyping in PASTURE/EFRAIM was performed in a Sequenom iPLEX assay using standard conditions (Sequenom, Inc., San Diego, CA, USA) at the Centre National de Génotypage (CNG, Evry Cedex, France). Details on primer sequences are available upon request from the corresponding author.

Flow cytometry of Tregs

After 24h incubation cells were washed and labelled with fluorochrome-conjugated monoclonal antibodies: 8µl of CD4-FITC, 4µl of CD25-PC5 (both Beckman Coulter). After surface labelling cells were fixed and permeabilized for FOXP3 intracellular staining with 10µl of FOXP3-PE (clone PCH101, eBioscience) and washed after incubation. The corresponding isotype conjugates IgG1-FITC, IgG1-PE (both Dako Cytomation, Glostrup, Denmark), and IgG2a-PC5 (Beckman Coulter) were used as controls.

mRNA and protein expression

In a central laboratory (Zurich, Switzerland), RNA was isolated using the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The mRNA was reverse-transcribed into cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed on the 7900HT Fast Real-Time PCR System using the Micro fluidic card TaqMan Array system of Applied Biosystems. The cycle

threshold (CT) describes the number of PCR cycles required for the fluorescence signal to exceed the detection threshold, which was set to the log-linear range of the amplification curve. The difference in CT values relative to *18S* rRNA and *B2M* was used to calculate the delta CT (Δ CT); a higher Δ CT corresponds to lower gene expression. mRNA expression data was analyzed using the comparative threshold method of Giulietti et al (2), a method expressing the measured number of PCR cycles of the participants relative to 1 participant. We chose as a reference the cord blood values for a non-farmer child whose expression of all genes was greater than the detection limit. The results are expressed as a gene expression multiplication factor compared with the reference. This method was described previously in (3). Protein sST2 levels were measured in sera of 4.5 year old children with ELISA according to manufacturer's instructions (MBL International Corporation, Nagoya, Japan).

Identification of polymorphisms in linkage disequilibrium with rs928413 and rs1342326 using HaploReg

The HaploReg v3 database was inquired to identify polymorphisms in linkage disequilibrium (LD) with rs928413 or rs1342326 ($r^2 \geq 0.8$). Functional annotations for each SNP region are suggested by HaploReg (histone marks, DNase hypersensitive sites and DNA-protein binding).

85 **REFERENCES**

- 86 1. Moffatt MF, Gut IG, Demenais F et al. A large-scale, consortium-based genomewide
87 association study of asthma. *N Engl J Med* 2010; **363**:1211–1221.
- 88 2. Giulietti A, Overbergh L, Valckx D et al. An overview of real-time quantitative PCR:
89 applications to quantify cytokine gene expression. *Methods* 2001; **25**:386–401.
- 90 3. Roduit C, Frei R, Depner M et al. Increased food diversity in the first year of life is
91 inversely associated with allergic diseases. *J Allergy Clin Immunol* 2014; **133**:1056–1064.

92

93 **Table S1. *IL-33* polymorphisms in relation to *TLR4* and *IL1RL1* mRNA expression and**
94 **sST2 protein expression in PASTURE/EFRAIM children**

			<i>TLR4</i> gene expression		
N	%	rs928413	GMR	95% CI	
251	45.5	AG/GG ref.	1.00		
301	54.5	AA	1.01	0.92	1.11
N	%	rs1342326	GMR	95% CI	
184	33.3	AC/CC ref.	1.00		
368	66.7	AA	1.00	0.91	1.10
			<i>IL1RL1</i> gene expression		
N	%	rs928413	GMR	95% CI	
251	45.5	AG/GG ref.	1.00		
301	54.5	AA	0.94	0.79	1.12
N	%	rs1342326	GMR	95% CI	
184	33.3	AC/CC ref.	1.00		
368	66.7	AA	0.89	0.74	1.08
			sST2 protein expression		
N	%	rs928413	GMR	95% CI	
177	45.6	AG/GG ref.	1.00		
211	54.4	AA	1.08	0.81	1.42
N	%	rs1342326	GMR	95% CI	
130	33.5	AC/CC ref.	1.00		
258	66.5	AA	1.09	0.81	1.46

95 Linear regression for mRNA gene and protein expression are expressed as geometric mean ratios
96 (GMR) compared to the reference group (ref.). CI = confidence interval