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Chapter 6

Effects of IL-33 on differentiated human Th2 cells stratified for *IL1RL1* asthma risk haplotypes

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Abstract

Background

Genetic variation at the IL1RL1 locus has consistently been associated with asthma. IL1RL1 encodes the IL-33 receptor, and is expressed on T-helper 2 (Th2) cells. Th2 cells are key players in chronic airway inflammation in asthma and respond to IL-33 by enhanced Th2 cell differentiation and increased production of Th2-cytokines such as IL-4, IL-5, and IL-13. However, it is unknown whether the response of Th2 cells to IL-33 is dependent on IL1RL1 genotype and/or presence of asthma. Therefore, we investigated if IL1RL1 asthma risk genotypes and presence of asthma influence the IL-33-induced response during Th2 cell activation in vitro.

Methods

We selected asthma patients and healthy controls based on genotype of asthma-associated IL1RL1 SNPs rs1420101, rs4988956, rs10192036, and rs10192157 and divided into 4 experimental groups: asthma patients carrying an asthma high risk or low risk haplotype, and healthy people carrying an asthma high risk or low risk haplotype. Of these people we isolated CD4 T-cells from PBMCs of peripheral blood. The CD4 T-cells from these groups were differentiated into Th2 cells, followed by CD3/CD28 activation in the absence or presence of IL-33. The primary read-out was gene expression by RNA-sequencing. Secondly, expression of IL-1RL1 and secreted cytokines were analysed at the protein level using flowcytometry and multiplexed bead analysis.

Results

Presence of IL-33 resulted in extensive changes in the gene expression profile of Th2 cells activated by CD3/CD28 crosslinking. The magnitude, but not the gene identity, of the IL-33 induced transcriptional changes was dependent on IL1RL1 haplotype as well as disease status, with the asthma risk haplotype and being asthmatic both conferring a stronger response to IL-33. Interestingly, we observed increased IL1RL1 gene expression levels upon IL-33 stimulation in risk haplotype carriers and asthma patients. At the protein level, IL-33 stimulation lead to an increased fraction of IL-1RL1 expressing Th2 cells, which was not dependent on haplotype or disease status.

Discussion & Conclusion

IL-33 had a profound effect on gene expression in activated Th2 cells. This effect was most pronounced in subjects carrying IL1RL1 asthma-associated genotypes and in asthma patients, while in healthy controls and carriers of the IL1RL1 protective haplotype the IL-33 induced response was of strongly reduced magnitude. The asthma risk haplotype of IL1RL1 contains rs1420101 (a pQTL affecting IL-1RL1 protein expression) and 3 non-synonymous SNPs in full LD (affecting the strength of IL-1RL1 signalling). Therefore, differences in both expression and signalling activity of the IL-1RL1 receptor may underlay the more

pronounced IL-33 induced Th2 activation in IL1RL1 asthma-risk haplotype carriers. The intriguing observation that disease status also influences the enhanced Th2 cell activation by IL-33 needs further mechanistic studies. Our results would suggest that asthma patients carrying the IL1RL1 risk haplotype may benefit most strongly from interventions targeted at the IL-33/IL-1RL1 pathway to reduce Th2 inflammation.

Keywords
Asthma, Th2 cells, IL-33, IL1RL1, genotype, gene expression

Introduction

Asthma is a common and heterogeneous disease, in which an interaction between genetic and environmental factors contributes to development of the disease. Genetic variants at the IL33 and IL1RL1 loci have been consistently associated with asthma, specifically childhood onset asthma, eosinophilia as well as type 2 high asthma (1-4). The Interleukin-33 (IL-33)/ Interleukin-1-Receptor-Like-1 (IL-1RL1) pathway can activate type-2 immunity (4,5). IL33 encodes the alarmin IL-33 which is expressed in bronchial epithelial as well as in endothelial cells. In response to environmental triggers such as allergen exposure, and viral or bacterial infection, bronchial epithelial cells release chemokines, cytokines and alarmins such as IL-33, TSLP and IL-25. These factors are important in the activation of the immune cascade resulting in hyperresponsiveness, remodelling and chronic inflammation of the airways (4-6). IL-33 can bind to the IL-1RL1 protein encoded by IL1RL1. IL-1RL1 has two main isoforms, a transmembrane receptor (IL-1RL1b or ST2L) that is part of the heterodimeric IL-33 receptor complex, and a soluble protein (IL-1RL1a or sST2) acting as a decoy receptor for IL-33. IL1RL1 is expressed by several structural cells (such as epithelial cells, fibroblasts, endothelial cells and smooth muscle cells) as well as a range of immune cells involved in asthma pathogenesis including mast cells, dendritic cells, macrophages, eosinophils, basophils, type-2 innate like helper cells (ILC2s), and Th2 cells (3,7,8).

Human Th2 cells constitutively produce IL-1RL1a and express low levels of IL-1RL1b on their cell surface, in contrast to Th1 and Treg cells (9). Th2 cells are key players in the initiation and maintenance of chronic airway inflammation characteristic of asthma (1,5). IL-33 has been shown to promote Th2 cell differentiation of naive human Th-cells and to enhance the production of Th2-cytokines such as IL-4, IL-5, and IL-13 (10). However, it is not known if genetic variation at the IL1RL1 locus affects the cellular response to IL-33 of Th2 cells.

Functional genetic studies have shown that the IL1RL1 locus harbours several independent genetic signals, some of which act as eQTLs or pQTLs, whereas others encode non-synonymous amino-acid changes within the signalling domain of the IL-1RL1 transmembrane receptor (3). So far, only few studies have directly assessed the functional consequences of asthma-associated genetic variation at the IL33 and IL1RL1 loci for the activity of the IL-33/IL-1RL1 pathway in primary cells relevant to the pathogenesis of asthma (3,11). We have recently

shown that overexpression of risk haplotypes of IL1RL1 in epithelial cells led to increased IL-33 induced signalling compared to non-risk haplotypes in vitro (3).
Understanding how IL1RL1 risk haplotypes affect asthma pathogenesis is critical for the development of precision medicine, facilitating the selection of patient groups for targeted treatment such as strategies directed at the IL-1RL1 pathway. Therefore, we study the functional consequences of the asthma-predisposing IL1RL1 haplotypes for IL-33 induced responses in Th2 cells. We hypothesize that asthma-associated IL1RL1 haplotypes enhance the response of Th2 cells to IL-33, resulting in increased activation of Th2 cells, as evident from Th2 cell transcriptomic responses, and increased expression of type 2 cytokines and pro-inflammatory molecules. To address the effect of disease status, we perform this study in asthma patients and healthy controls. To test our hypothesis, we performed a translational study investigating the effect of IL-33 on gene expression induced by CD3/CD28 crosslinking of in vitro differentiated Th2 cells from both asthma patients and matched controls, stratified for IL1RL1 asthma risk or protective genotype.

Material/Methods

Overall study design (see figure 1)

CD4 T cells were isolated from cryopreserved peripheral blood mononuclear cells (PBMCs) from both healthy subjects and asthma patients, selected on the basis of their IL1RL1 genotypes. The resting CD4+ T cells were subsequently differentiated into Th2 cells, followed by CD3/CD28 activation in the absence or presence of IL-33. RNA was isolated at 24h and protein analysis was performed at 72h. IL-33 induced effects were analysed in a genotype- and disease stratified way.

Patient selection, genotyping and SNP selection

Table 1 shows clinical and demographic characteristics of the asthma patients and controls investigated in this study. Sixteen asthma patients were selected from the 'Roorda' asthma cohort (230,231) (METc 2012/173), which is a long-term follow-up study of children with asthma. Asthma was doctor's diagnosed and confirmed by increased bronchial hyperresponsiveness after histamine challenge (BHR). Sixteen healthy subjects were selected from the 'NORM study' (190,224) (NCT00848406, METc2009/007), that was established based on inclusion of healthy volunteers with no BHR and negative for any chronic respiratory disorder. DNA of both patients and healthy controls was genotyped with an Illumina Omni Express Chip, as described previously (190,231). We selected four asthma-associated, likely functional *IL1RL1* SNPs based on our recent analysis (215,232). The first selected *IL1RL1* SNP, rs1420101, is a strong eQTL and pQTL for *IL1RL1* (26,215,232). The other three selected SNPs represent one genetic signal since these SNPs are in complete linkage disequilibrium (LD). These 3 SNPs (rs4988956, rs10192036, rs10192157) encode non-synonymous amino-acid changes that have been shown to influence IL-1RL1-

dependent signal transduction in epithelial cells (206,215,232). Genotypes were stratified into an asthma–low risk haplotype and asthma–high risk haplotype. The low-risk haplotype was defined as: rs1420101 (GG), rs4988956 (AA or AG), rs10192036 (AA or AC), and rs10192157 (AA or AG), whereas an asthma-high risk haplotype was defined as rs1420101 (AA), rs4988956 (GG), rs10192036 (CC), and rs10192157 (GG). This allowed selection of 8 asthma patients and 8 healthy subjects carrying the low-risk *IL1RL1* haplotype, and 8 asthma patients and 8 healthy subjects carrying the high-risk haplotype.

PBMC storage, $CD4^+$ T cell isolation, Th2 cell differentiation

PBMCs were isolated immediately after blood draw using Lymphoprep™ (Axis-Shield, Stemcell Technologies, #07801) as described before (233) and stored in liquid nitrogen until further use. PBMCs were thawed quickly (1 min in a 37 °C water bath), transferred in a 15mL tube containing warm thawing medium (500mL RPMI1640, 50mL FCS, 5mL Pen/Strep, and 50μM β-mercaptoethanol), centrifuged (375g, 7min, RT), resuspended in 5mL RPMI 10% FCS, and counted using the Beckman Coulter Counter.
Naïve $CD4^+$ T cells were isolated using a negative selection human $CD4^+$ T cell isolation kit according to manufacturers' protocol with LS columns (Miltenyi Biotec, #130-096-533). Subsequently, a human Th2 cell differentiation kit (CellXVivo, #CDK002) was used according to the manufacturer's protocol to obtain differentiated human Th2 cells in 13 days. In short, 250,000 naïve $CD4^+$ T cells/well were suspended in 1 mL human Th2 differentiation media (X-VIVO 15, 100U/mL Pen, 100μg/mL Strep, Th2 differentiation reagents)/well and plated in ☐CD3-precoated 24-well plates. Media was refreshed every three days by removing 900 μL and replenishing with 900 μL fresh Th2 medium. When cell densities up to $1x10^6$ cells/well were reached, cells were first split 1:10 and subsequent splits at 1:2. After 13 days, Th2 cells were harvested and counted.

Activation and IL-33 stimulation of Th2 differentiated cells

Differentiated Th2 cells were washed and resuspended in non-supplemented basal X-VIVO 15 medium for overnight culture, followed by polyclonal activation using 1μg/mL ☐CD3 (Purified NA/LE Mouse anti-Human CD3, 555329, BD Pharmingen) and 1μg/mL ☐CD28 (Purified NA/LE Mouse anti-Human CD28, 555725, BD Pharmingen) for 24h and 72h. Unstimulated controls remained on basal X-VIVO 15 medium. Simultaneously, to analyse the IL-33 induced responses, the differentiated Th2 cells were co-stimulated with 0 or 100ng/mL IL-33 (rhIL-33, 3625-IL-010, BioTechne) for the 24h and 72h respectively.

RNA isolation and RNA sequencing

To establish if *IL1RL1* genetic variants affect gene expression of differentiated Th2 cells, we collected cell pellets for mRNA isolation before CD3/CD28 activation, and 24h thereafter in the absence or presence of IL-33. Total RNA was isolated using 500μL TRIzol®Reagent (Life Technologies) according to manufacturer's protocol, as described before (234). To optimize RNA precipitation, 5 μg RNase-free glycogen was added as a carrier to the aqueous phase.

Precipitated and washed total RNA was resuspended in RNase-free water and yields were determined using the Nanodrop.
Total RNA was put at a minimum concentration of 4 ng/uL, and initial quality check of RNA was performed by capillary electrophoresis using the LabChip GX (PerkinElmer, Waltham, MA). Non-degraded RNA-samples with integrity scores RIN > 8 were selected for subsequent sequencing analysis. RNA sequencing libraries were prepared using the BioScientific NextFlex kit v3 (PerkinElmer, Waltham, MA). RNA sequencing was performed at the sequencing facility of the Genetics department of the UMCG on an Illumina NextSeq500 platform (SBS50 kit, high-output mode, Illumina, San Diego, CA) using default parameters for paired-end sequencing (2 × 100 bp).

Quality control of RNA sequencing data and quantification of expression

Quality control (QC) metrics were calculated for the raw sequencing data using the FastQC tool (version 0.11.3) (228). FastQ files were trimmed and aligned to build GRCh37.75 of the human reference genome using HISAT (version 0.1.5) allowing for 2 mismatches (225). QC metrics were calculated for the aligned reads using Picard-tools (version 1.130; http://picard.sourceforge.net): CollectRnaSeqMetrics, MarkDuplicates, CollectInsertSize-Metrics and SAMtools flagstat. In addition, we checked for concordance between sexlinked (XIST and Y-chromosomal genes), gene expression (inspection for outliers) and reported sex. Before gene quantification, SAMtools (version 1.2) was used to sort the aligned reads (226). The gene level quantification was performed by HTSeq (version 0.6.1p1, (227)) using Ensembl version 75 as gene annotation database.

Protein assays

For protein validation, supernatants of CD3/CD28 activated Th2 cells were analysed using Luminex after 72h of IL-33 stimulation. Total protein content of the cell culture supernatants were quantified using the Pierce™ BCA Protein Assay Kit (23225, ThermoFisher). Next, a premixed, custom-made magnetic bead-based multiplex assay for the Luminex® platform (LXSAHM, Biotechne) was used to quantify different analytes in the cell culture supernatants. We included B2-macroglobulin, CA15-MUC1, CXCL-10, Galectin-3, IL-2, IL-4, IL-5, IL-8, IL-13, IL-17, IL-18, MFG-E8, MIF, RAGE-AGER, and sST2. We performed the assay according to the manufacturer’s protocol. Data was analysed using GraphPad Prism 8.3.1.
Furthermore, we performed Flowcytometry (Beckman Coulter MoFlo Astrios) analysis of Th2 cells (antihuman CD4, BV 421 coloured, Biolegend #357424; antihuman CD25, APC Cy7 coloured, Biolegend #302614), treated with and without IL-33 for 72h and stained for cell surface IL-1RL1 expression (antimouse IL-33Rα, APC coloured, #145306) using FlowJo analysis software v10.

Statistical analyses

Differential gene expression:

After QC, differential gene expression (DGE) analyses of RNA sequencing data was performed

in R (Rstudio v3. 6.1) using the limma and edgeR packages (168). An average gene count of 10 counts/subject was taken as minimal gene expression for a gene to be included in the DGE analyses. Gene count data was log2 transformed using limma-voom (168). First, univariate analyses were performed assessing the effect of IL-33 treatment, haplotype and disease on DGE separately. We next applied a linearized model, with subject as blocking factor to allow paired analyses of the treatment effect for cells derived from the same subject comparing the condition with and without IL-33 treatment. In this model, haplotype and disease effects were assessed in a stratified way. Lastly, we compared the distribution of the induced fold change by IL-33 treatment between the different conditions and tested for differences in fold change using a T test. In all analyses, genes were considered statistically differentially expressed when the FDR adjusted p-value was <0.05.

Protein effects:

IL-33 treatment, haplotype and disease groups were compared for protein levels in a univariate way, using a non-paired T test, ANOVA or their non-parametric variants being the Mann-Whitney U test and Kruskall Wallis test when appropriate. The protein measurements were analysed using GraphPad Prism (8.3.1). A p-value<0.05 was considered statistically significant.

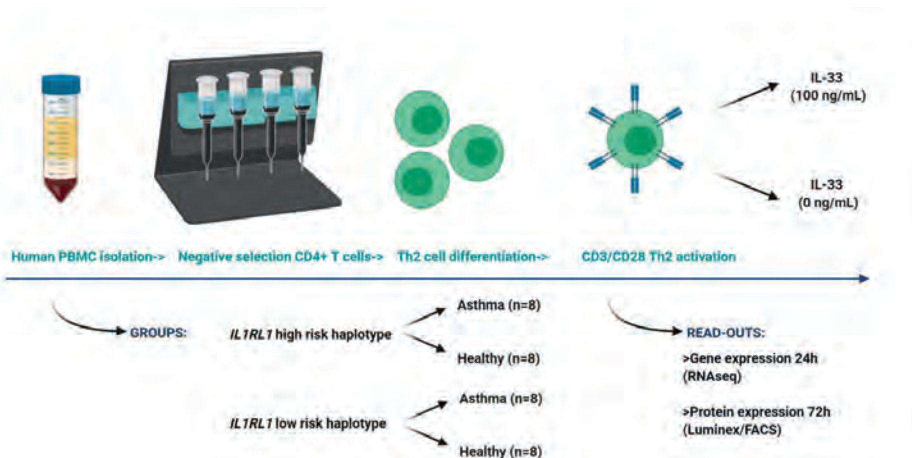


Figure 1: Overview of the study design.

Peripheral blood mononuclear cells (PBMCs) were isolated from asthma patients and healthy subjects of high risk and low risk *IL1RL1* haplotype. Resting CD4+ T cells were isolated from the PBMCs and differentiated to T-helper type 2 (Th2) cells *in vitro*. Th2 cells were CD3/28 activated in the presence or absence of IL-33 protein for 24h and 72h. Th2 cells were processed for RNA sequencing for expression analyses on RNA level (24h), and for Luminex and FACS for expression analyses on protein level (72h). Analyses were performed in a haplotype and disease stratified way. *IL1RL1* high risk haplotype= comprising asthma-associated *IL1RL1* risk genotypes, low risk= comprising asthma-associated *IL1RL1* protective genotypes.

Results

Cohort characteristics

A total of 16 subjects with high risk *IL1RL1* haplotypes, as well as 16 subjects carrying the low risk *IL1RL1* haplotype were included in the study. For both haplotype groups, half of these were asthma patients, while half of these were healthy subjects, resulting in an experimental design with 4 groups defined by a combination of disease state and haplotype, consisting of 8 donors each. Matching was performed as good as possible given the available PBMC donors, but the healthy control group contained fewer male participants compared to the asthma group, as well as healthy subjects were of younger age. General characteristics can be found in table 1.

Table 1: Study cohort

A-Asthma (Roorda) (230,231)

Feature	High risk haplotype (n=8)	Low risk haplotype (n=8)
Gender N (%male)	3 (37.5%)	7 (87.5%)
Age y mean (stdev)	49.5 (2.3)	50.2 (1.7)
FEV1 (pre, L) mean (stdev)	2.9 (0.5)	3.2 (0.8)
FEV1/FVC (%, pre) mean (stdev)	69.6 (6.6)	61.4 (12.6)
Blood eosinophils (10E9/L) mean (stdev)	0.21 (0.10)	0.12 (0.08)

B-Healthy (NORM)(190,224)

Feature	High risk haplotype (n=8)	Low risk haplotype (n=8)
Gender N (%male)	1 (12.5%)	3 (37.5%)
Age y mean (stdev)	32.6 (14.5)	36.8 (19.0)
FEV1 (pre, L) mean (stdev)	3.9 (0.9)	3.6 (0.5)
FEV1/FVC (%, pre) mean (stdev)	82.8 (6.9)	80.1 (2.9)
Blood eosinophils (10E9/L) mean (stdev)	0.21 (0.15)	0.11 (0.06)

RNA Sequencing

From the total set of 32 donors, we discarded three donors (one asthma and two healthy) during QC due to poor alignment (two) or outliers in gene expression (one). In addition, we checked for concordance between sex-linked (XIST and Y-chromosomal) gene expression and reported sex. All samples were concordant. This resulted in high quality RNA-seq data from 29 subjects (15 asthma and 14 healthy donors), that were analysed for IL-33 induced gene expression patterns.

Differential gene expression

IL-33 induces T cell regulatory and activation pathways

In vitro differentiated Th2 cells were CD3/CD28 activated in the absence (0 ng/mL) or presence of IL-33 protein (100 ng/mL) for 24h and differential gene expression (DGE) analysis was performed. First, we analysed the gene expression changes induced by CD3/CD28 stimulation (see supplemental figure I and supplemental table S1 for DGE pattern

upon CD3/CD28 activation and main text table 2 for pathway analysis of DGE upon CD3/CD28 stimulation). This paired analysis across all 29 donors revealed a total of 9185 genes that were significantly induced or repressed in CD3/CD28 stimulated Th2 cells compared to unstimulated cells (supplemental figure I A). No differences in CD3/CD28 activation were found between the *IL1RL1* haplotype groups or dependent on disease status (supplemental figure I B-E).

Next, we analysed the differential gene expression of CD3/CD28 activated Th2 cells in the absence versus the presence of IL-33, using a paired analysis. In the total group of 29 donors, this analysis revealed that presence of IL-33 during CD3/CD28 activation extensively changed the expression pattern of Th2 cells, by inducing 1568 genes and downregulating 2078 genes (figure 2) compared to matched activated Th2 cells without IL-33 (FDR-corrected p-value <0.05). Around 30% of all differentially expressed genes after CD3/CD28-mediated activation of Th2 cells were sensitive to the presence of IL-33. Gene ontology analysis of the differentially expressed genes revealed that the top 10 pathways most affected by the presence of IL-33 belonged to T cell activation and regulation of gene expression pathways, with Th1 cytokine production being the most significantly downregulated pathway (see table 3). This analysis indicates that presence of IL-33 during Th2 cell activation enhances the type-2 effector phenotype in agreement with previous studies (235,236).

IL-33 induced T cell activation is driven by IL1RL1 haplotype

The IL-33 dependent differential gene expression of stimulated Th2 cells was subsequently analysed in the two *IL1RL1* haplotype groups separately. Activated Th2 cells from subjects carrying the *IL1RL1* asthma high risk haplotype (n=14) showed a similar pattern of differential gene expression upon IL-33 stimulation (see figure 3a) compared to those observed in the total dataset (figure 2). In contrast, in activated Th2 cells from subjects with the *IL1RL1* asthma low risk genotype, no genes were found to be differentially expressed upon IL-33 stimulation at the FDR-corrected $p < 0.05$ level (figure 3a, n=15). To directly test whether the IL-33 driven gene expression profile is different between the two haplotype groups, we contrasted the IL-33 dependent differences in gene expression between the two haplotype groups (figure 3b) (i.e. substracting the IL-33 effect in low risk from the IL-33 effect in high risk). This direct comparison did not identify any genes that were significantly differentially induced or repressed by IL-33 between the two haplotype groups. Therefore, we hypothesized that the difference between the two haplotype groups was not based on a qualitatively different response to IL-33, but instead reflected a quantitative difference, with the high-risk haplotype group having a much stronger IL-33 induction or repression of gene expression than the low-risk haplotype group. To test this hypothesis, we compared the distribution of the effect sizes of IL-33 addition on gene expression for all genes in the dataset between the two *IL1RL1* haplotypes (figure 3b). This analysis revealed a significantly larger fold change in gene expression induced by IL-33 in activated Th2 cells from *IL1RL1* asthma high-risk haplotype carriers than in those from low-risk carriers. The average fold change due to IL-33 in high-risk haplotype carriers was -0.038 (n=14), whereas the average

fold change in low risk haplotype carriers was -0.016 (n=15). The 95% confidence interval of difference in means between the two haplogroups comprised -0.028: -0.016, t=-6.80, p=1.09e-11.

IL-33 induced T cell activation is also influenced by disease status

Next, we asked whether the IL-33 induced response was different between Th2 cells from asthma patients and those from healthy controls. To this end, we first analysed the IL-33 responsive genes in activated Th2 cells from asthma patients and healthy controls separately. In this analysis, we found a total of 1247 genes to be upregulated and 1617 downregulated after IL-33 exposure during CD3/CD28 activation of Th2 cells from asthma patients (figure 4a). In contrast, only 10 genes were significantly differentially expressed after IL-33 exposure during activation of Th2 cells from healthy subjects (figure 4a). When directly contrasting the IL-33 effect for disease status (i.e. substracting the IL-33 effect in healthy from the IL-33 effect in asthma), no genes were differentially expressed (figure 4b). To test whether the effect size of IL-33 exposure on gene expression was the explanation for the observed difference between Th2 cells from asthma donors and healthy controls, we again plotted the distribution of the effect size of IL-33 on gene expression levels (figure 4b), now comparing Th2 cells of asthma patients versus healthy subjects. Here, we do not observe a significant difference in the effect size between the two disease groups. However, as any disease effect may be masked by the presence of both haplotypes, we further separated the disease groups in high-risk haplotype carriers and low risk haplotype carriers. In supplemental figure II we show that the effect size of IL-33 on gene expression changes was larger in Th2 cells from asthmatics compared to those from healthy donors when stratified for *IL1RL1* haplotype. This suggests that in addition to a haplotype effect, disease status also affects the sensitivity of Th2 cells to IL-33.

IL1RL1 is differentially expressed upon IL-33 stimulation dependent of haplotype and disease status

Since we noticed a different response to IL-33 stimulation of CD3/CD28-activated Th2 cells as a function of both *IL1RL1* haplotype and disease status, we hypothesized that the level of *IL1RL1* expression might be different in IL-33 stimulated Th2 cells based on haplotype and/or disease status. Indeed, when specifically looking into *IL1RL1* expression levels, we noticed an upregulation of (total) *IL1RL1* RNA levels (figure 5, total *IL1RL1*) upon IL-33 activation that was significantly larger in high-risk haplotype carriers, and in asthma patients compared to low-risk carriers and healthy donors, respectively.

Protein studies

IL-33 stimulation increased the fraction of IL-1RL1 expressing Th2 cells

To evaluate whether the observed increases in *IL1RL1* gene expression was also present at the protein level, we performed flowcytometric analysis of cell surface IL-1RL1 expression on CD3/CD28 activated Th2 cells cultured in the presence and absence of IL-33 for 72 hours.

We observed that IL-33 stimulation led to an increase in the fraction of IL-1RL1 expressing Th2 cells (figure 6), both overall (6a) and when analysed stratified for haplotype (6b) or disease status (6c). The change in fraction of IL-1RL1 expressing Th2 cells by IL-33 was not different between the haplotype groups or between the disease groups (see supplemental figure III).

IL-33 stimulation did not alter the level of soluble IL-1RL1 or Th2 cytokines in supernatants of activated Th2 cells

We next investigated protein expression of IL-33 stimulated Th2 cells in supernatants, including soluble IL-1RL1, Th2 cytokines and a selection of proteins differentially expressed at the RNA level upon IL-33 treatment. The selection included B2-macroglobulin, CA15-MUC1, CXCL-10, Galectin-3, IL-2, IL-4, IL-5, IL-8, IL-13, IL-17, IL-18, MFG-E8, MIF, RAGE-AGER. We did not observe an effect of IL-33 stimulation on soluble IL-1RL1 expression, nor on soluble protein levels of the other studied protein. (See supplemental figure IV).

Table 2: Pathways differentially expressed upon CD3/CD28 activation of Th2 cells

GO term	Ontology	N	Down	Up	P.Down	P.Up
GO:0044237	cellular metabolic process	4280	1162	3118	0.48	4.69E-102
GO:0008152	metabolic process	4559	1533	3026	0.49	2.99E-93
GO:0071704	organic substance metabolic process	4284	1267	3017	0.49	2.62E-87
GO:0006807	nitrogen compound metabolic process	3896	989	2907	0.49	7.42E-87
GO:0044238	primary metabolic process	4100	1111	2989	0.50	7.78E-87
GO:0043170	macromolecule metabolic process	3518	1418	2100	0.50	2.44E-78
GO:0034641	cellular nitrogen compound metabolic process	2163	367	1796	0.50	5.42E-73
GO:0044260	cellular macromolecule metabolic process	2930	1054	1876	0.50	5.59E-68
GO:0006139	nucleobase-containing compound metabolic process	1790	785	1005	0.50	8.24E-64
GO:0050907	detection of chemical stimulus involved in sensory perception	485	187	298	0.50	3.30E-63

Gene Ontology (GO)-pathway analysis of the significantly differentially expressed genes upon CD3/CD28 activation of Th2 cells (n=29). The top 10 of most significant GO terms upon CD3/CD28 activation are shown. Fisher exact test was used as statistical test to determine significantly altered GO-pathways (biological pathways and molecular function).

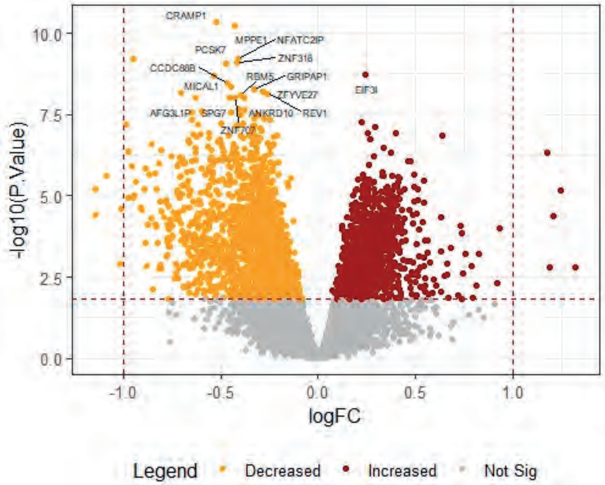


Figure 2: IL-33 causes differential gene expression in CD3/CD28 activated Th2 cells

IL-33 treatment effect on Th2 cell gene expression-in the total population (n=29)

Vulcano plot of -log10 (Pvalue) against LogFoldChange (logFC) for the change in gene expression induced by IL-33 stimulation of Th2 cells, total population.

3640 genes were differentially expressed (out of 11741 tested); 1562 upregulated (brown dots) and 2078 downregulated genes (orange dots) (Total population n=29 subjects.)

Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

Table 3: Pathways differentially expressed upon IL-33 stimulation of Th2 cells

GO term	Ontology	N	Down	Up	P.Down	P.Up
GO:2000556	positive regulation of T-helper 1 cell cytokine production	159	39	18	1.34E-13	0.994704
GO:2000554	regulation of T-helper 1 cell cytokine production	151	37	17	1.36E-19	0.994082
GO:0044212	transcription regulatory region DNA binding	382	59	93	0.593392	5.87E-17
GO:0071622	regulation of granulocyte chemotaxis	769	114	172	0.792514	6.14E-15
GO:1902680	positive regulation of RNA biosynthetic process	686	101	157	0.80164	2.91E-14
GO:0002291	T cell activation via T cell receptor contact with antigen bound to MHC molecule on antigen presenting cell	708	102	161	0.86683	5.49E-12
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	774	110	174	0.907911	1.08E-10
GO:0002286	T cell activation involved in immune response	513	69	119	0.943525	4.87E-19
GO:0001067	regulatory region nucleic acid binding	383	59	93	0.602176	3.90E-17
GO:0071496	cellular response to external stimulus	161	19	42	0.938735	4.05E-17

Gene Ontology (GO)-pathway analysis of the significantly differentially expressed genes after IL-33 exposure of Th2 cells (n=29) during CD3/CD28 stimulation. The top 10 of most significant GO terms after IL-33 stimulation are shown. Fisher exact test was used as statistical test to determine significantly altered GO-pathways (biological pathways and molecular function).

A.

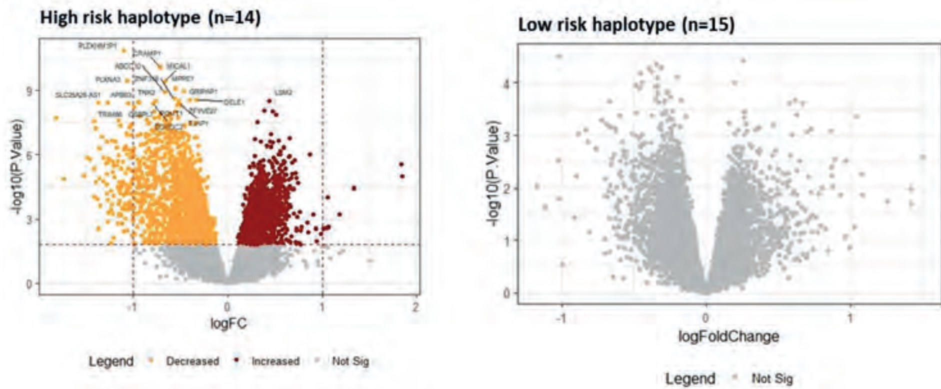


Figure 3: Effect of IL-33 treatment on gene expression of Th2 cells is driven by IL1RL1 haplotype

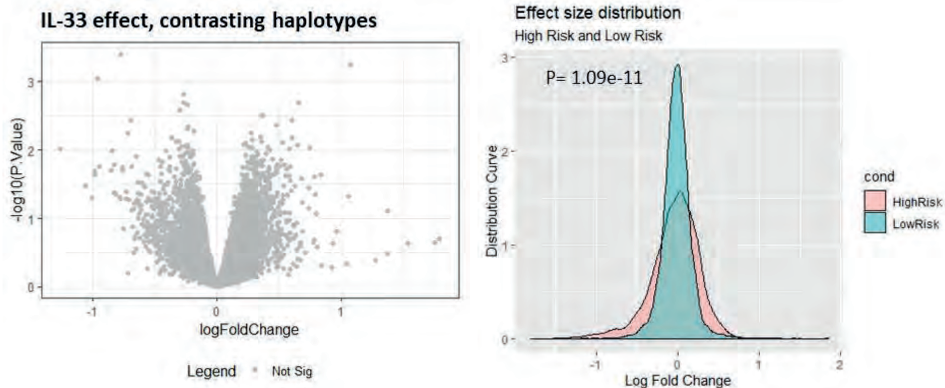
A) Vulcano plots of -log10 (Pvalue) against LogFoldChange (logFC) for the change in gene expression induced IL-33 stimulation of Th2 cells, stratified on haplotype.

Left: high risk haplotype 3844 genes were differentially expressed (out of 11741 tested); 1729 upregulated (brown dots) and 2115 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=14 subjects)

Right: low risk haplotype 0 genes were differentially expressed (out of 11741 tested); (FDR-adjusted p-value <0.05, n=15 subjects)

Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

B.



Left: Differential gene expression pattern shown for the IL-33 effect directly comparing (contrasting) haplotype high risk and low risk carriers.

11741 genes were tested, of which none were significantly differently induced or repressed between the two haplotype groups upon IL-33 treatment. (FDR-adjusted p-value >0.05). *X-axis: logFold change in gene expression upon IL-33 treatment; Y-axis: significance level of effect as –log10 P-values.*

Right: The distribution of effect sizes (fold change) upon IL-33 treatment in haplotype high risk and low risk carriers. Difference in mean fold change was compared using T-test statistics:

Average fold change high risk haplotype: -0.0382; Average fold change low risk haplotype: -0.0162; 95% confidence interval of difference in means: -0.028: -0.016, p-value = 1.09e-11. *X-axis: effect size as logFold change in gene expression upon IL-33 treatment; Y-axis: frequency distribution of effect sizes.*

A.

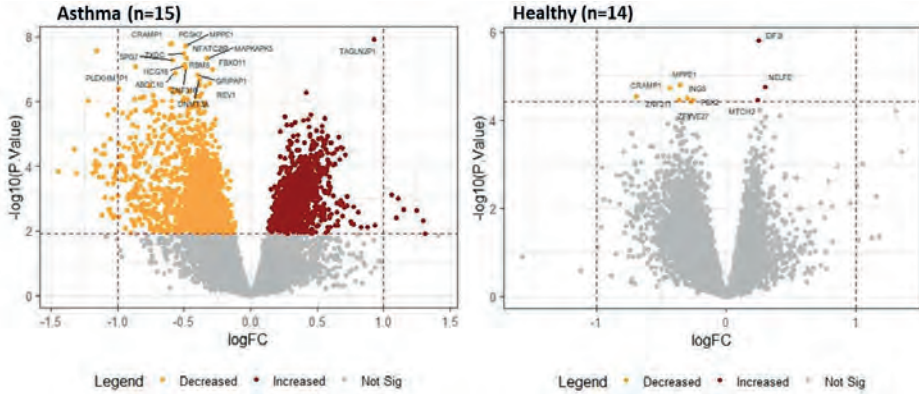
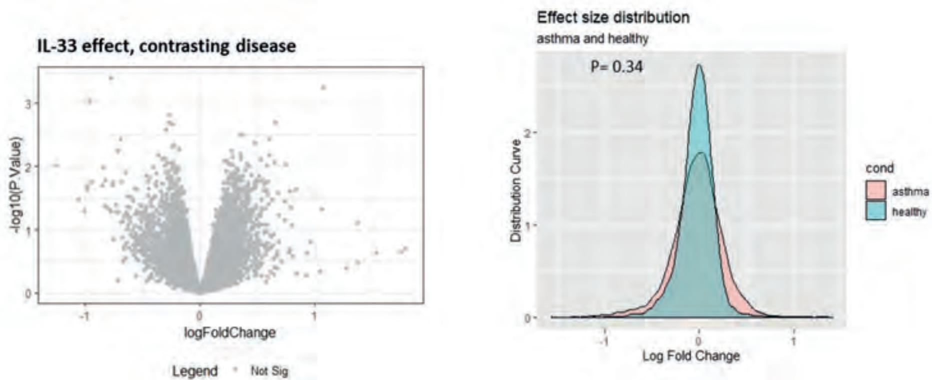


Figure 4: Effect of IL-33 treatment on gene expression of Th2 cells stratified on disease status
A) Vulcano plots of -log10 (Pvalue) against LogFoldChange (logFC) for the change in gene expression induced IL-33 stimulation of Th2 cells, stratified on disease.
Left: asthma- 2864 genes were differentially expressed (out of 11741 tested); 1247 upregulated (brown dots) and 1617 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=15 subjects)
Right: healthy- 10 genes were differentially expressed (out of 11741 tested); 3 upregulated (brown dots) and 7 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=14 subjects)
Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

B.



B)
Left: Differential gene expression pattern shown for the IL-33 effect directly comparing (contrasting) asthma and healthy
11741 genes were tested, of which none were significantly differently induced or repressed between asthma and healthy upon IL-33 stimulation. (FDR-adjusted p-value >0.05). *X-axis: logFold change in gene expression upon IL-33 stimulation; Y-axis: significance level of effect as –log10 P-values.*
Right: The distribution of effect sizes (fold change) upon IL-33 stimulation in haplotype asthma and healthy
Difference in mean fold change was compared using T-test statistics:
Average fold change asthma: -0.0249; Average fold change healthy: -0.0278; 95% confidence interval of difference in means: -0.00295: 0.00863, p-value = 0.34. *X-axis: effect size as logFold change in gene expression upon IL-33 stimulation; Y-axis: frequency distribution of effect sizes.*

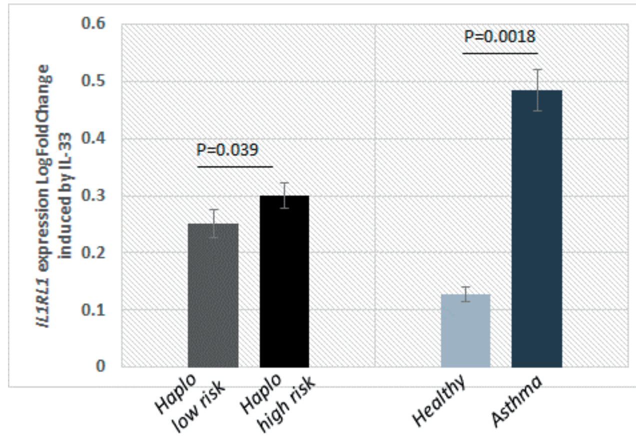


Figure 5: IL-33 induces IL1RL1 expression in Th2 cells to a larger extent in IL1RL1 risk haplotype carriers and asthma patients

Shown is the log fold change of IL1RL1 gene expression (RNA) induced by the presence of IL-33 during Th2 cell activation in (left) haplotype groups and (right) disease groups. Groups were compared using T- test statistics.

8a
The percentage of IL-1RL1 expressing Th2 cells is induced by IL-33
P=2.03E-4
% of IL1RL1 expressing TH2 cells
No IL-33
IL-33 100ng/mL
Treatment

8b
The percentage of IL-1RL1 expressing Th2 cells is induced by IL-33
low risk
P=1.17E-2
high risk
P=9.03E-3
% of IL1RL1 expressing TH2 cells
No IL-33
IL-33 100ng/mL
No IL-33
IL-33 100ng/mL
Treatment

8c
Percentage of IL-1RL1 expressing Th2 cells is induced by IL-33
healthy
P=1.16E-4
asthma
P=2.78E-2
% of IL1RL1 expressing TH2 cells
No IL-33
IL-33 100ng/mL
No IL-33
IL-33 100ng/mL
Treatment

Figure 6: IL-33 induces the percentage of IL-1RL1 expressing Th2 cells

Th2 cells were stimulated with IL-33 for 72h (red dots), then stained for IL-1RL1 protein expression and analysed by Flowcytometry. The percentages of IL-1RL1 expressing Th2 cells upon IL-33 exposure are plotted above. Total population: (6*a, no IL-33 mean 29.1+/-11.6%, IL-33 mean 37.7+/-14.8%; p=2.03E-4*) and when analysed stratified for haplotype (6b, low risk haplotype *no IL-33 mean 31.4+/-8.9%, IL-33 mean 40.9+/-15.3%; p=1.17E-2;* high risk haplotype *no IL-33 mean 26.4+/-14.2%, IL-33 mean 33.4+/-13.8%; p=9.03E-3*) or stratified for disease (6c, healthy *no IL-33 mean 29.6+/-13.2%, IL-33 mean 38.7+/-14.2%; p=1.17E-4;* asthma *no IL-33 mean 28.8+/-10.8%, IL-33 mean 36.8+/-15.9%; p=2.78E-2*). Statistics: paired T test. Grey dotted line: average percentage (%) of IL-1RL1 expressing Th2 cells.

Discussion

Summary of main results

In this study, we investigated our hypothesis that asthma-associated *IL1RL1* haplotypes enhance the response of Th2 cells to IL-33, resulting in increased activation of Th2 cells. Here, we found that *IL1RL1* asthma risk haplotypes and disease status significantly affect transcriptional responses of Th2 cells to IL-33. The IL-33 driven changes in gene expression mapped to pathways involved in Th1 cell cytokine production (suppressed by IL-33) and gene expression regulation of Th2 cell pathways (enhanced by IL-33), including the IL-4/IL-5 pathway. The higher sensitivity to IL-33 in high risk haplotype carriers and asthma patients might be mediated by increased signalling capacity of the transmembrane IL-1RL1 receptor, or due to increased expression of the receptor as a consequence of genotype effects and/or disease priming effects. At the protein level, the fraction of (transmembrane) IL-1RL1 expressing Th2 cells was increased by IL-33, irrespective of *IL1RL1* haplotype or disease status. For the soluble IL-1RL1 levels in the supernatants of the Th2 cells, we did not find an influence of IL-33 stimulation, disease status or *IL1RL1* haplotype.

IL-33 effects on Th2 cells are affected by IL1RL1 asthma risk haplotype

Genetic and functional studies support a role for the IL-33/IL-1RL1 pathway in asthma pathogenesis (26,206,215,232,237). Genetic association studies have repeatedly found SNPs at the *IL33* and *IL1RL1* loci associated with asthma (1,22,25,26). The asthma-associated *IL1RL1* SNP rs1420101, that is part of our currently studied *IL1RL1* haplotype, regulates mRNA and protein in lung tissue and bronchial epithelial cells and serum (189,215,216,232,238). The T-allele of this SNP is associated with increased risk of asthma and lower lung function (26,212,215,232), as well as with higher RNA levels of the transmembrane *IL1RL1* in lung tissue (189,232), and with lower RNA levels of the soluble *IL1RL1* in bronchial epithelial cells (232). This SNP is also a known pQTL of the soluble (protein) decoy receptor IL-1RL1a, with the asthma-risk allele of rs1420101 associating with lower soluble IL-1RL1 levels in serum and in supernatants of bronchial epithelial cells (27,232,239,240). Altogether, the asthma risk allele of rs1420101 is associated with increased expression levels of the transmembrane receptor and reduced levels of the soluble decoy *IL1RL1*, a functional antagonist of IL-33 signalling, likely contributing to an increased sensitivity to IL-33. Coding effects of asthma-associated *IL1RL1* SNPs have also been recognized, including 5 SNPs in full LD located in exon 11 of the transmembrane form of *IL1RL1*. These SNPs encode non-synonymous amino acid changes in the TIR homology domain, shown to modulate IL-1RL1b receptor signalling in cell reporter systems (26,206,232). Only few studies used primary cells relevant to asthma pathogenesis to investigate functional effects of disease-associated *IL1RL1* SNPs. One study reported of blood eosinophils from people carrying the exon 11 *IL1RL1* protective haplotype to have reduced IL-33–mediated IL-8 secretion (206). Since rs1420101 and these exon 11 SNPs are in moderate LD, it is possible to define asthma risk haplotypes based on these two genetic signals, as was done in our study, containing both the e/pQTL SNP rs1420101 and

three of the non-synonymous SNPs. To the best of our knowledge, functional effects of these *IL1RL1* SNPs have not been studied in primary Th2 cells before.
We report that the sensitivity of Th2 effector cells for IL-33 is strongly dependent on the haplotype of the *IL1RL1* receptor, with almost no response in the low risk haplotype, but a strong response in the asthma susceptible *IL1RL1* haplotype. This is one of the first reports to show a dominant effect of this *IL1RL1* asthma risk haplotype on IL-33 induced responses in primary cells of an asthma-relevant cell type depending on endogenous *IL1RL1*, rather than overexpression of engineered receptor variants. The higher sensitivity to IL-33 in *IL1RL1* asthma risk haplotype carriers could be mediated by increased signal transduction of the asthma risk haplotype, as a consequence of the 3 non-synonymous coding SNPs encoding a functionally altered signalling domain in the cytoplasmic part of the transmembrane receptor. Previously, we have shown that asthma-risk allele carriers of these SNPs have induced signalling activity upon IL-33 stimulation in HEK-SEAP cells (232). Alternatively, or additionally, the more pronounced effects of IL-33 in risk haplotype carriers may be explained by higher expression of *IL1RL1* mediated via the QTL SNP rs1420101. Indeed, on RNA level we found an induction of *IL1RL1* expression by IL-33 which was to a greater extent in risk haplotype carriers. However, on protein level, although IL-33 generally induced an increase in the fraction of IL-1RL1 expressing Th2 cells, this effect was not dependent on *IL1RL1* haplotype. Also, we did not find a difference in soluble IL-1RL1 protein levels in the Th2 supernatants upon IL-33 stimulation. This may indicate that our observations are not affected by changes in IL-1RL1-a levels; alternatively it may well be that the timepoint was not optimal to detect protein effects (72h) as compared to the RNA effects (24h).

IL-33 effects on Th2 cells are affected by disease status

The IL-33 effect on gene expression in Th2 cells was not only affected by haplotype, but also by disease status. In our studies, IL-33 exposure had a strong effect on gene expression in asthmatic Th2 cells, but not in Th2 cells obtained from healthy donors. In this case, however, there was no significant difference in the IL-33 induced effect size on gene expression between the two disease groups while we had seen that IL-33 had a larger effect size in risk haplotype carriers. Therefore, we also studied the disease effect on the IL-33 mediated response in the separate haplotype groups, as the disease effect may be masked by the presence of both haplotypes. Indeed, when separated in high and low risk haplotype carriers, IL-33 had a stronger effect on the size of gene expression changes in Th2 cells from asthmatics than from healthy people. This suggests that in addition to a haplotype effect, also disease status affects the cells' sensitivity to IL-33. Again, this may be mediated via altered *IL1RL1* expression, as here we also noticed that the increase in *IL1RL1* RNA expression due to IL-33 exposure was larger in cells derived from asthmatics. How this would work mechanistically would require more experimental studies, but a potential mechanism might be epigenetic programming of gene expression in the CD4 T cells obtained from asthmatic donors (241,242). For example, it has been recognized that asthma and allergy status affects epigenetic modifications in different cells from the immune system and respiratory tract (242), including epigenetic

effects on the pattern of Th1 and Th2 cell differentiation, regulatory T cell differentiation, and Th17 development (243). If (epigenetic) programming in asthmatic donors would have a dominant effect on the Th2 cells , these cells might generally express higher levels of inflammatory molecules upon activation. To check this, we compared general activation of Th2 cells due to antiCD3/CD28 exposure between the disease groups. We found that CD3/CD28 activation of Th2 cells induced a large change in gene expression, but both the identity of differentially expressed genes and the effect sizes did not differ between Th2 cells obtained from healthy and asthmatic donors. This suggests that the increased sensitivity of Th2 cells from asthma patients may be specific for IL-33 and is not due to a generally increased sensitivity to stimulation. Interestingly, Dijk *et al.* (215) recently studied the potential link between asthma-associated SNPs at the *IL1RL1* locus, asthma disease status and *IL1RL1* methylation patterns. Asthma SNPs at *IL1RL1* were strongly associated with *IL1RL1* methylation, while asthma disease status did not associate with *IL1RL1* methylation status. However, this study was performed in whole blood and not in specific cell types. Therefore, the effect of asthma disease status on (*IL1RL1*) methylation patterns in Th2 cells would need to be analysed in specific cell types, in order to see whether or not this could explain the increased sensitivity to IL-33 in Th2 cells derived from asthmatics.

IL-33 induced effects are linked to Th2 biology

The above findings underline a potential link between *IL1RL1* haplotypes and IL-33 biology in Th2 activation in asthma. Top immune-related genes that were differentially expressed upon IL-33 stimulation included genes encoding Th2 cytokines IL-4 and IL-5, but also IL-17, IL-18 and IL-2. Interestingly, especially in *IL1RL1* risk haplotype carriers, IL-33 also induced differential expression of *STAT2* and *STAT6* (supplemental), which are key transcription factors in IL-4 mediated Th2 signalling pathways (244). Moreover, in *IL1RL1* risk haplotype carriers, the gene encoding the critical Th2 cell transcription factor *GATA3* was differentially expressed upon IL-33 stimulation of activated Th2 cells. GATA3 is found to be involved in the regulation of *IL1RL1* expression by binding to an enhancer located 12kb upstream of the *IL1RL1* transcription start site (244). These RNA data indicate that the IL-33 responsive Th2 cells were enforced in their effector phenotypes. Validation of these findings at the protein level, however, failed to confirm these observations, which might be due to the timepoint of protein measurement (72h after activation). For short-acting molecules such as cytokines, it may well be a too late a timeframe for finding protein differences (245-247).

Clinical implications

The current study suggests that strong haplotype effects exist for *IL1RL1* affecting the sensitivity of Th2 cells to IL-33 in asthma as reflected by its larger effect on gene expression in asthma-risk haplotype carriers. This could have implications for developing targeted drugs in asthma, but might as well have potential predictive use. Since the asthma-associated alleles of the 3 non-synonymous SNPs that we studied are known to increase IL-1RL1 signalling activity, these SNPs could be used to select asthma patients that would potentially

have a pronounced response to drugs targeted at the IL-33/IL-1RL1 pathway. Especially drugs directed to inhibit the transmembrane IL-1RL1 receptor would in theory be beneficial in patients carrying these non-synonymous SNPs. Finding ways to select subpopulations of asthma for targeted drugs is especially relevant in light of the current clinical trials that study anti-IL-33 drugs (248,249), and definition of the likely responder group in such a clinical trial would be of great value.

Strengths and limitations

Although we took a next step in investigating the functionality of asthma-associated genetic variation by using primary immune cells from patients and studying endogenous *IL1RL1*, this design has several strength and limitations. A strength is that the naturally occurring haplotypes reflect true combinations of genetic variation and therefore incorporates potential SNP-context effects as compared to SNP mutation strategies that do not, making it more representative. However, it is well-known that the LD pattern at the *IL1RL1* locus is complex (26,239), and the SNPs that we have studied with a focus on the *IL1RL1* pathway may in fact be causal SNPs with functionality for its neighbouring genes (e.g. *IL1R1*, *IL18R1*, *IL18RAP*) with which these SNPs are in high LD. Studying these SNPs in populations with a different LD pattern as was done recently in the African American population might provide a way forward (250). Moreover, the authors realize that, although we carefully tried to match the disease and haplotype groups on clinical parameters, there were fewer males in the healthy group, which may act as a potential confounder, but in light of our stratified analyses we had limited number to further study sex effects separately. Furthermore, future studies would benefit from investigating isoform specific effects of *IL1RL1* SNPs on *IL1RL1* expression, as RNA differences in the variant encoding the soluble decoy IL-1RL1a versus the transmembrane form IL-1RL1b may have different mechanistic implications.

In conclusion, the current study shows that IL-33 may activate Th2 cells differently in subjects carrying asthma-associated risk genotypes in *IL1RL1* and in asthma patients, which might partly been driven by differences in IL-1RL1 signalling and expression upon Th2 activation. This may form a bridge between association of the *IL1RL1* gene with asthma and Th2 biology in asthma pathogenesis and may help to select subgroups of asthma based on genotype and gene expression for targeted treatment in asthma.

Acknowledgements

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To take home

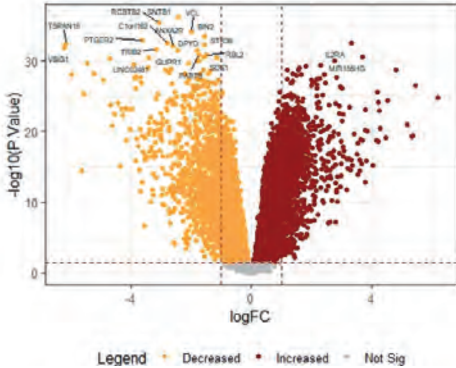
∞ Th2 cells from people carrying asthma-associated haplotypes in *IL1RL1* may be more prone to IL-33 induced inflammation, which may partly run via altered IL-1RL1 signalling partly via altered expression of the IL-1RL1 receptor.

Supplemental see also online

i. Gene expression data:

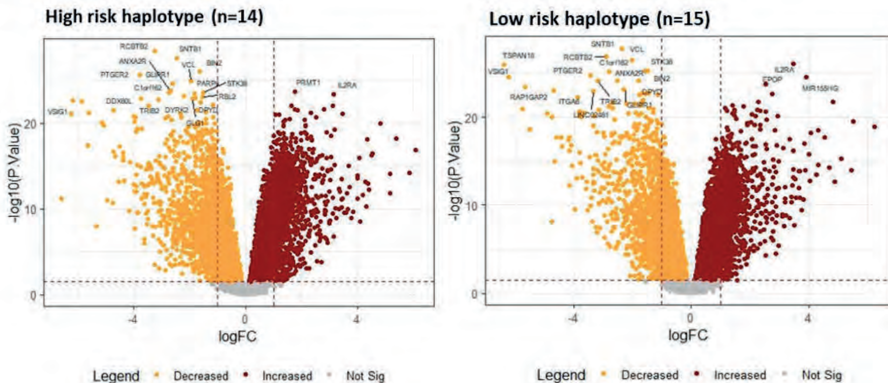
Figure I: Effect of CD3/CD28 activation on gene expression of Th2 cells

A) Total population (n=29)



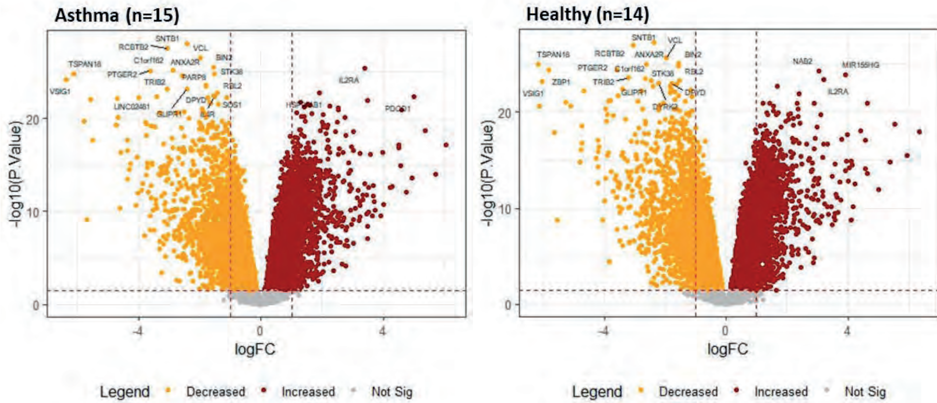
Vulcano plot of -log10(Pvalue) against LogFoldChange(logFC) for the change in gene expression induced by CD3/CD28 activation of Th2 cells, total population.
9185 genes were differentially expressed (out of 12038 tested); 4934 upregulated (brown dots) and 4251 downregulated genes (orange dots). (Total population n=29 subjects.)
Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

B) Stratified on haplotype



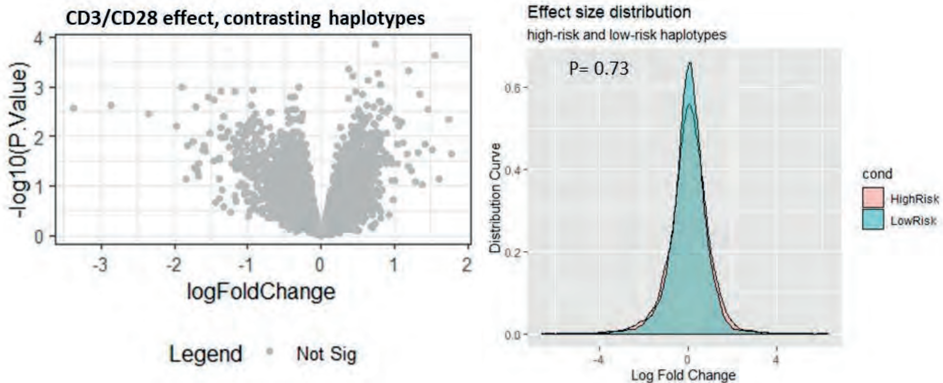
Vulcano plots of -log10(Pvalue) against LogFoldChange(logFC) for the change in gene expression induced by CD3/CD28 activation of Th2 cells, stratified on haplotype.
Left: high risk haplotype 8314 genes were differentially expressed (out of 12038 tested); 4504 upregulated (brown dots) and 3810 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=14 subjects)
Right: low risk haplotype 7852 genes were differentially expressed (out of 12038 tested); 4290 upregulated (brown dots) and 3562 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=15 subjects)
Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

C) Stratified on disease



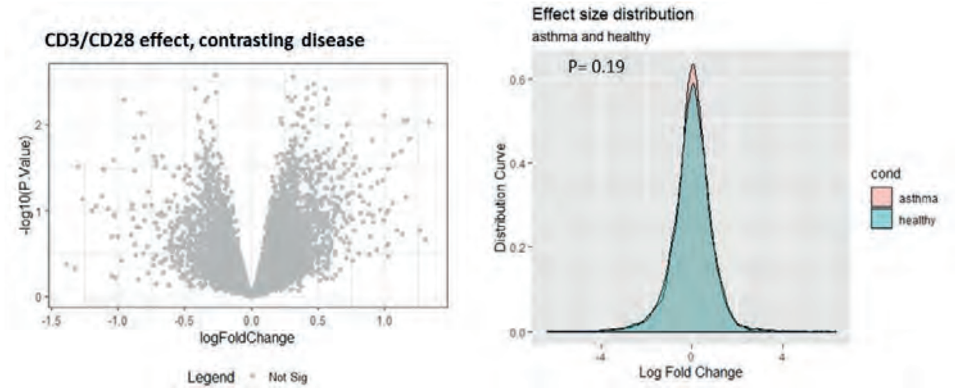
Vulcano plots of -log10(Pvalue) against LogFoldChange(logFC) for the change in gene expression induced by CD3/CD28 activation of Th2 cells, stratified on disease.
Left: asthma- 7924 genes were differentially expressed (out of 12038 tested); 4372 upregulated (brown dots) and 3552 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=15 subjects)
Right: healthy 8185 genes were differentially expressed (out of 12038 tested); 4374 upregulated (brown dots) and 3811 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=14 subjects)
Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

D) no differences of CD3/CD28 effect between haplotypes



Left: Differential gene expression pattern shown for the CD3/CD28 effect directly comparing (contrasting) high risk and low risk haplotype carriers. 12038 genes were tested, of which none were significantly differently induced or repressed between Th2 cells from the two haplotype groups upon CD3/CD28 activation. (FDR-adjusted p-value >0.05).
X-axis: logFold change in gene expression upon CD3/CD28 activation; Y-axis: significance level of effect as –log10 P-values.
Right: The distribution of effect sizes (fold change) upon CD3/CD28 activation in haplotype high risk and low risk carriers.
Difference in mean fold change was compared using T-test statistics:
Average fold change high risk haplotype: 0.0167; Average fold change low risk haplotype: 0.0128; 95% confidence interval of difference in means: -0.019: 0.026, p-value = 0.73. *X-axis: effect size as logFold change in gene expression upon CD3/CD28 activation; Y-axis: frequency distribution of effect sizes.*

E) no differences of CD3/CD28 effect between asthma and healthy



Left: Differential gene expression pattern shown for the CD3/CD28 effect directly comparing (contrasting) asthma and healthy

12038 genes were tested, of which none were significantly differently induced or repressed between asthma and healthy Th2 cells upon CD3/CD28 activation. (FDR-adjusted p-value >0.05). *X-axis: logFold change in gene expression upon CD3/CD28 activation; Y-axis: significance level of effect as –log10 P-values.*

Right: The distribution of effect sizes (fold change) upon CD3/CD28 activation in haplotype asthma and healthy

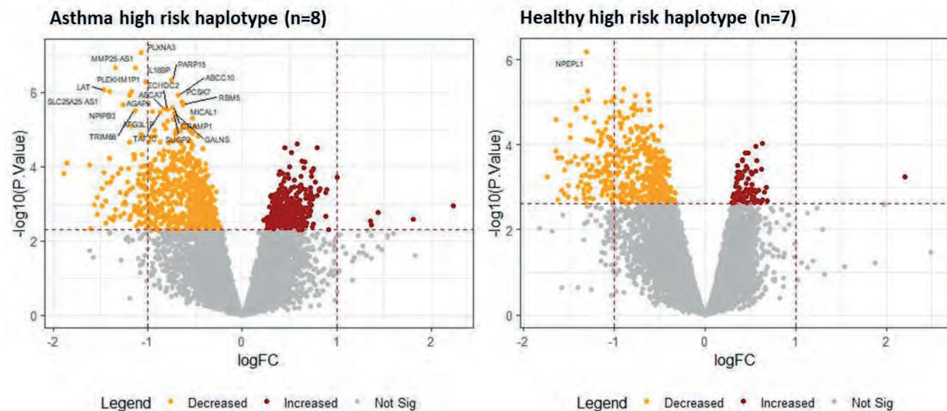
Difference in mean fold change was compared using T-test statistics:

Average fold change asthma: 0.0213; Average fold change healthy: 0.0064; 95% confidence interval of difference in means: -0.00748: 0.0373,

p-value = 0.19. *X-axis: effect size as logFold change in gene expression upon CD3/CD28 activation; Y-axis: frequency distribution of effect sizes.*

Figure II: In separate IL1RL1 high and low risk haplotypes, disease status affects the IL-33 effect on gene expression of Th2 cells

A.



A) high risk haplotype

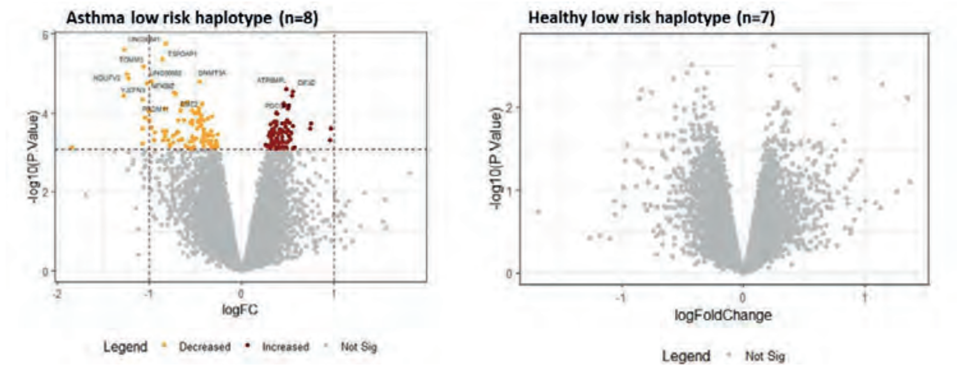
Left: asthmatic donors / high risk haplotype carriers only - 1211 genes were differentially expressed (out of 11757 tested); 420 upregulated (brown dots) and 791 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=8 subjects)

Right: healthy donors / high risk haplotype carriers only - 557 genes were differentially expressed (out of 11757 tested); 85 upregulated (brown dots) and 472 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=7

subjects)

Vulcano plots of -log10(Pvalue) against LogFoldChange(logFC) for the change in gene expression induced IL-33 stimulation of Th2 cells are shown. Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

B.



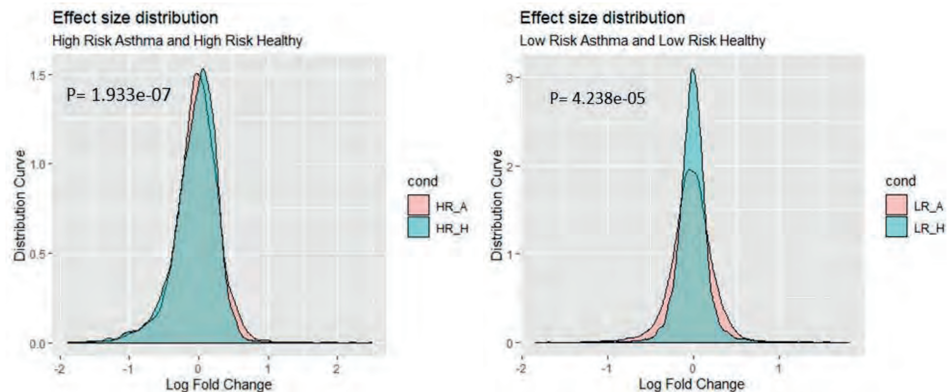
B) low risk haplotype

Left: asthmatic donors / low risk haplotype carriers only - 188 genes were differentially expressed (out of 11757 tested); specifically 80 upregulated (brown dots) and 108 downregulated genes (orange dots) (FDR-adjusted p-value <0.05, n=8 subjects)

Right: healthy donors / low risk haplotype carriers only - 0 genes were differentially expressed (out of 11757 tested) (FDR-adjusted p-value <0.05, n=7 subjects)

Vulcano plots of -log10(Pvalue) against LogFoldChange(logFC) for the change in gene expression induced IL-33 stimulation of Th2 cells are shown. Horizontal dotted line represents the p-value where the FDR-adjusted p-value <0.05, the vertical dotted lines show logFoldchange=-1 and logFoldchange=1 respectively.

C.



C) Distribution of IL-33 induced effect sizes

Left: The distribution of effect sizes (fold change) upon IL-33 treatment in asthma-high risk and healthy-high risk donors.

Difference in mean fold change was compared using T-test statistics:

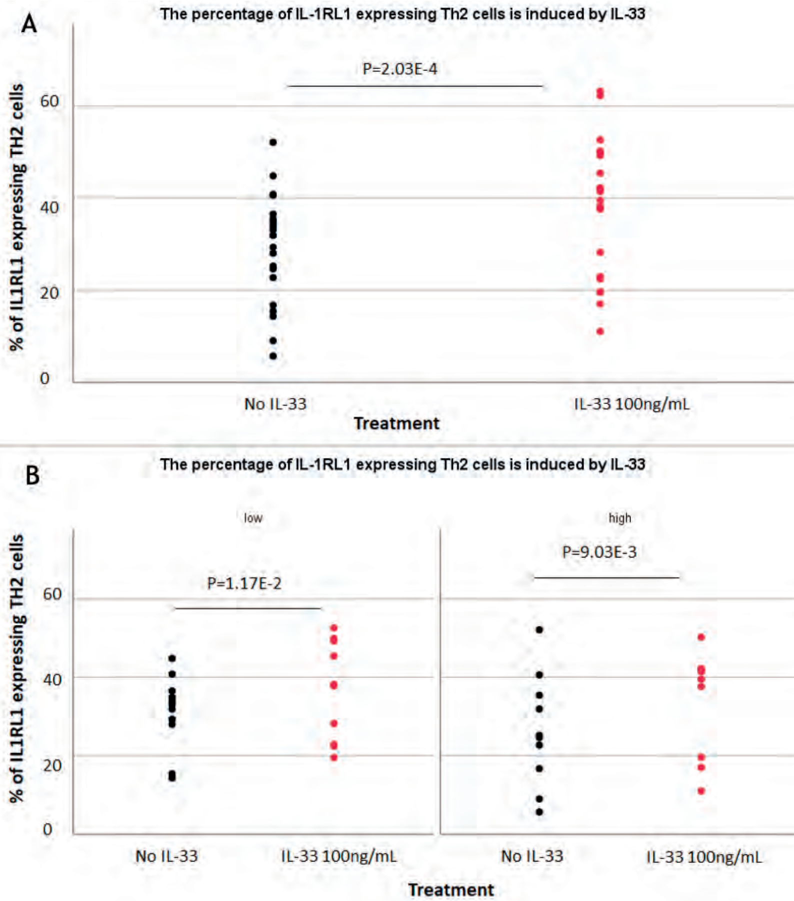
Average fold change asthma high risk haplotype (HR_A): -0.0511; Average fold change healthy high risk haplotype (HR_H): -0.0291

95% confidence interval of difference in means: 0.0137: 0.0302, p-value = 1.933e-07. *X-axis: effect size as logFold change in gene expression upon IL-33 treatment; Y-axis: frequency distribution of effect sizes.*

Right: The distribution of effect sizes (fold change) upon IL-33 treatment in asthma-low risk and healthy-low risk carriers.
Difference in mean fold change was compared using T-test statistics:
Average fold change asthma low risk haplotype (LR_A): -0.0233; Average fold change healthy low risk haplotype (LR_H) : -0.0119
95% confidence interval of difference in means: -0.0168: -0.0059, p-value = 4.238e-05. *X-axis: effect size as logFold change in gene expression upon IL-33 treatment; Y-axis: frequency distribution of effect sizes.*

ii. FACS data: percentage of IL-1RL1 expressing Th2 cells upon IL-33 exposure

Figure III:



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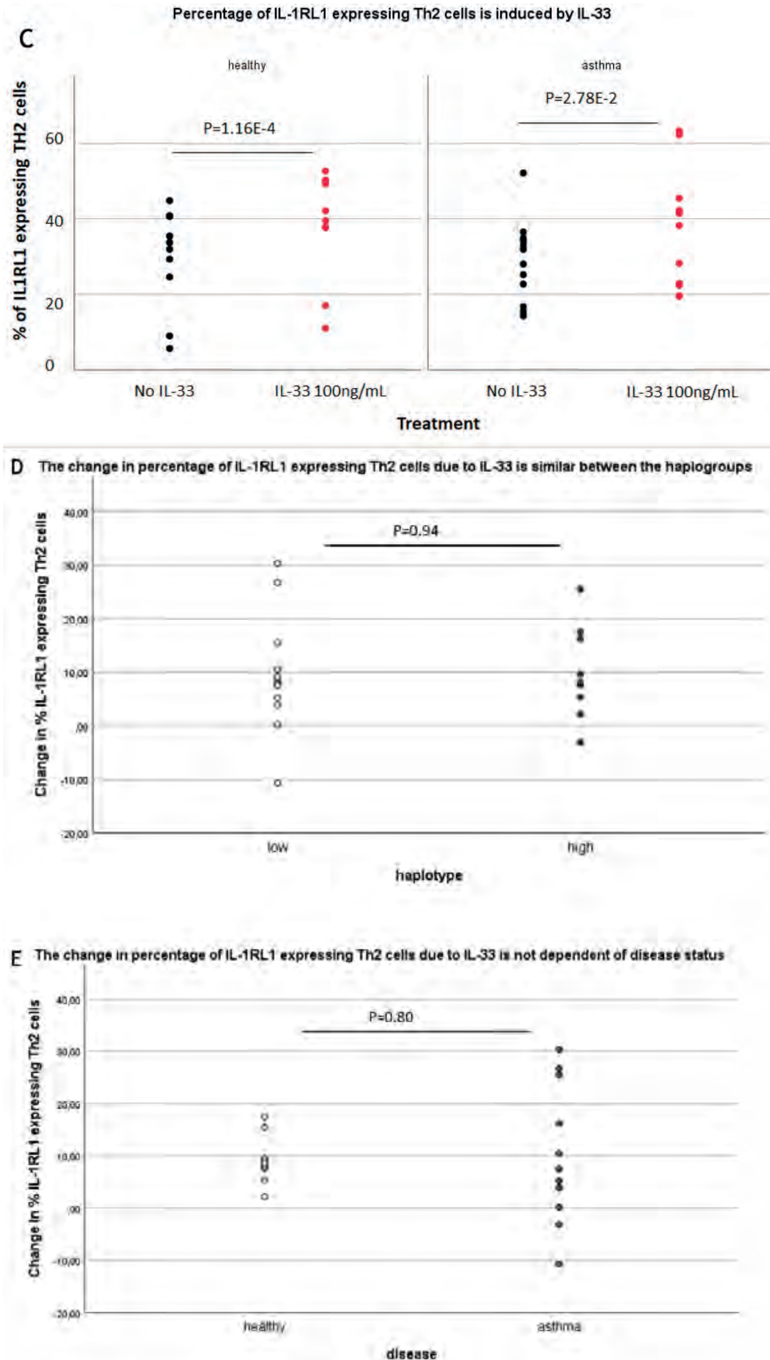


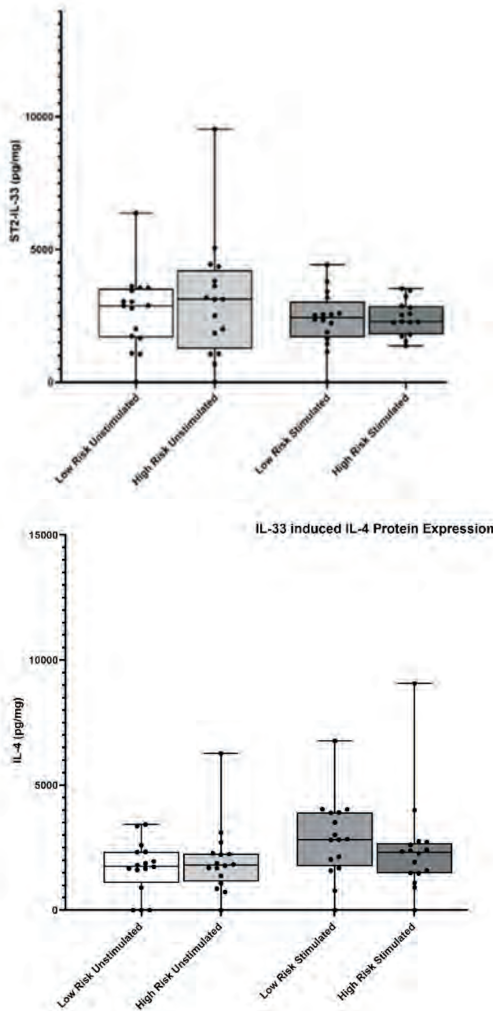
Figure III: IL-33 induces the percentage of IL-1RL1 expressing Th2 cells

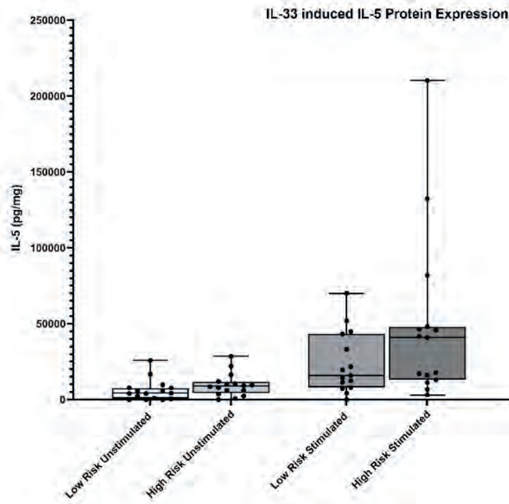
Th2 cells were stimulated with IL-33 for 72h, then stained for IL-1RL1 protein expression and FAC sorted. IL-33 induced the percentage of IL-1RL1 expressing TH2 cells, unstratified (*A, no IL-33 mean 29.1+/-11.6%, IL-33 mean 37.7+/-14.8%; p=2.03E-4*) and when analysed stratified for haplotype (B, low risk haplotype *no IL-33 mean 31.4+/-8.9%, IL-33 mean*

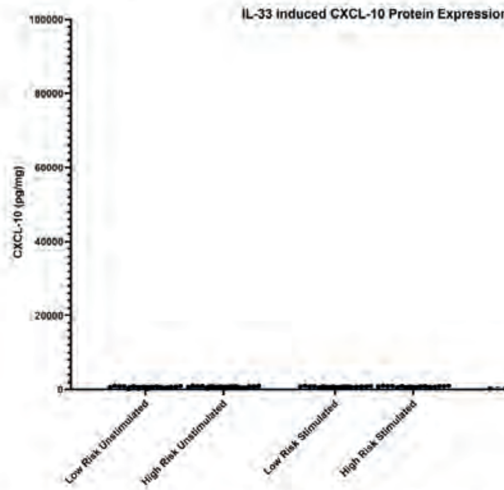
40.9+/-15.3%; p=1.17E-2; <u>high risk haplotype</u> *no IL-33 mean 26.4+/-14.2%, IL-33 mean 33.4+/-13.8%; p=9.03E-3*) or stratified for disease (C, <u>healthy</u> *no IL-33 mean 29.6+/-13.2%, IL-33 mean 38.7+/-14.2%; p=1.17E-4;* <u>asthma</u> *no IL-33 mean 28.8+/-10.8%, IL-33 mean 36.8+/-15.9%; p=2.78E-E2*). The absolute change in percentage of IL-1RL1 expressing Th2 cells due to IL-33 was not different for the two haplogroups: (D, <u>low risk haplotype</u> mean % difference+/-stdev 9.54+/-10.9%, <u>high risk haplotype</u> mean % difference+/-stdev 9.87+/-8.7%, p=0.94), nor dependent of disease status (E, <u>healthy</u> mean % difference+/-stdev 9.12+/-4.7%, <u>asthma</u> mean % difference+/-stdev 10.20+/-13.2%, p=0.80). Statistics: paired T test in case of IL-33 treatment, 2-tailed T-test for comparison haplotype/disease effects.

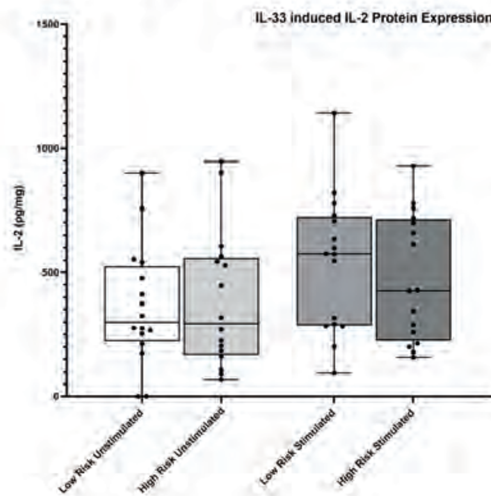
iii. Luminex data:

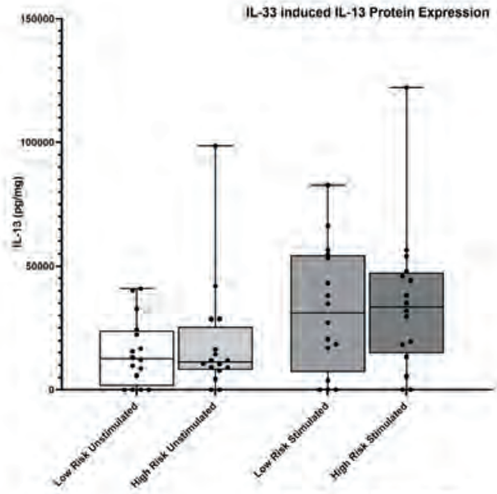
Figure IV:

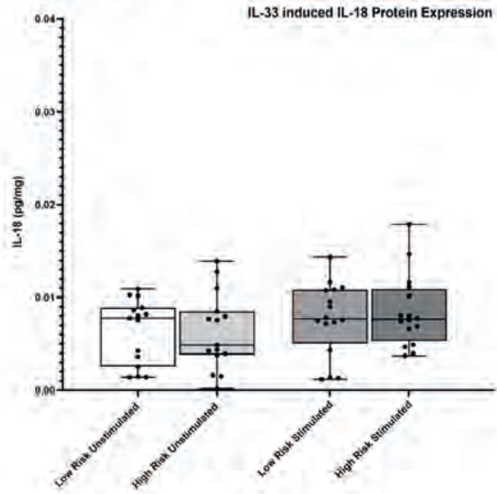


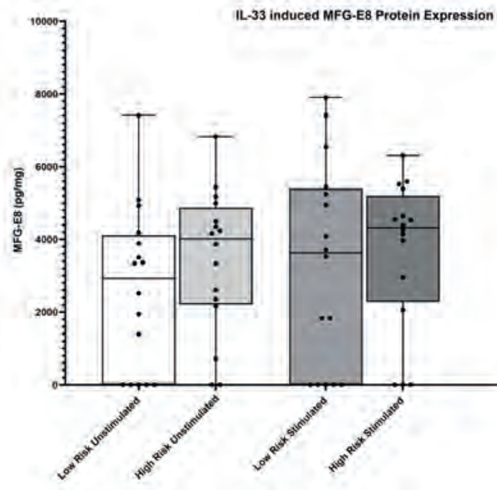

IL-33 induced IL-5 Protein Expression
IL-5 (pg/mg)
250000
200000
150000
100000
50000
0
Low Risk Unstimulated
High Risk Unstimulated
Low Risk Stimulated
High Risk Stimulated


IL-33 induced CXCL-10 Protein Expression
CXCL-10 (pg/mg)
100000
80000
60000
40000
20000
0
Low Risk Unstimulated
High Risk Unstimulated
Low Risk Stimulated
High Risk Stimulated


IL-33 induced IL-2 Protein Expression
IL-2 (pg/mg)
1500
1000
500
0
Low Risk Unstimulated
High Risk Unstimulated
Low Risk Stimulated
High Risk Stimulated







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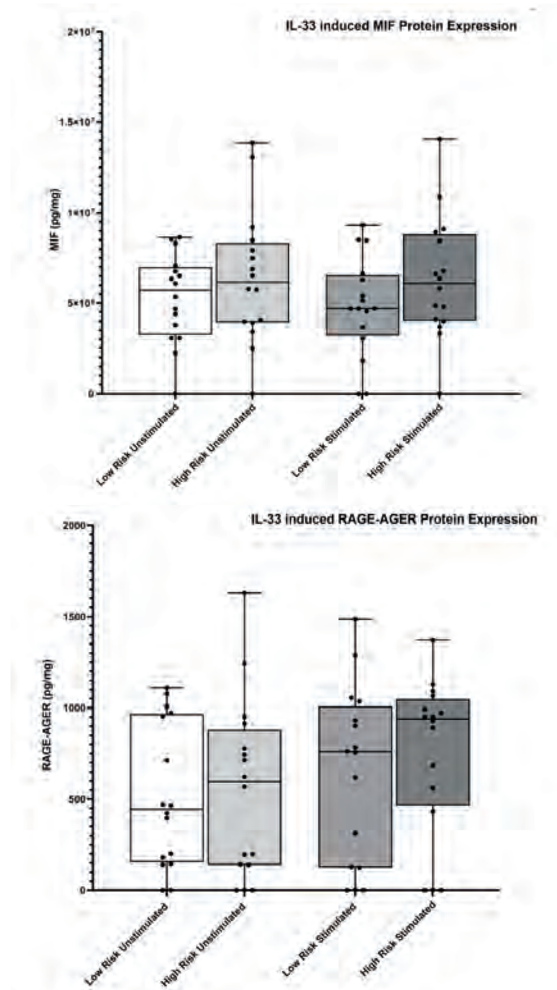
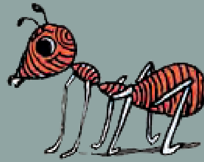
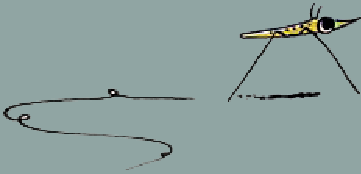


Figure IV: Effect of IL-33 stimulation on soluble protein expression of Th2 cells
CD3/CD28-activated TH2 cells were stimulated with 100ng/mL IL-33 for 72h and extracellular soluble protein levels determined in cell supernatants using Luminex. Candidate proteins were selected based on these being significantly differentially expressed on RNA level following IL-33 stimulation, with a minimum logfold change of 2; as well as were considered relevant for TH2 cell biology. These included B2-macroglobulin, CA15-MUC1, CXCL-10, Galectin-3, IL-2, IL-4, IL-5, IL-8, IL-13, IL-17, IL-18, MFG-E8, MIF, RAGE-AGER. For none of these proteins a significant difference was found as consequence of IL-33 exposure, nor a haplotype effect was found. (P>0.05)

C (IL33 × IL1RL1)





Part III

Clinical translation of the IL-33/IL-1RL1 pathway in asthma and allergy