Received Date : 14-Dec-2015

Revised Date : 08-Feb-2016

Accepted Date : 25-Feb-2016

Article type : Original Article: Epidemiology and Genetics

This is the author manuscript accepted for publication and has 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/all.12869](https://doi.org/10.1111/all.12869)

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Short title: *IL6R*-associated asthma risk genes

Identification of *STOML2* as a putative novel asthma risk gene associated with *IL6R*

Joana A. Revez,^a Melanie C Matheson,^b Jennie Hui,^{c-f} Svetlana Baltic,^g AAGC collaborators,^h Alan James,^{i,j} John W. Upham,^k Shyamali Dharmage,^b Philip J. Thompson,^g Nicholas G. Martin,^a John L. Hopper^b and Manuel A.R. Ferreira^a

Affiliations

^a QIMR Berghofer Medical Research Institute, Brisbane, Australia.

^b Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Australia.

^c PathWest Laboratory Medicine of Western Australia (WA), Nedlands, Australia

^d School of Population Health, The University of WA, Nedlands, Australia

^e School of Pathology and Laboratory Medicine, The University of WA, Nedlands, Australia

^f Busselton Population Medical Research Institute, Sir Charles Gairdner Hospital, Perth, Australia

^g Institute for Respiratory Health, University of WA, Perth, Australia.

^h Australian Asthma Genetics Consortium (AAGC) collaborators are listed in the Supplementary Material

ⁱ School of Medicine and Pharmacology, University of Western Australia, Nedlands, Australia

^j Department of Pulmonary Physiology, West Australian Sleep Disorders Research Institute, Nedlands, Australia

^k School of Medicine, Translational Research Institute, The University of Queensland, Australia

Corresponding author:

Manuel A R Ferreira, PhD

QIMR Berghofer Medical Research Institute

Locked Bag 2000, Royal Brisbane Hospital,

Herston QLD 4029, Australia

Phone: +61 7 3845 3552

Fax: +61 7 3362 0101

Email: manuel.ferreira@qimrberghofer.edu.au

Abstract

Background: Functional variants in the interleukin-6 receptor gene (*IL6R*) are associated with asthma risk. We hypothesized that genes co-expressed with *IL6R* might also be regulated by genetic polymorphisms that are associated with asthma risk. The aim of this study was to identify such genes.

Methods: To identify genes whose expression was correlated with that of *IL6R*, we analyzed gene expression levels generated for 373 human lymphoblastoid cell lines by the Geuvadis consortium, and for 38 hematopoietic cell types by the Differentiation Map Portal (DMP) project. Genes correlated with *IL6R* were then screened for nearby single nucleotide polymorphisms (SNPs) that were significantly associated with both variation in gene expression levels (eSNPs) and asthma risk.

Results: We identified 90 genes with expression levels correlated with those of *IL6R* and that also had a nearby eSNP associated with disease risk in a published asthma GWAS (N=20,776). For 16 (18%) genes, the association between the eSNP and asthma risk replicated with the same direction of effect in a further independent published asthma GWAS (N=27,378). Amongst the top replicated associations (FDR<0.05) were eSNPs for four known (*IL18R1*, *IL18RAP*, *BCL6* and *STAT6*) and one putative novel asthma risk gene, Stomatin-like protein 2 (*STOML2*). The expression of *STOML2* was negatively correlated with *IL6R*, while eSNPs that increased the expression of *STOML2* were associated with increased asthma risk.

Conclusion: The expression of *STOML2*, a gene that plays a key role in mitochondrial function and T-cell activation, is associated with both IL-6 signaling and asthma risk.

Keywords: allergy, IL-6, IL-6R, mitochondria, SLP2

Introduction

The interleukin 6 receptor gene (*IL6R*) has been implicated in asthma pathophysiology both by human genetic association studies (1-3) and experimental animal models of asthma (4, 5). A common single nucleotide polymorphism (SNP) in *IL6R* was first identified as a risk factor for asthma in a meta-analysis of genome-wide association studies (GWAS) including 57,800 individuals (1). In that study, asthma risk was estimated to increase by 1.09-fold for each copy of the rs4129267:T allele, which has a frequency of 36% in Europeans. A second

independent but less common SNP in *IL6R* (rs12083537, minor allele frequency of 19%) has also been reported to associate with asthma risk (OR=1.05 (2)). Notably, both rs4129267 and rs12083537 are associated with variation in serum protein levels of the soluble form of IL-6R (sIL-6R) (2, 6).

Variation in serum sIL-6R levels is thought to arise due to at least three mechanisms: (1) proteolytic shedding of the membrane bound form of IL-6R (mIL-6R) (7), which accounts for most variation in sIL-6R levels; (2) differential splicing of exon 9, which produces an isoform that directly encodes sIL-6R (8); and (3) release of microvesicle-associated IL-6R (9). The first two mechanisms are increased by the rs4129267:T asthma risk allele (6, 10). Increased shedding most likely arises because this allele is in phase (linkage disequilibrium [LD] $r^2 = 0.99$ in Europeans) with the 358Ala amino-acid variant that is located within the main mIL-6R cleavage site (11) and that increases receptor shedding by ADAM proteases (10). As a result, the asthma rs4129267:T predisposing allele is associated with increased sIL-6R serum levels and decreased mIL-6R expression. This strongly suggests that increased asthma risk is associated with increased IL-6 trans-signaling, which is mediated by sIL-6R, and decreased IL-6 classic signaling, mediated by mIL-6R (12).

The hypothesis that IL-6 trans-signaling contributes to disease pathophysiology has been experimentally demonstrated in mouse models of experimental asthma. Doganci et al. (4) showed that ovalbumin (OVA) -sensitized mice treated with sgp130-Fc, a fusion protein that inhibits trans- but not classic IL-6 signaling, had significantly lower eosinophilia and Th2 cytokine levels in bronchoalveolar lavage fluid (BALF) after OVA challenge, when compared to control mice. Similarly, Ullah et al. (5) found that both neutrophilia and eosinophilia induced by cockroach challenge were attenuated by sgp130-Fc, which was mirrored in significant decreases in IL-13, IL-17A and IL-17F levels in BALF. Furthermore, inhibition of IL-6 signaling had no protective effect in house dust mite challenged mice, which had lower levels of sIL-6R in the airways and so had reduced activation of IL-6 trans-signaling. These findings are consistent with the observation in the human genetic association studies that increased sIL-6R levels are associated with increased asthma risk.

Therefore, there is unambiguous evidence from both human and animal studies that dysregulation of *IL6R* expression contributes to the pathophysiology of a subset of individuals with asthma. On the other hand, there is some evidence that *IL6R* expression is associated with that of other immune and inflammatory genes (13, 14) but, to our knowledge, this possibility has not been studied systematically to date. In this study, we first analyzed two publicly available gene expression datasets to search for genes whose expression was

correlated with that of *IL6R* (henceforth referred to as “*IL6R*-associated genes”); this information was then integrated with results from both asthma and transcriptome GWAS to identify the subset of *IL6R*-associated genes that are likely to be genetic risk factors for asthma. Identifying disease risk genes that are also associated with *IL6R* might provide new clues into the broader gene network that underlies the pathological effect of IL-6 signaling dysregulation in asthma.

Methods

Our analytical procedure is summarized in Figure 1 and described in detail below.

Analysis of gene expression levels in 373 Europeans studied by the Geuvadis consortium

To identify genes with expression levels correlated with those of *IL6R*, we first analyzed RNA-seq data generated by the Geuvadis consortium for human lymphoblastoid cell lines (LCLs) (15). LCLs are derived from peripheral blood B-cells and so represent a practical *in vitro* model to study gene expression patterns relevant to immune-related conditions.

We downloaded from the European Bioinformatics Institute (EBI) RPKM-normalized gene expression levels for 53,934 transcripts measured in LCLs of 462 unrelated individuals from the 1000 Genomes Project (accession E-GEUV-1, file GD660.GeneQuantRPKM.txt.gz). Transcripts expressed in >90% of individuals (N=18,702) were selected and distributions normalized using a rank-based inverse-normal transformation. We restricted our analysis to samples of European ancestry (N=373) and unique transcripts (unambiguous match between HGNC symbol and corresponding Ensembl ID) annotated in GENCODE (16) (N=15,440). The association between *IL6R* expression levels and the expression of each of the other 15,440 genes was then tested using linear regression, with covariates included in the model to adjust for the effects of sex, population (CEU, GBR, FIN, TSI), sequencing center and gene GC content (see below). Sixteen principal components (PCs) were also included as covariates to account for the effect of unmeasured confounders, as described below. These analyses were performed in R version 3.2.2.

Gene GC content can affect RNA-seq-derived expression levels differently in different individuals (17, 18). We tested this possibility and indeed found that in some individuals, increased gene GC content was associated with increased gene expression, while in others the reverse was true (**Supplementary Figure 1**). To correct for this potential confounder, we calculated a GC correction per individual as follows. From the 18,702 genes

expressed in more than 90% of individuals, we (1) excluded a subset of 3,756 highly expressed genes (RPKM>21.48); (2) normalized the distribution of the remaining genes with a rank-based inverse-normal transformation; and (3) for each individual determined a correction value as the regression slope obtained from the regression of gene expression levels on gene GC content (for two examples, see **Supplementary Figure 1**). GC content for each gene was obtained from the Conditional Quantile Normalization R package for 15,277 genes (17). To correct for the effects of unmeasured confounders, PC analysis was applied to the matrix of quantile-normalized gene expression levels using the prcomp function of the R stats package (19), and the first 16 PCs were then extracted and included as covariates when testing the association between *IL6R* expression levels and the expression levels of other genes.

The top 10% of genes with expression most associated with that of *IL6R* were then selected for subsequent analyses. For a justification of how/why we selected a cut-off of 10% (instead of e.g. 5%), see section “Selection of cut-off to prioritize *IL6R*-associated genes” below.

Analysis of gene expression levels in 38 cell types from the DMAP project

We used a second independent approach to identify genes associated with *IL6R*; this approach differed from the analysis of the Geuvadis data in that we searched for genes correlated with *IL6R* within an individual but across immune cell types (e.g. cell types with increased expression of *IL6R* also have increased expression of gene X), instead of within a cell type but across individuals (e.g. individuals with increased expression of *IL6R* in LCLs also have increased expression of gene X).

A file (DMap_data.gct) containing normalized gene expression levels for 8,968 genes measured in 38 hematopoietic cell populations isolated from four to seven individuals was downloaded from the Differentiation Map Portal (DMAP) project (20) website. We then selected 7,372 unique genes annotated in GENCODE (16), and measured the association of their expression with *IL6R* expression using the cosine distance metric, as implemented in the GeneNeighbors module of GenePattern (21). As for the Geuvadis data, the top 10% of genes most associated with *IL6R* expression (based on the absolute of the distance metric) were selected for subsequent analyses.

Identification of SNPs associated with asthma risk and variation in gene expression

Not all genes associated with *IL6R* will contribute to asthma pathophysiology. To identify the subset of *IL6R*-associated genes whose expression might be causally-related to asthma risk, we combined information from published GWAS of asthma and published GWAS of gene expression levels. Specifically, for each gene X (e.g. *BCL6*) that is associated with *IL6R*, we (1) extracted summary association statistics (effect, risk allele, *P*-value) for SNPs within 1 Mb of the gene boundaries from the Ferreira et al. GWAS (22), which included 6,685 asthmatics and 14,091 controls; (2) selected the SNP with the most significant association with asthma (amongst those with $P < 0.05$) in that 1 Mb interval; (3) identified all genes whose expression was previously reported to associate (typically, but not always, with $FDR < 0.05$ imposed by the original study) with this SNP (or a proxy, $r^2 > 0.8$) in 11 published GWAS of gene expression levels conducted in cells/tissues relevant to asthma (15, 23-32); and (4) retained that SNP for further analysis if one of the genes known to be regulated by this SNP was also gene X (i.e. *BCL6*). In other words, we selected a subset of *IL6R*-associated genes that are regulated by a nearby SNP that associates with asthma risk. We considered an alternative more comprehensive analytical strategies (selection of all SNPs associated with asthma in step (2) above), but found that this would result in hundreds of SNPs being carried forward to validation, which would decrease power given the multiple testing burden (see below).

Selection of cut-off to prioritize *IL6R*-associated genes for downstream analyses

In the first step of our analytical procedure (Figure 1A), we used a 10% cut-off to select a group of genes whose expression was most strongly associated with that of *IL6R*. The choice of cut-off determined the number of genes selected for downstream analysis, and so also the number of SNPs that were moved to validation (see below). As such, an important consideration when deciding what cut-off to use was the power provided by the validation study to replicate a significant association after accounting for multiple SNP testing. Cut-offs of 5% and 10% resulted in 37 and 61 SNPs reaching the validation step, respectively. Based on the specific effect sizes and minor allele frequencies observed for these SNPs in the discovery GWAS, we estimated the power provided by the validation study to replicate the associations at a Bonferroni-corrected threshold of 0.0014 (0.05/37) and 0.0008 (0.05/61), respectively. The validation study was adequately powered to replicate 22 of the 37 (59%) SNP associations obtained with the 5% cut-off and 35 of the 61 (57%) associations obtained with the 10% cut-off. Therefore, the ability to replicate the associations with these two sets of

SNPs was comparable between the two cut-offs (59% vs. 57%). The 10% cut-off maximised the opportunity to discover new risk SNPs, and so we selected that cut-off.

Validation of genetic associations with asthma risk

The previous analyses identified a set of SNPs found to regulate an *IL6R*-associated gene and that were also associated with asthma risk in the Ferreira et al. GWAS (22). We refer to these SNPs that are associated with variation in the expression of a nearby gene as “eSNPs”.

Some of these might represent novel risk variants for asthma. To test this possibility, we investigated whether these eSNPs were also associated with asthma risk in an independent study, the GABRIEL consortium GWAS (33). After excluding overlapping samples (N=1,207, Busselton cohort), results were based on the analysis of 12,077 asthmatics and 15,301 controls. A reproducible association was defined as a significant ($P < 0.05$) and consistent (i.e. same direction of effect as in the Ferreira et al. GWAS (22) association in this analysis. The proportion of eSNPs for *IL6R*-associated genes with a reproducible association with asthma out of all eSNPs tested in the GABRIEL is denoted in the section below by $f_{IL6R-genes}$. To obtain an overall measure of association between an eSNP and asthma risk, we used METAL (34) to meta-analyze results from the Ferreira et. al GWAS and the GABRIEL GWAS using a fixed-effects model.

Comparison with a random selection of genes

We applied the same analytical approach described above (**Figure 1**) to a random set of 1,000 genes, instead of *IL6R*. This allowed us to estimate the extent to which the enrichment of significant associations between asthma risk and eSNPs for *IL6R*-associated genes was because eSNPs in general – and not just those specific to *IL6R*-associated genes – are more likely to be disease-associated than randomly selected SNPs (35). To this end, we (1) selected a random gene tested in both the Geuvadis and DMAP datasets; (2) applied steps A through D (**Figure 1**) to identify genes co-expressed with that random gene (instead of *IL6R*); and (3) identified the proportion of eSNPs for genes co-expressed with that random gene that had a reproducible association asthma risk (and denote this fraction by $f_{random-genes}$). This procedure was repeated for 1,000 different random genes. We then calculated the mean $f_{random-genes}$ across the 1,000 replicates, and the proportion of replicates where $f_{random-genes} \geq f_{IL6R-genes}$. This proportion represents the (empirically-derived) probability of observing reproducible eSNP

associations with asthma risk more often than observed for *IL6R*-associated genes when selecting a random gene of interest.

Results

Identification of genes with expression levels associated with those of *IL6R*

To identify genes that were associated with *IL6R*, we used two complementary approaches (**Figure 1 A**). First, we tested the association between *IL6R* expression and that of 15,440 genes measured with RNA-seq in LCLs of 373 individuals of European descent by the Geuvadis consortium (15). In this analysis, the expression of 1,020 genes (6.6%) was associated with the expression of *IL6R* at a $P < 0.05$. The most associated genes ($N = 1,544$; top 10%) are listed in **Supplementary Table 1** and include notable genes such as *ATP8B2* and *IL18R1*, which are respectively a gene located near *IL6R* and a gene located in an established asthma risk locus (36).

The previous analysis aimed to identify genes associated with *IL6R* when comparing expression levels measured in a single cell type across different individuals. In a separate approach, we analyzed data from the DMAP project (20) to search for genes whose expression values were correlated with *IL6R* across 38 different hematopoietic cell populations. In this analysis, the expression of 3,643 genes (49%) was associated with that of *IL6R* at $P < 0.05$. This excess of significant associations likely arises because cell types analyzed by the DMAP project derive from a common hematopoietic progenitor cell and many belong to the same lineage, and so their transcriptional profiles are highly correlated. (20) As for the analysis of the Geuvadis data, we then selected the top 10% of genes with expression patterns most associated with *IL6R* for subsequent analysis ($N = 737$, **Supplementary Table 1**). Amongst the most *IL6R*-associated genes were for example *LY96* and *TLR2*, two genes involved in the regulation of inflammation (37, 38).

After combining the lists of genes most associated with *IL6R* in these two independent approaches (top 10% of each), we obtained 2,203 genes (**Figure 1 A**), including those identified (1) in the Geuvadis dataset only ($N = 1,466$); (2) in both the Geuvadis and DMAP datasets ($N = 78$) and (3) in the DMAP dataset only ($N = 659$).

Identification of asthma risk SNPs near *IL6R*-associated genes

The analysis above identified 2,203 genes with expression levels associated with *IL6R*, either across different individuals and/or cell types. We hypothesized that the expression of some of

these genes might be causally related to asthma. If so, we would expect that genetic polymorphisms that regulate the expression of these genes would be associated with asthma risk. To test this possibility, for each gene, we first identified the nearby (± 1 Mb) single nucleotide polymorphism (SNP) with significant ($P < 0.05$) and strongest association with asthma risk in a recently published asthma GWAS (22), that included 6,685 cases and 14,091 controls. For twelve genes there were no significant SNPs within 1 Mb, and so these were not considered further. The resulting list of 2,191 SNPs was further pruned by removing duplicate (i.e. same SNP selected for two or more genes; $N = 1,122$) or correlated (linkage disequilibrium $r^2 > 0.1$; $N = 79$) SNPs, leaving for further analysis 990 independent SNPs that were associated with asthma risk and located near an *IL6R*-associated gene.

Regulation of gene expression by asthma risk SNPs near *IL6R*-associated genes

We then investigated if the association between each of these 990 independent SNPs and asthma risk could arise because these SNPs regulate the expression of the nearby *IL6R*-associated gene. Of the 990 SNPs, 358 (36%) were associated with the expression of at least one nearby gene in published GWAS of gene expression levels (which we refer to as eSNPs), including 84 (8.5%) that were eSNPs for the actual gene that was correlated with *IL6R* (Supplementary Table 2).

Replication of the association between asthma and eSNPs for *IL6R*-associated genes

Eighty four eSNPs were associated with both asthma risk in the Ferreira et al. GWAS (22) and expression levels of an *IL6R*-associated gene in published GWAS of gene expression. To validate the association observed between asthma risk and these 84 eSNPs, we analyzed publically available results from the GABRIEL asthma GWAS (33). After excluding samples that overlapped with the Ferreira et al. GWAS, results were available for 12,077 asthmatics and 15,301 controls. Of the 84 eSNPs, 61 were tested in the GABRIEL GWAS and for 14 of these (23%) there was a significant ($P < 0.05$) and consistent (same direction of effect) association (Table 1 and Figure 1), when only about two reproducible associations were expected at this significance level by chance alone given multiple testing ($61 \text{ [eSNPs tested]} \times 0.05 \text{ [} P < 0.05 \text{]} \times 0.5 \text{ [same direction]} = 1.5$).

Of the 14 eSNPs (representing 16 genes; Figure 2) with a reproducible association with asthma risk in the replication analysis, four were significant after controlling for the 61 SNPs tested ($\text{FDR (39)} < 0.05$). Three of these are established risk variants for allergic disease, specifically those regulating the expression of *IL18R1/IL18RAP* (33), *BCL6* and

STAT6 (40). On the other hand, rs7039317 (combined discovery and replication analysis OR = 1.10 for the T allele, $P=2 \times 10^{-6}$; Supplementary Table 3), an eSNP for *STOML2*, has not previously been reported to be associated with the risk of asthma or other allergic diseases. Therefore, *STOML2* represents a putative novel risk gene for asthma.

Association between *STOML2* expression, *IL6R* expression and asthma risk

To further characterize the association observed between the expression of *STOML2* and that of *IL6R* in the DMAP project, we examined the expression of both genes across the 38 hematopoietic cell types studied. There was a negative correlation between *IL6R* and *STOML2* expression (**Figure 3**); for example, central memory CD4⁺ T Cells and neutrophilic metamyelocytes had high *IL6R* expression and low *STOML2* expression, whereas megakaryocytes and erythroids had low *IL6R* expression and high *STOML2* expression.

We also combined results from the asthma and transcriptome GWAS to predict the direction of effect of *STOML2* expression on asthma risk. The rs7039317:T allele that was associated with increased asthma risk was associated with increased expression of *STOML2* in whole-blood (29) (**Table 2**), suggesting that increased *STOML2* expression has a predisposing effect on asthma.

Other genes near *STOML2* regulated by rs7039317

Many eSNPs are known to be associated with the expression of multiple genes, as we observed for rs10197862, an eSNP for both *IL18R1* and *IL18RAP*. Although rs7039317 was only associated with the expression of a single gene that was correlated with *IL6R*, it could nonetheless be associated with the expression of other nearby genes unrelated to the IL-6 signaling pathway. When we queried results from 11 published GWAS of gene expression levels, there were an additional three genes whose expression was correlated with this SNP, namely *VCP* ($P=2 \times 10^{-17}$; (27, 29)), *PIGO* ($P=3 \times 10^{-6}$; (29)) and *DNAJB5* ($P=6 \times 10^{-4}$; (29)). Therefore, in addition to *STOML2*, these three genes represent putative target genes for rs7039317 and so might also be related to asthma pathophysiology.

Comparison with a random selection of genes

The aim of this study was to identify asthma risk genes amongst *IL6R*-associated genes. This was assessed by identifying eSNPs with a reproducible association with asthma risk amongst eSNPs for *IL6R*-associated genes. We found 14 such eSNPs amongst 61 eSNPs tested (23%), a significant enrichment over the 2.5% expectation ($0.05 [P<0.05] \times 0.5$ [same direction] =

0.025). It is possible that this enrichment of significant asthma associations amongst the eSNPs tested arose mostly because eSNPs in general are more likely to be disease-associated than randomly selected SNPs (35). To test this, we applied steps A through D (**Figure 1**) to a random gene of interest in place of *IL6R*, and repeated this analysis 1,000 times (see Methods for details). On average, we found that 17.4% (SD = 4.7%) of eSNPs for genes co-expressed with a given random gene had a reproducible association with disease risk, a 1.3-fold decrease when compared to 23% for the list of *IL6R*-associated genes, but this difference was not statistically significant (empirical $P=0.120$; see Methods for details). The observation that the enrichment of reproducible associations between eSNPs and asthma risk is comparable whether we considered *IL6R* or a random selection of genes does not imply that the associations are false-positives. Instead, it is consistent with previous studies that demonstrate that eSNPs are more likely to be associated with human traits than frequency-matched SNPs that are not related to gene expression (35). This observation is important to interpret our results but does not detract from the identification of eSNPs that are related to asthma risk as well as the expression of *IL6R*-associated genes.

Discussion

In this study, we investigated the possibility that genes whose expression is associated with that of *IL6R* might also be causally related to asthma pathophysiology.

To identify genes with expression levels associated with that of *IL6R*, we used two complementary approaches, each with its own strengths and weaknesses. The RNA-seq dataset generated by the Geuvadis consortium (15) for 373 unrelated individuals provided a unique opportunity to identify genes that share transcriptional regulatory mechanisms with *IL6R* constitutively in LCLs; for example, these could be shared regulatory elements (e.g. enhancers) or transcription factors. The top three genes most correlated with *IL6R* expression in this analysis were: *FYN* (negative correlation), which regulates mast cell function (41), B-cell development (42) and is phosphorylated upon IL-6 binding to IL-6R (43); *CD180* (positive correlation), which belongs to the Toll-like receptor family and is involved in innate immune response to mycobacteria (44); and *ATP8B2* (positive correlation), which is located in close proximity (53 kb) to *IL6R*, suggesting that both genes might share a nearby regulatory element. Our results demonstrate that the expression of these genes is to some extent coordinated with that of *IL6R* in LCLs, and is consistent with the previously suggested role for *IL6R* in Treg development (4), innate immunity (45) and inflammation (46). The major caveat of this analysis was the use of an immortalized cell line that, although derived

from a relevant cell type (B-cells), provides a very limited representation of gene expression patterns that are relevant for asthma.

To partly address the limitation of using LCLs to uncover *IL6R*-associated genes that are relevant for asthma, we also analyzed publicly available gene expression patterns measured in 38 different hematopoietic cell populations by the DMAP project (20). The strengths of this approach included the use of primary cells collected from volunteers (instead of cell lines) and the opportunity to identify genes associated with *IL6R* across cell types within individuals (instead of within a cell type across individuals). As such, the two approaches used (analyses of Geuvadis and DMAP data) shared the same aim but were conceptually distinct. An association between *IL6R* and another gene across cell types within an individual could arise, for example, if differentiation into specific cell lineages from a common progenitor requires temporal coordination of the expression of both genes (e.g. simultaneous expression; expression of one gene but not the other).

In this second approach, the proportion of genes with expression associated with that of *IL6R* (49%) at $P < 0.05$ far exceeded the 5% nominal expectation. However, this is perhaps not too unexpected given that the cell types analyzed by the DMAP project not only derive from a common hematopoietic progenitor cell (hematopoietic stem cells $CD133^+ CD34^{dim}$), but also include cell types that belong to the same lineage: for example, CD8 T cells and CD4 T cells, early B cells and pro B cells. As highlighted in the original DMAP publication (20), the global transcription profiles from cell types of related lineages are highly correlated.

The top three genes whose expression was most closely associated with that of *IL6R* in the DMAP project were (all with a positive correlation): *CD4*, a cell surface antigen that is expressed on subsets of T cells, as well as on monocytes and macrophages, and plays an important role in T-helper cell development and activation (47); *VIPRI*, a high-affinity G protein-coupled receptor for vasoactive intestinal peptide, a neuropeptide that controls both innate and adaptive immunity (48, 49) and that has long been linked to asthma (50); and *RGL1*, a downstream effector protein of the Ras pathway of IL-6 signal transduction.

The clear literature links between IL-6 signaling and the function of some of the top *IL6R*-associated genes in the Geuvadis and DMAP analyses, suggests that both approaches were indeed able to identify specific components of the IL-6 signaling transduction pathway, its upstream regulators or downstream target genes. As for *IL6R*, we hypothesized that genetic dysregulation of the expression of these genes could affect asthma risk, for example by interfering with cellular differentiation or function in response to IL-6 stimulation. To address this hypothesis, we identified the subset of *IL6R*-associated genes whose expression

was regulated by a SNP that was also a risk factor for asthma in a published GWAS (22). This list (90 genes) included genes directly relevant for both IL-6 signaling and asthma, for example *TLR1* (51), *IL17RA* (52, 53) and *NDFIP1* (54), amongst others.

Due to multiple testing, the SNP associations with asthma for some of these 90 genes were likely to represent false-positive findings. To identify those with a reproducible association, we extracted results for these SNPs from an independent asthma GWAS (33) and found that for 16 genes the SNP association with asthma was both significant and consistent. As such, this represents the group of genes which our analyses most convincingly link to both asthma etiology and IL-6 signaling. Five of these genes were identified at a significance threshold that controls for multiple SNP testing in the replication study ($FDR < 0.05$): *IL18R1*, *IL18RAP*, *BCL6*, *STAT6* and *STOML2*. The first four, but not *STOML2*, are known target genes of SNPs that have an established (i.e. genome-wide significant) association with allergic disease (33, 40).

IL18R1 and *IL18RAP* encode the alpha and beta chains of the IL-18 receptor; IL-18 signaling through this receptor can activate IL-6 production (55), and IL-18 serum levels have been found to be positively correlated with sIL-6R levels (56). Therefore, together with these studies, our results indicate that the IL-18 and IL-6 signaling pathways are associated, and that this could in part be achieved by the coordinated transcription of *IL6R*, *IL18R1* and *IL18RAP*.

BCL6 is a transcription factor with a critical role in the activation and differentiation of germinal center (GC) B cells (57) and CD4 T cell differentiation into T follicular helper (Tfh) cells (58). IL-6 stimulation is required for *BCL6* expression, while in turn *BCL6* induces *IL6R* expression (59). Thus, the expression of both genes is associated during specific B and T cell differentiation programs. Our finding that *BCL6* expression is positively correlated with *IL6R* expression across hematopoietic cell types further supports the importance of coordinated transcription of these genes during normal cell lineage commitment. The association between SNPs that regulate the expression of these two genes and asthma risk suggests that their effect on asthma pathophysiology might be related to dysregulation of GC B cell and/or Tfh cell differentiation.

STAT6 is a transcription factor that plays a key role in adaptive immunity, by mediating IL-4 and IL-13 signaling through their cognate receptors (60), but also in innate immunity (61). Few studies have reported an association between IL-6 and *STAT6*, suggesting that these two signaling pathways might be mostly independent. However, in macrophages, IL-6 can induce the expression of the IL-4 receptor and augment IL-4-induced

STAT6 signaling (62). As such, *IL6R* and *STAT6* co-expression might be important for innate immune responses. Consistent with this possibility, in the DMAP project, the cell types with highest expression of both *IL6R* and *STAT6* were monocytes (not shown), the precursors for macrophages.

Lastly, results from our analyses indicate that *STOML2* is a putative novel asthma risk gene whose expression is negatively associated with that of *IL6R*. The overall statistical evidence we found for an association between the *STOML2* eSNP and asthma risk ($P=2\times 10^{-6}$) would be considered suggestive and not genome-wide significant in the context of a GWAS. As such, validation of this association in a well powered independent study is required to unambiguously confirm this eSNP as a novel risk factor for asthma.

STOML2 encodes for a protein that is mostly expressed in mitochondria, is required for the correct development of cell respiratory chain complexes (63), and promotes T cell activation (64). In our study, we found that the eSNP associated with increased *STOML2* expression was associated with an increased disease risk, suggesting that *STOML2* may have a pro-inflammatory effect in asthma. This is consistent with the observation that T cells from *STOML2*-deficient mice have decreased IL-2 production in response to cell activation, and this translated into reduced CD4⁺ T cell responses (65). In the DMAP project, CD4⁺ T cells had high expression of *IL6R* and low expression of *STOML2*. We speculate that in individuals with the rs7039317:T allele that increases *STOML2* expression (29), mitochondria biogenesis and function in CD4⁺ T cells is increased, which results in stronger T cell responses to allergens, thereby increasing asthma risk. Functional studies that formally test this hypothesis are warranted.

Interestingly, IL-6R blockade has been shown to attenuate the development of cachexia by promoting mitochondrial biogenesis and dynamics (66), which are induced by *STOML2* (65). These observations are consistent with our finding of a negative correlation between *IL6R* and *STOML2* expression and, collectively, suggest that *STOML2* is part of a gene network that underlies the effect of IL-6 on muscle loss. Of note, skeletal muscle dysfunction is common in chronic obstructive pulmonary disease (67) and is induced by chronic intake of corticosteroids (68).

In conclusion, we identified 16 genes whose expression was associated with *IL6R* and that were regulated by common polymorphisms that had a reproducible association with asthma risk. This list included 5 known and 11 putative new asthma risk genes, of which *STOML2* had the strongest SNP association with asthma. These genes provide new clues into

broader gene networks that are associated with IL-6 signaling and that contribute to asthma pathophysiology.

Acknowledgments

We thank all study participants, including customers of 23andMe who answered surveys, as well as the employees of 23andMe, who together made this research possible. This work was supported in part by the National Human Genome Research Institute of the National Institutes of Health (R44HG006981) and the National Health and Medical Research Council of Australia (613627 and APP1036550). J.A.R. is supported by a doctoral grant (SFRH/BD/92907/2013) from Fundação para a Ciência e Tecnologia, Portugal.

Author contributions

J.A.R. and M.A.R.F. conducted the analyses and wrote the manuscript. M.C. M., J.H., S.B., A.J., P.J.T., J.W.U., S.D., N.G.M and J.L.H. were involved in data collection and analysis for the asthma GWAS. All authors read and approved the manuscript for publication.

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Figure 1. Flowchart of IL6R co-expression analysis. Steps taken for the identification of genes with expression (i) associated with that of IL6R and (ii) regulated by asthma risk SNPs.

Figure 2. Genes that are co-expressed with *IL6R* and have a reproducible association with asthma or allergies. Underlined genes were significant at FDR<5% in the replication analysis. Distance of genes to *IL6R*, and of eSNPs to genes, represent strength of association (i.e. shorter distances represent stronger association).

Figure 3. Expression of *IL6R* and *STOML2* across 38 hematopoietic cell types. High gene expression levels are portrayed in red and low gene expression levels in white.

Abbreviations: DDENDA2, Myeloid Dendritic Cell; TCELLA6, Naïve CD4+ T Cells; TCELLA7, Effective Memory CD4+ T Cell; GMP, Granulocyte-Monocyte Progenitor; TCELLA2, Naïve CD8+ T Cells; GRAN3, Neutrophil; MONO2, Monocyte; MONO1, Colony Forming Unit-Monocyte; GRAN2, Neutrophilic Metamyelocyte; TCELLA8, Central Memory CD4+ T Cell; ERY4, Erythroid 4; ERY3, Erythroid 3; HSC1, Hematopoietic Stem Cell 1; MEGA1, Colony Forming Unit-Megakaryocytic; HSC3, Hematopoietic Stem Cell 3; ERY5, Erythroid 5; ERY1, Erythroid 1; MEP, Megakaryocyte/Erythroid Progenitor; MEGA2, Megakaryocyte; BCELLA4, Mature B Cell Class Switched; PRE_BCELL3, Pro B Cells; NKA4, NKT; DENDA1, Plasmacytoid Dendritic cell; ERY2, Erythroid 2; PRE_BCELL2, Early B Cells; CMP, Common Myeloid Progenitor; EOS2, Eosinophil; GRAN1, Colony Forming Unit-Granulocyte; BASO1, Basophil; TCELLA4, Central Memory CD8+ T Cell; NKA1, Mature NK Cell 1; TCELLA1, Effective Memory RA CD8+ T Cell; TCELLA3, Effective Memory CD8+ T Cell; BCELLA3, Mature B Cell; BCELLA1, Naïve B Cells; NKA2, Mature NK Cell 2; NKA3, Mature NK Cell 3; BCELLA2, Mature B Cell Class Able To Switch

Table 1. Fourteen SNPs that are eSNPs for genes that are co-expressed with *IL6R* and also have a reproducible association with asthma risk.

No.	eSNP	Chr:bp	Co-expression with <i>IL6R</i>			Ferreira, 2014		GABRIEL		
			Gene	Cor	Study	OR, allele	P-value	Proxy SNP	OR*, allele	P-value
1	rs10197862	2:102350089	<i>IL18R1</i>	-0.202	G	1.24, A	4x10⁻¹¹	rs13431828	1.2, C	2x10⁻⁹
	rs10197862	2:102350089	<i>IL18RAP</i>	-0.143	G	1.24, A	4x10⁻¹¹	rs13431828	1.2, C	2x10⁻⁹
2	rs1464510	3:188394766	<i>BCL6</i>	0.324	D	0.91, A	0.0001	rs1559810	0.92, A	0.0001
3	rs7039317	9:34964538	<i>STOML2</i>	-0.346	D	1.11, T	0.0003	-	1.09, T	0.0010
4	rs324011	12:57108399	<i>STAT6</i>	0.415	D	1.09, T	0.0001	rs167769	1.07, T	0.0022
5	rs4833095	4:38798089	<i>TLR1</i>	0.448	D	1.2, T	5x10 ⁻¹²	-	1.07, T	0.0044
6	rs2357792	16:50734311	<i>NOD2</i>	0.648	D	1.08, A	0.0011	rs6500331	1.06, G	0.0050
7	rs13416555	2:8301605	<i>ID2</i>	0.324	D	1.12, C	1x10 ⁻⁵	rs10178845	1.06, G	0.0120
8	rs6511788	19:12369223	<i>MAN2B1</i>	0.425	D	1.07, T	0.0088	-	1.06, T	0.0160
9	rs11265424	1:160530060	<i>SLAMF1</i>	0.109	G	0.91, A	0.0005	rs1055880	0.95, C	0.0178
10	rs2435206	17:45980745	<i>NSF</i>	0.374	D	1.08, T	0.0014	rs2435211	1.05, T	0.0197
11	rs1253118	14:59497301	<i>RTN1</i>	0.556	D	1.07, T	0.0051	rs9323348	1.05, T	0.0210
12	rs13212921	6:27237643	<i>BTN2A1</i>	0.329	D	1.14, T	0.0002	rs13219354	1.08, C	0.0224
13	rs11000805	10:73903593	<i>NDST2</i>	0.349	D	1.07, C	0.0145	rs17741873	1.06, T	0.0316
14	rs9289837	3:151388204	<i>P2RY13</i>	0.452	D	0.88, T	1x10 ⁻⁵	rs7637803	0.95, T	0.0416
	rs9289837	3:151388204	<i>MED12L</i>	0.129	G	0.88, T	1x10 ⁻⁵	rs7637803	0.95, T	0.0416

Highlighted in light grey are SNPs that had not previously been reported to associate with asthma risk. SNPs with significant association with asthma after correction for multiple testing (FDR<0.05) in the replication analysis are represented with bold font. *The OR is reported for the allele that is on the same haplotype as

(i.e. in phase with) the allele reported for the Ferreira, 2014 GWAS SNP. Proxy SNPs were chosen based on an $r^2 > 0.8$. Abbreviations: BP, base pair; Chr, chromosome; D, DMAP; G, Geuvadis; OR, Odds Ratio.

Table 2. Effect of asthma-associated SNPs on gene expression.

Asthma association (Ferreira 2014 GWAS)			Gene expression association						
SNP rs ID	Effect		eSNP in LD with asthma SNP		Gene	P-value	Study	Tissue	Effect on expression*
	allele	OR	rs ID	r^2					
rs10197862	A	1.24	rs10197862	1.00	<i>IL18RAP</i>	2.8×10^{-137}	(29)	Whole blood	Decreased
rs10197862	A	1.24	rs950881	0.95	<i>IL18R1</i>	0.00025	(29)	Whole blood	Decreased
rs11000805	C	1.07	rs17741873	0.86	<i>NDST2</i>	2.7×10^{-10}	(29)	Whole blood	Increased
rs11265424	A	0.91	rs6670721	0.81	<i>SLAMF1</i>	3.0×10^{-15}	(29)	Whole blood	Decreased
rs1253118	T	1.07	rs956901	0.87	<i>RTN1</i>	0.00160	(29)	Whole blood	Decreased
rs13212921	T	1.14	rs13217285	0.92	<i>BTN2A1</i>	1.7×10^{-15}	(15)	LCLs	N/A
rs13416555	C	1.12	rs891058	0.99	<i>ID2</i>	6.1×10^{-08}	(27)	PBMCs	Increased
rs1464510	A	0.91	rs9864529	0.81	<i>BCL6</i>	5.2×10^{-10}	(27)	PBMCs	Increased
rs2357792	A	1.08	rs7342715	0.89	<i>NOD2</i>	3.5×10^{-11}	(27)	PBMCs	Decreased
rs2435206	T	1.08	rs2435211	0.96	<i>NSF</i>	9.4×10^{-05}	(24)	LCLs	N/A
rs324011	T	1.09	rs12368672	0.89	<i>STAT6</i>	9.8×10^{-198}	(29)	Whole blood	Decreased
rs4833095	T	1.20	rs12233670	0.97	<i>TLR1</i>	2.8×10^{-57}	(31)	Whole blood	Increased
rs6511788	T	1.07	rs10411986	0.98	<i>MAN2B1</i>	5.2×10^{-06}	(15)	LCLs	N/A
rs7039317	T	1.11	rs10972275	0.93	<i>STOML2</i>	9.6×10^{-05}	(29)	Whole blood	Increased

rs9289837	T	0.88	rs13327359	1.00	<i>MED12L</i>	2.4×10^{-37}	(29)	Whole blood	Decreased
rs9289837	T	0.88	rs7637803	0.85	<i>P2RY13</i>	2.0×10^{-14}	(29)	Whole blood	Decreased

* Corresponding to the eSNP allele that is on the same haplotype as the asthma effect allele. Abbreviations: LD, linkage disequilibrium; N/A, not available; OR, odds ratio; SNP, single nucleotide polymorphism.

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Author/s:

Revez, JA;Matheson, MC;Hui, J;Baltic, S;James, A;Upham, JW;Dharmage, S;Thompson, PJ;Martin, NG;Hopper, JL;Ferreira, MAR

Title:

Identification of STOML2 as a putative novel asthma risk gene associated with IL6R

Date:

2016-07-01

Citation:

Revez, J. A., Matheson, M. C., Hui, J., Baltic, S., James, A., Upham, J. W., Dharmage, S., Thompson, P. J., Martin, N. G., Hopper, J. L. & Ferreira, M. A. R. (2016). Identification of STOML2 as a putative novel asthma risk gene associated with IL6R. ALLERGY, 71 (7), pp.1020-1030. <https://doi.org/10.1111/all.12869>.

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