Functional CTLA-4 variants associate to both allergic asthma and rhinitis potentially by modulating naïve regulatory T cells

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To the editor,

A genome-wide linkage study previously indicated the chromosome 2q region containing the cytotoxic T-lymphocyte protein 4 (*CTLA-4*) gene might be a candidate asthma locus (*1*). However, prior reports of associations between *CTLA-4* genetic variants and asthma are conflicting and inconclusive (*2-4*). This study aimed to determine the role of *CTLA-4* single nucleotide polymorphisms (SNPs) on allergy risk by carrying out genetic association and functional analyses.

We identified 5 tag-SNPs (rs733618, rs4553808, rs16840252, rs231775, and rs3087243) in *CTLA-4* from the Hapmap Chinese Han population (CHB, Figure S1). These tag-SNPs were genotyped in a cohort of 1703 Singapore Chinese adults (age: 22.2 ± 5.6, 42% male, table S1). Of these 5*CTLA-4* tag-SNPs genotyped, rs3087243 has the highest significance level of associations with AR without asthma (p = 4.69 x 10^{-3}, OR = 1.38), asthma without AR (p = 3.49 x 10^{-4}, OR = 1.58), and AR with asthma (p = 6.67 x 10^{-4}, OR = 1.52, **Table 1**).

Next, we determined if rs3087243 has a functional effect on*CTLA-4* . We extracted *CTLA-4* mRNA expression data from 31,300 whole blood samples constituting 36 different cohorts collected by the eQTLgen consortium. In these cohorts, meta-analysis showed a strong correlation between allele "A" of rs3087243 and increasing*CTLA-4* mRNA expression (meta p -value = 2.67E-69, combined$Z$ -score = 17.60, **Figure 1A**). From the HapMap CHB data, tag-SNP rs3087243 is in a strong linkage disequilibrium (r^2 = 1) with rs11571316 that is located ˜1.4 kb upstream of the *CTLA-4* gene (**Figure S1**). We cloned -1686 bp to +110 bp of the *CTLA-4*gene to conduct an *in vitro* luciferase assay and showed a significantly higher *CTLA-4* promoter activity associated with allele "A", as compared to allele "G" of rs3087243 (p< 0.01, **Figure 1B and 1C**). This suggests rs11571316 might represent a functional SNP that influences the *CTLA-4*promoter activity and causes the apparent genetic association between the tag-SNP rs3087243 and allergy.

Lastly, when analysing the *CTLA-4* surface expression of T cells by flow cytometry we demonstrated a functional effect of rs11571316 on protein levels in T regulatory (Treg) cells. On peripheral blood mononuclear cell (PBMC) samples from a Singapore Chinese cohort (n = 201, age: 21.0 ± 2.3, 58% male) we observed robust CTLA-4 expression on Treg cells (gated for CD4+ CD25+ Foxp3+), while only lower amounts of the

protein were detected on CD25-Foxp3- T effector (Teff) cells (**Figure S2**). Compared to memory-type Treg (CD45RA-) the basal levels in naïve-type Treg cells (CD45RA+) were also lower. However, in this subset we observed a dosage effect of increasing *CTLA-4* protein levels from the "CC" to "TT" genotype of rs11571316 (ANOVA $p = 8.5 \times 10^{-10}$,**Figure 1D**). Besides this strong effect on naïve Treg, nominal association between rs11571316 genotype and *CTLA-4* protein levels was only observed in T effector (Teff) cells (ANOVA $p = 0.011$,**Figure S3**). This suggests that rs11571316 might regulate *CTLA-4* protein levels mainly in naïve Treg cells and contribute to the development of allergy. In agreement with this, prior reports have also demonstrated an important role of Treg cells in the control of airway inflammation in asthmatic patients (*5*). Also, a separate linkage block in 2q was previously reported to modulate the mRNA expression of *ICOS* among Treg and Teff subsets and associated with the risk of atopy and asthma (*6*). Therefore, we speculated an interplay between *ICOS* and *CTLA-4* in these T cell subsets might contribute to the pro-inflammatory condition that underlies allergy manifestation (**Figure S4**).

In conclusion, the present study identified a functional genetic signal that was associated with allergy via modulating *CTLA-4* expression in Treg cell subsets. Our data highlight a possible mechanism for how a targeted immune dysregulation can affect the risk for multiple disease phenotypes. The naïve Treg phenotype warrants further investigation as a potential target for therapeutic intervention in affected patients by harnessing strategies to attenuate *CTLA-4* expression on these cells.

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Table 1: Genetic association of CTLA-4 tag SNPs to allergic rhinitis and asthma in Singapore Chinese population

Logistic regression test was performed adjusting for age and gender. CI: Confidence Interval; Logistic P: adjusted logistic regression p-value; OR: adjusted odds ratio, using minor allele as the reference category. *Logistic P < 0.05 is considered significant association

Reference/Phenotype SNP	Reference/Phenotype Allele	AR without Asthma (N=455) vs Non-atopic Non-allergic c Logistic P
rs733618	G/A	**3.04E-03***
rs4553808	G/A	9.08E-01
rs16840252	T/C	7.96E-01
rs231775	A/G	**9.24E-03***
rs3087243	A/G	**4.69E-03***

Figure Legend

Figure 1. Functional association between 2q variants and *CTLA-4* expression. (A) Meta-analysis of whole blood eQTL association between *CTLA-4* mRNA expression and rs3087243 genotypes, using 31,300 samples constituting 36 different cohorts from the eQTLgen consortium. **(B)** Schematic illustration of the*CTLA-4* gene region with the relative location of SNPs. The cloned region and haplotypes used for the *in vitro* luciferase assay were also indicated. **(C)** Relative luciferase units (RLUs) of the*CTLA-4* promoter construct were compared across haplotypes. Promoter-less luciferase plasmid pGL4.10 (empty vector, EV) was used as a background control. Lipo: Lipofectamine transfection reagent.**(D)** Protein expression of *CTLA-4* in naïve-type CD45RA+ T regulatory (Treg) cells was compared across rs11571316 genotypes. An independent t-test was conducted to determine statistical significance in **(C)** and **(D)** that was defined as p -value < 0.05.