

1 **Interaction of T-cell and antigen presenting-cell co-stimulatory genes in childhood IgE**

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39 **Short title: co-stimulatory genes and childhood serum IgE (max 45 characters)**

40
41 **Word count: 2959 (max 3000)**

43 **Abstract**

44 It is likely that multiple genes contribute to IgE production. Co-stimulatory molecules are crucial
45 for the cross-talk between antigen presenting cells and T-lymphocytes which drives the IgE
46 response. We evaluated gene-gene interactions of haplotype tagging polymorphisms in a pathway
47 of 24 co-stimulatory genes in relation to serum IgE levels. We assessed this at ages 1-2 years and
48 6-8 years in 3,062 Dutch children from a pooled data set of three birth cohorts PIAMA,
49 PREVASC and KOALA. Single- and multilocus associations with serum IgE levels (3rd vs. 1st
50 tertile) were evaluated by Chi²-tests and multidimensionality-reduction method (MDR) in co-
51 stimulatory genes *VTCN1*, *TNFRSF4*, *TNFRSF18*, *TNFRSF14*, *TNFSF18*, *TNFSF4*, *CD28*,
52 *CTLA4*, *ICOS*, *PDCD1*, *BTLA*, *CD80*, *CD86*, *HLA-G*, *CD274*, *PDCD1LG2*, *CD276*, *LILRA4*,
53 *LILRB1*, *LILRB2*, *LILRB4*, *CD40*, *ICOSLG*, and *CD40LG*. We found multiple statistically
54 significant single ^(S) and multilocus ^(M) associations for the genes *VTCN1*SM, *TNFSF18*SM,
55 *TNFSF4*^S, *CD28*^S, *CTLA4*^M, *ICOS*^S, *BTLA*^M, *CD80*^M, *CD86*SM, *CD274*SM, *PDCD1LG2*^M,
56 *LILRA4*SM, *LILRB4*^M, and *CD40*SM with serum IgE. Two-locus interactions of *CD86* with *VTCN1*
57 and *CD274* with *LILRA4* were confirmed by logistic regression. In conclusion, serum IgE levels
58 are regulated by multiple gene-gene interaction effects in the co-stimulatory pathway. We suggest
59 using research strategies that model multiple gene-gene interactions in genetic studies. (200
60 words)

61

62 **Key words:** birth cohort, co-stimulation, genetic interaction, IgE, MDR

63 Introduction

64 Atopic diseases asthma, hay fever and atopic dermatitis are complex in origin, *i.e.* development of
65 these diseases results from an interplay between genetic variants and environmental factors. It is
66 increasingly clear that genes exert only a small or even no effect on the development of atopy on
67 their own, and that they may act synergistically ⁽¹⁾. To identify which genes contribute to the
68 development of atopic disease by association analyses, a research strategy thus should be applied
69 that is capable to combine single nucleotide polymorphisms in multiple genes. A promising
70 method designed for studying multifactorial diseases by computational algorithms is the
71 Multifactor Dimensionality Reduction (MDR) method ⁽²⁾.

72 A core feature of atopy is the presence of increased total serum IgE. A key control point for IgE
73 synthesis and regulation is the necessity for co-stimulation to activate T-lymphocytes. T-
74 lymphocytes recognize antigens by T-cell receptor binding to the antigen presenting MHC class II
75 molecules on antigen presenting cells. Subsequent cross-talk through co-stimulatory molecules
76 integrating various positive and negative signals is necessary for differentiation and activation of
77 the T-lymphocytes. Activated T-lymphocytes can direct an immune response and when directed
78 towards a Th2 response, they induce B-lymphocytes to produce IgE. Due to their pivotal role in
79 fine tuning T-lymphocyte differentiation and activation, genes encoding co-stimulatory receptors
80 and ligands are promising candidate genes for atopic disease. Indeed, results of previous single
81 candidate gene studies have suggested that polymorphisms in co-stimulatory genes, *i.e.* *CD86* ⁽³⁾,
82 *CD40* ⁽⁴⁾, *CTLA4* ⁽⁵⁾, *HLA-G* ⁽⁶⁾, *ICOS* ⁽⁷⁾ and *PDCDI* ⁽⁸⁾ may influence susceptibility to atopic
83 disease.

84 We hypothesized that polymorphisms in genes encoding co-stimulatory receptors and ligands
85 influence susceptibility to elevated IgE levels. We evaluated haplotype tagging SNPs of co-
86 stimulatory pathway genes in relation to serum IgE levels in a combined dataset of 3,062 children
87 participating in three Dutch birth cohorts (Allergenic study ⁽⁹⁾) at ages 1-2 years and 6-8 years. In

88 addition to studying main effects of these genes, we investigated two-, three- and four-locus gene-
89 gene interaction with respect to serum IgE levels using MDR.

90 **Subjects and methods**

91 *Study populations*

92 The Allergenic study includes three prospective Dutch birth cohorts of similar design, *i.e.* PIAMA
93 ⁽¹⁰⁾, PREVASC ^(11;12) and KOALA ⁽¹³⁾. Genetic studies were approved by local medical ethics
94 committees of participating institutes. All parents provided written informed consent.

95

96 *IgE measurements*

97 Total IgE levels were determined in capillary or venous blood collected at age 1 and 8 years in
98 PIAMA, age 1, 2, and 6 in PREVASC, and age 1 and 2 in KOALA (Sanquin Research,
99 Amsterdam). Total IgE levels were measured by radioimmunoassay as described previously ⁽¹⁴⁻¹⁶⁾
100 and expressed as international units per milliliter (1 IU representing 2.4 ng of IgE). Total IgE
101 measurements were clustered at age 1-2 years and 6-8 years and analysed in tertiles, see online
102 repository.

103

104 *SNP selection and genotyping*

105 Haplotype tagging SNPs were selected from the HapMap database ⁽¹⁷⁾ or from the Innate
106 Immunity web site ⁽¹⁸⁾ depending on the largest number of SNPs with a minor allele frequency >
107 0.1 available in each database. Additionally, the biomedical literature was screened for SNPs
108 within the candidate genes known to have functional impact or to be associated with asthma or
109 atopy. Genomic DNA was extracted from buccal swabs or blood using standard methods ⁽¹⁹⁾.
110 DNA was amplified by using REPLI-g UltraFast technology (QiagenTM). Genotyping was
111 performed by Competitive Allele-Specific PCR using KASParTM genotyping chemistry,
112 performed under contract by K-Biosciences. Quality of genotype data was guaranteed by
113 standards of K-Biosciences and verified by comparing the genotyping results in genomic versus
114 amplified DNA in a subset of children.

115

116 *Statistical methods*

117 All SNPs were analysed for Hardy-Weinberg equilibrium (HWE) using χ^2 statistics ($p > 0.01$). We
118 used χ^2 tests to analyse whether genotypes in this pathway were associated with elevated serum
119 IgE levels at 1-2 years and at 6-8 years (highest vs. lowest tertile) by using a co-dominant model.
120 For SNPs with a $p < 0.10$, AIC (Akaike Information Criterion) was evaluated to determine the best
121 fitting genetic model (additive, dominant or recessive). Odds ratios (ORs) and 95% confidence
122 intervals (CI) were calculated by logistic regression analysis. SNPs located on the X-chromosome
123 were analyzed in boys and girls separately. Calculations were performed using SPSS 14.0
124 statistical software and considered significant if $p < 0.05$ (two sided).

125 Haplotypes were constructed from the SNPs available in each gene and frequency distributions
126 among cases and controls were estimated by the expectation-maximization algorithm. Differences
127 in these frequency distributions were evaluated by a log-likelihood ratio test (in house software).
128 Since the haplotype tagging SNP selection uses multi-marker predictors to capture all information
129 of the gene (*i.e.* aggressive tagging), we also analysed SNPs that were captured by multiple SNPs
130 by constructing haplotypes.

131 Gene-gene interactions were analysed using Multifactor Dimensionality Reduction (MDR)
132 (version 1.0.0). The MDR approach has been described previously ⁽²⁰⁾, see online repository. The
133 significance of the average prediction error was calculated using MDR permutation test and a p -
134 value < 0.05 was considered significant. Logistic regression analyses were performed to confirm
135 significant 2-way interaction results from MDR analyses, if the interaction term was significant (in
136 a multiplicative model) the best fitting genetic model e.g. dominant or recessive was analysed.

137 **Results**

138 *Study population*

139 3,062 children were genotyped and 2,927 Dutch children were selected for genetic analyses (table
140 1). Children who were not from Dutch origin (5.7%) were excluded from further analyses because
141 inclusion of non-Caucasians may result in spurious genetic effects due to population stratification.
142 Cut-off values determined by tertiles of serum IgE (online supplement table E1) identified 503
143 cases and 541 controls at age 1-2 years, and 307 cases and 308 controls at age 6-8 years.

144

145 *SNP selection and genotyping*

146 145 SNPs of 24 genes relevant to co-stimulation, *VTCN1* (also named *B7-H4/ B7x*), *TNFRSF4*
147 (*OX40*), *TNFRSF18* (*GITR*), *TNFRSF14* (*HVEM*), *TNFSF18* (*GITRL*), *TNFSF4* (*OX40L*), *CD28*,
148 *CTLA4*, *ICOS*, *PDCD1* (*PD-1*), *BTLA*, *CD80* (*B7-1*), *CD86* (*B7-2*), *HLA-G*, *CD274* (*B7-H1/PD-*
149 *L1*), *PDCD1LG2* (*PD2L*), *CD276* (*B7-H3*), *LILRA4* (*ILT7*), *LILRB1* (*ILT2*), *LILRB2* (*ILT4*),
150 *LILRB4* (*ILT3*), *CD40*, *ICOSLG*, and *CD40LG* (*CD40L*), were selected for genotyping (figure 1
151 and table E2 in the Supplementary Data). Five SNPs failed amplification; one SNP was
152 monomorphic (rs7602383, *ICOS*). Genotypes for three SNPs, rs1181390 (*CD28*), rs9848900
153 (*CD86*), and rs7565639 (*PDCD1*) deviated from Hardy-Weinberg equilibrium in controls (at 1-2
154 years and/or at 6-8 years of age) and were not considered for further analyses leaving 136 SNPs
155 for SNP and haplotype analysis. MDR analyses were performed with all 136 SNPs and a selection
156 of 54 SNPs, after exclusion of 80 SNPs that were in LD ($D' > 0.8$) with one or more SNPs
157 (Supplementary Data table E2).

158

159 *Single SNP analysis*

160 A total of 8 SNPs in 4 genes were significantly associated with serum IgE at age 1-2 years at
161 either the allele or genotype level, and 13 SNPs in 7 genes at age 6-8 years ($p < 0.05$, table 2). SNPs
162 in *CD40* and *LILRA4* were associated with IgE in both age groups, but with different SNPs, *i.e.*

163 rs3746821 and rs3745419 at age 1-2 years and rs3765459 and rs2241384 at age 6-8. Interestingly,
164 the associated SNPs in both genes showed high LD ($D'=0.8$; and $r^2=0.03$ for both SNP
165 combinations). None of the single SNP associations remained significant when corrected for
166 multiple comparisons using false discovery rate (data not shown).

167

168 *Haplotype analysis*

169 We constructed haplotypes combining all tagging SNPs for each gene and haplotypes that
170 captured non-genotyped SNPs (presented in supplemental data table E3). One *CD86* haplotype
171 was significantly more prevalent in cases than controls at age 1-2 years (10 vs. 6%, $p=0.01$). This
172 *CD86* haplotype comprehends the haplotype that was constructed of rs2681415 and rs2681411 to
173 tag the non-genotyped SNPs rs9872438 and rs2681408. The combined minor alleles of these
174 SNPs were significantly more prevalent in cases compared to controls at age 1-2 years (12 vs. 7%,
175 $p=0.0002$). Another haplotype, consisting of two minor alleles for *ICOSLG* rs2070561 and
176 rs3746963, was less prevalent among cases compared to controls at age 6-8 years (16 vs. 22%,
177 $p=0.04$).

178

179 *MDR analyses*

180 Table 3 shows the best multilocus models for elevated serum IgE detected by MDR when
181 considering one, two, three, and four loci in all co-stimulatory genes. At age 1-2 years, the
182 prediction error of each model was statistically significant ($p=0.03$, 0.02, 0.02, and 0.02
183 respectively) based on 1,000 permutations. At 6-8 years, the 2-, 3-, and 4-locus models showed
184 statistical significance ($p=0.04$, 0.02, and 0.01 respectively). At both ages multiple synergistic
185 interactions between SNPs were observed, as indicated by the red and orange colour in the
186 dendrograms (Figure 2).

187 The gene-gene interaction effects of the best 2-locus models were confirmed by conventional
188 logistic regression analysis. At age 1-2 years, the best 2-locus model, *i.e.* rs10804556 (*CD86*) and

189 rs12030415 (*VTCNI*), showed a dendrogram without synergistic effect (figure 2a). The
190 information gain (entropy based) calculated for this pair of SNPs indicated redundancy, which
191 may be interpreted as the two SNPs acting in parallel redundant ways to increase IgE. Logistic
192 regression revealed that individuals with minor alleles of each SNP had an increased risk to
193 develop an elevated serum IgE level, and this risk did not further increase in individuals having
194 the minor alleles of both SNPs. A borderline statistically significant interaction existed between
195 the SNPs in logistic regression analysis (p-value for interaction 0.058, figure 3a). At age 6-8 years,
196 the dendrogram showing the relation between rs4143815 (*CD274*) and rs2241384 (*LILRA4*)
197 indicated synergistic interaction (figure 2b). Logistic regression revealed that individuals carrying
198 one or two minor alleles of either SNP had a decreased risk to develop elevated serum IgE. In
199 contrast, this decreased risk was not observed in individuals having one or two minor alleles of
200 both SNPs, as indicated by a statistically significant interaction (p-value for interaction 0.004,
201 figure 3b).

202

203 *Integrating analytical strategies*

204 Table 4 shows a summary of both the single SNP, the multilocus, and the haplotype associations
205 of the co-stimulatory pathway with IgE at age 1-2 and 6-8 years. When considering associations at
206 a gene level, 3 genes, *i.e.* *CD86*, *CD274*, and *LILRA4* showed significant associations with serum
207 IgE at both ages 1-2 years and at 6-8 years. Three genes, *i.e.* *BTLA*, *CTLA4* and *VTCNI* showed
208 association at 1-2 years of age, but not at 6-8 years. Another 5 genes, *i.e.* *CD276*, *ICOS*, *LILRB4*,
209 *TNFSF4*, and *TNFSF18* showed association at 6-8 years, but not at 1-2 years.

210 Interestingly, 12 SNPs, in the genes *BTLA*, *CD40*, *CD80*, *CD86*, *CD274*, *CD276*, *CTLA4*,
211 *LILRA4*, *LILRB4*, and *VTCNI* associated in multilocus models and did not associate with IgE in
212 the single SNP or haplotype analyses. Thus, multilocus analyses by MDR identified gene
213 variations that associated with elevated serum IgE without having a main effect.

214 **Discussion**

215 This study evaluated single SNPs, haplotypes, and multilocus associations of haplotype tagging
216 SNPs in a pathway of co-stimulatory genes and their association with the predisposition to
217 elevated serum IgE levels at ages 1-2 and 6-8 years. We expand current knowledge by showing
218 that within a biological pathway multiple gene combinations contribute to serum IgE levels. We
219 found multiple multilocus associations which showed statistical significance. One, two, three, and
220 four loci models were found to better predict the presence of elevated serum IgE than would be
221 expected by chance. Some polymorphisms were not significantly associated when tested in a
222 single SNP analysis, yet they were significantly associated in the multilocus models. This stresses
223 the importance of applying research strategies that model multiple interactions in genetic
224 association studies.

225 It has been well established that multiple genes are involved in the predisposition to elevated
226 serum IgE ^(1;21-23). In comparison to other studies that used the MDR approach in atopic disease,
227 we found many statistically significant multilocus models ⁽²¹⁻²³⁾. This is most likely because we
228 evaluated genes in a biological pathway, whereas previous studies selected genes based on earlier
229 reported associations with atopic disease.

230 Some of the evaluated genes have previously been described to associate with atopic phenotypes
231 in single gene association studies, *i.e.* *CD86* ⁽³⁾, *CD40* ⁽⁴⁾, *CTLA4* ^(5;24), *HLA-G* ⁽⁶⁾, *ICOS* ⁽⁷⁾ and
232 *PDCDI* ⁽⁸⁾ yet other genes were found not to be associated with atopy such as *CD28* ⁽⁵⁾, and
233 *LILRB4* ⁽²⁵⁾. A summary of these association studies is presented in the Supplementary Data table
234 E4. We here confirm main effects of the genes *CD86*, *CD40*, and *ICOS* with respect to serum IgE
235 levels. In addition, we show that *LILRB4*, which did not appear to have a main effect on IgE levels
236 in single gene association studies ^(5;25), does affect IgE levels in interaction with other gene
237 polymorphisms. Although we did not find main effects for *CTLA4*, *HLA-G* and *PDCDI*, our
238 results are not in contradiction with previous studies, because the associated atopic phenotypes
239 were different, *e.g.* specific IgE to grass pollen (*PDCDI*), or asthma and airway

240 hyperresponsiveness (*HLA-G*), or the studies evaluated different subgroups of individuals, *e.g.*
241 females (*CTLA-4*), or an adult asthma population (*CTLA-4*).

242 IgE production is known to rise during childhood and the influence of certain genes may be age-
243 specific ⁽²⁶⁾. We therefore tested associations with IgE at two different age groups. The single and
244 multilocus models were different between the age groups 1-2 and 6-8 years, suggesting that genes
245 in this pathway have indeed age-specific effects on IgE development. However in our unbiased
246 approach by applying MDR analysis, only the best models for each data set are given by MDR.
247 Since multiple loci in this pathway may be associated with IgE development, these loci may
248 compete with each other causing different results in each age group. Therefore we conclude that
249 MDR is not suitable for comparison between data sets in different age groups.

250 Of the interactions identified, MDR indicated that the type of interaction in most of the models
251 was synergistic. The negative gene-gene interactions in the logistic regression of the 2-locus
252 models suggest that polymorphisms of these genes counteract each other's effect. This seems to be
253 biologically plausible, because by counteracting effects of small genetic variations, the immune
254 system would prevent itself from derailment by small genetic changes. Further biological
255 interpretation of multilocus models is rather speculative, but current data provide a first suggestion
256 that activation of allergen-specific T-lymphocyte responses can take place by modifying co-
257 stimulatory signals. Thus the genes under study are likely important homeostatic regulators of T-
258 lymphocyte activation and subsequent IgE production.

259 Our results support the hypothesis that multiple gene-gene interactions are involved in IgE
260 regulation by fine-tuning of lymphocyte responses. Activation of T-lymphocytes requires, besides
261 TCR-MHCII/peptide complex recognition, additional secondary signals provided by co-
262 stimulatory molecules expressed on antigen presenting cells (APCs). The interaction between
263 CD28 on T-lymphocytes and its two ligands B7-1 (CD80) and B7-2 (CD86) on APCs is
264 considered to be the master co-stimulatory pathway for optimal T-cell responses ⁽²⁷⁾. CD86 is
265 constitutively expressed on APCs at low levels and rapidly upregulated upon stimulation, whereas

266 CD80 is inducible and expressed later than CD86. In contrast to the stimulatory signals provided
267 by CD28, interaction of CD80 or CD86 with the CD28 homolog CTLA-4 induces signals that
268 down-regulate T-cell activation. CTLA-4 is constitutively expressed only on CD4⁺CD25⁺
269 regulatory T-lymphocytes and is induced on activated T-lymphocytes and CTLA-4 signalling
270 plays an important role in regulating the intensity of allergic disease ⁽²⁸⁾.

271 Strengths of our study are its large sample size and prospective follow-up, which enabled us to
272 evaluate the influence of co-stimulatory pathway polymorphisms in two age groups that represent
273 different stages of the developing immune system in early childhood and primary school age. We
274 have previously published the successful identification of important genetic mechanisms in the
275 development of childhood atopy in our Allergenic cohort ⁽⁹⁾, thus showing it's high potential for
276 genetic studies. Furthermore, the selection of haplotype tagging SNPs has made it very unlikely
277 that we have missed important signals from genes.

278 To appreciate our results we should also consider some potential limitations to our study. First,
279 environmental influences were not considered in our analyses. Several studies have shown that
280 environmental influences can be of great importance in the development of atopy and we
281 recommend considering these in future research. As a result of recruitment strategies, our study
282 represents a selected population with a relatively high number of children with atopic parents and
283 our results may not be fully representative of the general population. Secondly, the proportion of
284 the cohorts that participated in each age group was variable, *i.e.* in age group 1-2 years all cohorts
285 contributed to the IgE measurements whereas at age 6-8 only IgE measurements of PREVASC
286 and PIAMA could be evaluated. As a result, age groups may have been subject to different
287 selection effects or variable environmental exposures and may therefore not be completely
288 comparable. It is therefore important to note that several genes were associated with IgE at 1-2 as
289 well as 6-8 years, internally replicating our results.

290 None of the single SNP associations remained significant when corrected for multiple
291 comparisons using false discovery rate. However, due to linkage disequilibrium between the

292 evaluated single SNPs, the statistical tests performed are not completely independent and
293 correction may be overly conservative. We tested the best models obtained from MDR analysis for
294 significance based on 1000 permutations. To assess if our results in the pooled cohorts at age 1-2
295 and 6-8 years are valid, we investigated whether we could replicate the observed two-way
296 interaction in our separate cohorts. Indeed we found a similar and significant interaction in 2
297 different cohorts at age 1-2 years in logistic regression analyses, p-values for interaction being
298 0.008 and 0.03 for the PREVASC and KOALA cohort respectively. We were unable to replicate
299 the borderline significant interaction we observed in the full cohort at age 6-8 years in the two
300 separate cohorts. This interaction was observed in the PIAMA (n=437) but not the PREVASC
301 cohort that contained lower numbers of individuals (n=130).

302 The SNPs found to be associated in this study were based on haplotype selection, hence their
303 functional role is not clear. Furthermore, MDR is a new technique that can be considered as an
304 unbiased data-mining approach. Thus, this study can be viewed as hypothesis generating. The
305 selected important genes in the co-stimulatory pathway can now guide replication studies and
306 functional analyses. This may ultimately lead to novel targets for early prevention of atopy
307 development.

308 In conclusion, serum IgE levels are regulated by multiple gene-gene interaction effects of many
309 genes in the co-stimulatory pathway. The genetic interactions we observed occur in a biologically
310 plausible way. Our results implicate that investigation of genetic contribution to complex traits
311 will not be possible without analytical approaches that consider effects of multiple interacting loci
312 in one gene as well as in multiple genes.

313 **Acknowledgements**

314 The authors would like to thank the children and parents of the PIAMA, PREVASC and KOALA
315 study for their participation. In addition we acknowledge the field workers, secretaries and
316 scientific collaborators dedicated to the PIAMA, PREVASC and KOALA cohorts, Marcel
317 Bruinenberg for his advice on DNA isolation, processing and genotyping, Ilja Nolte for her advice
318 on haplotype analyses, and Antoon van Oosterhout for his advice on gene selection. We thank
319 Jason H. Moore for his helpful comments to our questions with respect to MDR analyses.
320 This study was financially supported by ZonMW grant number 912-03-031. G.H. Koppelman is
321 supported by a Zon-Mw VENI grant, number 91656091.

322 **Web Resources**

HapMap database

URL: <http://www.hapmap.org>; data release 19. phase II. October 2005

Innate Immunity web site. Innate Immunity PGA. NHLBI Program in Genomic Applications

URL: <http://www.innateimmunity.net/data/homology>; October 2005

323 K-Biosciences. Cambridge. UK

324 URL: <http://www.kbiosciences.co.uk>

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405 **Table 1.** Characteristics of participating children in the Allergenic birth cohort.

Characteristics	PIAMA	PREVASC	KOALA	P^a
Participants in genetic study (number)	1,037	374	1,651	- ^b
Ethnicity (% Dutch origin)	95.1	95.7	95.2	-
Boys (%)	51.2	49.2	50.6	0.80
Total serum IgE				
1 year (IU/ml) ^c	7.1 (2.0-17.0) N=369	8.6 (3.5-19.4) N=226	6.0 (2.6-12.5) N=699	0.002
2 years (IU/ml) ^c	n.a. ^d	11.7 (4.2-28.7) N=358	12.0 (3.7-38.0) N=704	0.80
6 years (IU/ml) ^c	n.a.	22.5 (7.6-67.0) N=218	n.a.	-
8 years (IU/ml) ^c	64.9 (23.0-240.0) N=748	n.a.	n.a.	-

406 ^a P = p-value based for comparison between cohorts by Chi-square test or analysis of variance
407 where appropriate; ^b - = not tested; ^c geometric mean (interquartile range); ^d n.a. = not available.

408 **Table 2.** SNPs significantly associated with increased serum IgE, at allele and genotype level
 409 (p<0.05).

Gene	rs number	Association at allele level 1-2 years					Association at genotype level 1-2 years			
		MAF ^a (controls / cases)	Allele	OR ^b	(95% CI) ^c	P ^d	Genotype	OR ^b	(95% CI) ^c	P ^e
<i>CD86</i>	rs10804556	0.18 / 0.24	G	1.46	1.14-1.87	0.003	A:A	1.00		0.002
							G:A	1.56	1.19-2.04	
							G:G	1.79	0.97-3.28	
<i>CD86</i>	rs2681415	0.10 / 0.15	G	1.35	1.00-1.82	0.008	A:A	1.00		0.004
							G:A	1.67	1.23-2.27	
							G:G	1.34	0.54-3.33	
<i>VTCNI</i>	rs10047089	0.45 / 0.50	A	1.25	1.05-1.49	0.01	G:G	1.00		0.05
							G:A	1.29	0.96-1.72	
							A:A	1.53	1.08-2.17	
<i>VTCNI</i>	rs12030415	0.24 / 0.29	A	1.31	1.07-1.60	0.05	G:G	1.00		0.003
							G:A	1.05	0.81-1.37	
							A:A	2.46	1.47-4.12	
<i>CD86</i>	rs1915087	0.32 / 0.36	C	1.32	1.07-1.62	0.01	T:T	1.00		0.04
							C:T/C:C	1.50	1.02-2.20	
<i>CD86</i>	rs11717893	0.27 / 0.24	-	-	-	-	T:T	1.00		0.04
							C:T	0.98	0.76-1.27	
							C:C	0.50	0.29-0.86	
<i>CD40</i>	rs3746821	0.11 / 0.09	-	-	-	-	G:G/G:T	1.00		0.04
							T:T	0.26	0.07-0.93	
<i>LILRA4</i>	rs3745419	0.17 / 0.19	-	-	-	-	T:T/A:T	1.00		0.04
							A:A	2.31	1.04-5.17	
Gene	rs number	Association at allele level 6-8 years					Association at genotype level 6-8 years			
		MAF ^a (controls / cases)	Allele	OR ^b	(95% CI) ^c	P ^d	Genotype	OR ^b	(95% CI) ^c	P ^e
<i>CD274</i>	rs2297136	0.42 / 0.50	T	1.35	1.07-1.69	0.01	C:C	1.00		0.03
							C:T	1.25	0.86-1.83	
							T:T	1.89	1.19-3.02	
<i>TNFSF18</i>	rs2236876	0.23 / 0.28	T	1.32	1.02-1.72	0.04	C:C	1.00		0.05
							C:T	1.52	1.09-2.13	
							T:T	1.34	0.66-2.71	
<i>LILRA4</i>	rs17836364	0.15 / 0.20	A	1.37	1.01-1.84	0.04	G:G	1.00		0.04
							G:A/A:A	1.44	1.02-2.04	
<i>ICOS</i>	rs4521021	0.19 / 0.25	C	1.34	1.02-1.76	0.04	T:T	1.00		0.02
							C:T/C:C	1.5	1.08-2.08	
<i>TNFSF18</i>	rs975074	0.51 / 0.45	G	0.79	0.62-1.00	0.05	T:T	1.00		0.04
							G:T/G:G	0.66	0.45-0.98	
<i>CD28</i>	rs1181390	0.37 / 0.32	-	-	-	-	C:C	1.00		0.04
							C:A	1.15	0.80-1.66	
							A:A	0.63	0.40-0.99	
<i>CD274</i>	rs10975123	0.19 / 0.16	-	-	-	-	C:C/C:T	1.00		0.02
							T:T	0.26	0.08-0.78	
<i>TNFSF4</i>	rs11811856	0.26 / 0.30	-	-	-	-	C:C	1.00		0.05
							C:G	0.97	0.69-1.36	
							G:G	2.19	1.14-4.22	
<i>CD40</i>	rs3765459	0.21 / 0.25	-	-	-	-	G:G/G:A	1.00		0.02
							A:A	2.53	1.19-5.39	
<i>TNFSF18</i>	rs723858	0.19 / 0.23	-	-	-	-	A:A	1.00		0.05
							A:T/T:T	1.39	1.00-1.94	
<i>LILRA4</i>	rs2241384	0.22 / 0.18	-	-	-	-	C:C	1.00		0.04
							C:T/T:T	0.71	0.50-0.99	
<i>CD274</i>	rs2297137	0.26 / 0.21	T	0.75	0.58-0.99	0.04	-	-	-	-
<i>CD274</i>	rs4143815	0.31 / 0.25	C	0.77	0.59-0.99	0.04	-	-	-	-

410 ^a MAF = minor allele frequency; ^b OR = odds ratio calculated by logistic regression; ^c 95% confidence
 411 interval; ^d P-value determined by Chi-square test (1 df); ^e P-value determined by logistic regression

412 **Table 3.** Results of MDR analysis evaluating all co-stimulatory genes.

Loci ^a	Best model		Mean CV ^b consistency	Mean prediction error (%)	P ^c
	Gene(s)	SNP(s)			
<i>1-2 years</i>					
1	<i>CD86</i>	rs10804556	9.4	45.3	0.03
2	<i>CD86 and VTCN1</i>	rs10804556 rs12030415	6.4	43.4	0.02
3	<i>VTCN1 BTLA</i>	rs9288953 rs7023227			
	<i>CD274</i>	rs11805655	4.6	42.8	0.02
4	<i>VTCN1 CD40</i>	rs9288953 rs745307			
	<i>CD86 CTLA4</i>	rs2332096 rs231806	1.2	39.9	0.006
<i>6-8 years</i>					
1	<i>ICOS</i>	rs4521021	5.4	45.2	0.09
2	<i>CD274 LILRA4</i>	rs4143815 rs2241384	2.6	42.3	0.04
3	<i>TNFSF18 LILRA4</i>	rs975074 rs2241385			
	<i>LILRB4</i>	rs3745871	5.6	39.8	0.02
4	<i>CD276 CD80</i>	rs11072430 rs610902			
	<i>CD80 CD86</i>	rs7648642 rs4308217	1.4	38.1	0.006

413 ^aNumber of loci considered; ^bCV=cross-validation; ^cSignificance of prediction error (empirical p-
414 value based on 1000 permutations).

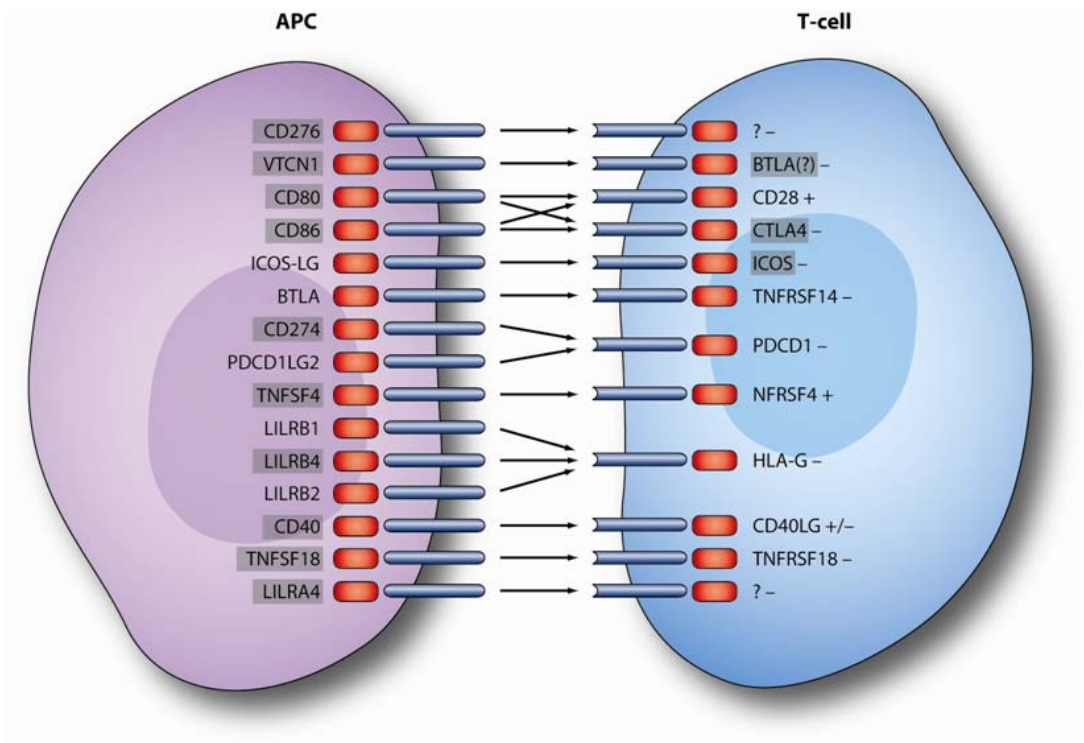
415 **Table 4.** Summary of single and multilocus associations (p-values) in the co-stimulatory pathway
 416 at ages 1-2 years and 6-8 years.

Gene	Rs number	Association with IgE at 1-2 years					Association with IgE at 6-8 years						
		Allele	Genotype	Association by MDR				Allele	Genotype	Association by MDR			
				1 locus	2 locus	3 locus	4 locus			1 locus	2 locus	3 locus	4 locus
<i>BTLA</i>	rs7023227			0.02									
<i>CD28</i>	rs1181390		0.04										
<i>CD40</i>	rs3746821		0.03										
<i>CD40</i>	rs3765459							0.02					
<i>CD40</i>	rs745307											0.006	
<i>CD80</i>	rs610902												0.006
<i>CD80</i>	rs7648642												0.006
<i>CD86</i>	rs10804556	0.003	0.002	0.03	0.02								
<i>CD86</i>	rs2681415	0.008	0.003										
<i>CD86</i>	rs1915087	0.01	0.04										
<i>CD86</i>	rs11717893		0.04										
<i>CD86</i>	rs2332096											0.006	
<i>CD86</i>	rs4308217												0.006
<i>CD274</i>	rs10975123								0.02				
<i>CD274</i>	rs2297137							0.04					
<i>CD274</i>	rs4143815							0.04		0.04			
<i>CD274</i>	rs2297136							0.01	0.03				
<i>CD274</i>	rs11805655											0.02	
<i>CD276</i>	rs11072430												0.006
<i>CTLA4</i>	rs231806												0.006
<i>TNFSF18</i>	rs2236876							0.04	0.05				
<i>TNFSF18</i>	rs975074							0.05	0.04			0.02	
<i>TNFSF18</i>	rs723858								0.05				
<i>ICOS</i>	rs4521021							0.04	0.02				
<i>LILRA4</i>	rs17836364							0.04	0.04				
<i>LILRA4</i>	rs3745419		0.03										
<i>LILRA4</i>	rs2241384								0.04	0.04			
<i>LILRA4</i>	rs2241385												0.02
<i>LILRB4</i>	rs3745871												0.02
<i>TNFSF4</i>	rs11811856								0.04				
<i>VTCN1</i>	rs10047089	0.01	0.05										
<i>VTCN1</i>	rs12030415	0.05	0.002		0.02								
<i>VTCN1</i>	rs9288953					0.02	0.006						

418 **Figure legends**

419 **Figure 1.** Hypothetical scheme of co-stimulatory receptor and ligand pairs evaluated in this study.
420 Genes that show association with serum IgE are boxed. APC=antigen presenting cell; *BTLA* = B
421 and T lymphocyte attenuator; *CD40LG* = CD40 antigen ligand; *CTLA4* = cytotoxic T-lymphocyte-
422 associated antigen 4; *TNFSF18* = tumor necrosis factor (ligand) superfamily, member 18; *HLA-G*
423 = HLA-G histocompatibility antigen, class I, G; *ICOS* = inducible T-cell co-stimulator; *ICOSLG* =
424 inducible T-cell co-stimulator ligand; *LILRA4* = leukocyte immunoglobulin-like receptor,
425 subfamily A (with TM domain), member 4; *LILRB1* = leukocyte immunoglobulin-like receptor,
426 subfamily B (with TM and ITIM domains), member 1; *LILRB2* = leukocyte immunoglobulin-like
427 receptor, subfamily B (with TM and ITIM domains), member 2; *LILRB4* = leukocyte
428 immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4; *PDCD1*=
429 programmed cell death 1; *PDCD1LG2* = programmed cell death 1 ligand 2; *TNFRSF14* = tumor
430 necrosis factor receptor superfamily, member 14; *TNFRSF18* = tumor necrosis factor receptor
431 superfamily, member 18; *TNFRSF4* = tumor necrosis factor receptor superfamily, member 4;
432 *TNFSF4* = tumor necrosis factor (ligand) superfamily, member 4; *VTCN1* = V-set domain
433 containing T cell activation inhibitor 1; ? = receptor unknown; - = inhibitory signalling effect; +
434 positive signalling effect; Information adapted from ⁽²⁹⁻³⁰⁾.

Figure 1



435

436

437 **Figure 2.** Interaction dendrogram visualizing the information gain (entropy based) associated with
 438 considering SNP interactions in the co-stimulatory pathway at (a) 1-2 years and (b) 6-8 years of
 439 age. The color of the line connecting pairs of SNPs indicates the degree of synergy (positive
 440 information gain i.e. the SNPs combined provide a different (stronger or weaker) effect or
 441 redundancy (negative information gain). The shorter the line the stronger the interaction.

Figure 2 (a)

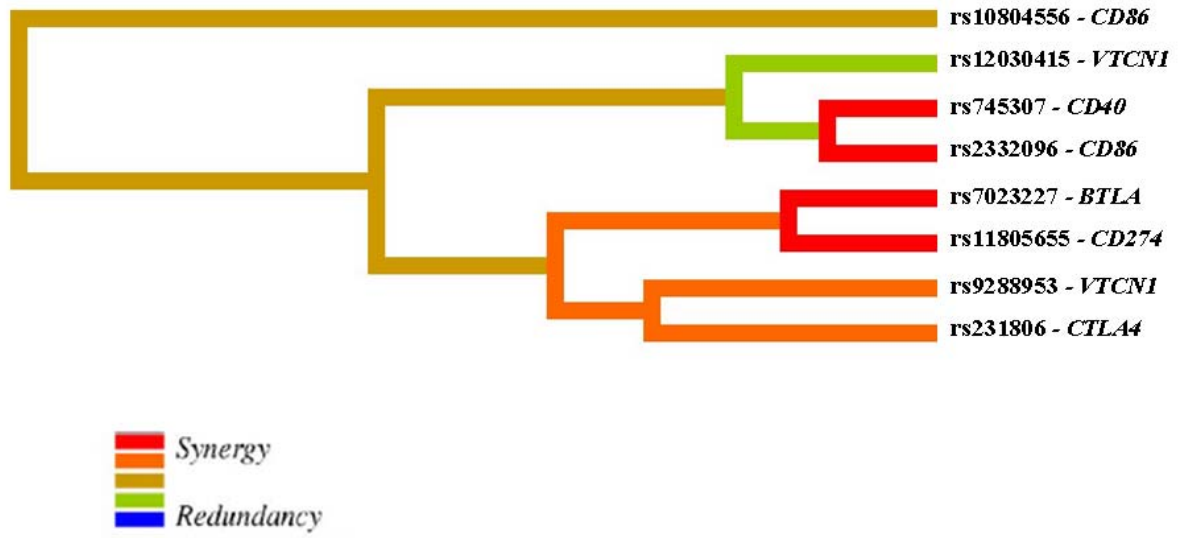
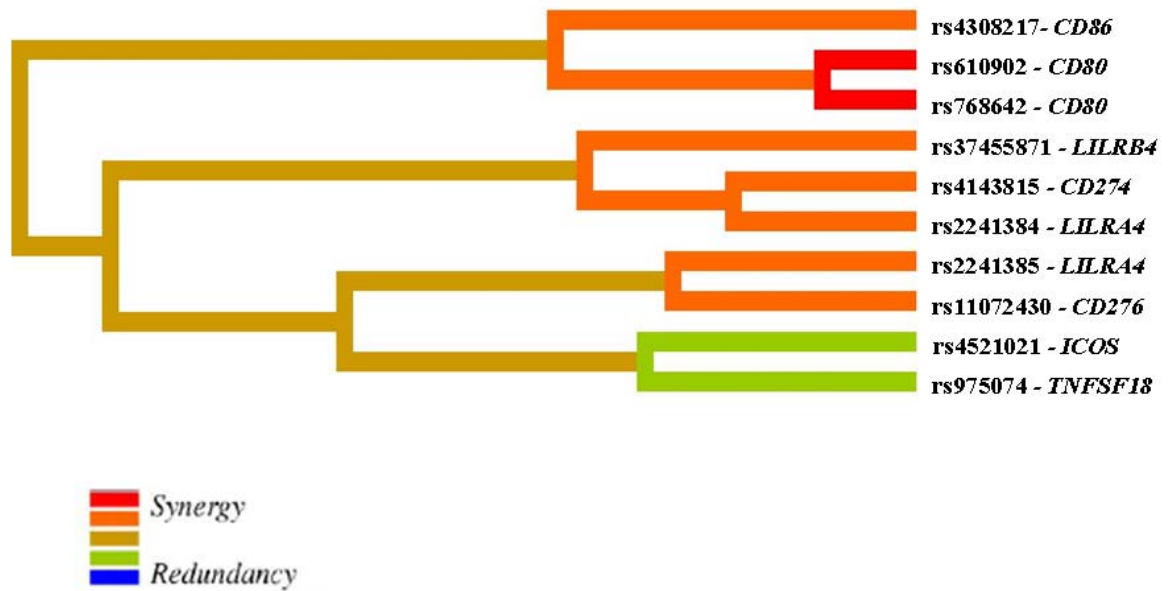


Figure 2 (b)



443

444

445 **Figure 3.** Two locus associations between co-stimulatory molecules by logistic regression.

446 (a) best 2 locus model at 1-2 years; and (b) best 2 locus model at 6-8 years; OR= odds ratio and

447 95% confidence interval; * p-value for interaction.

Figure 3 (a)

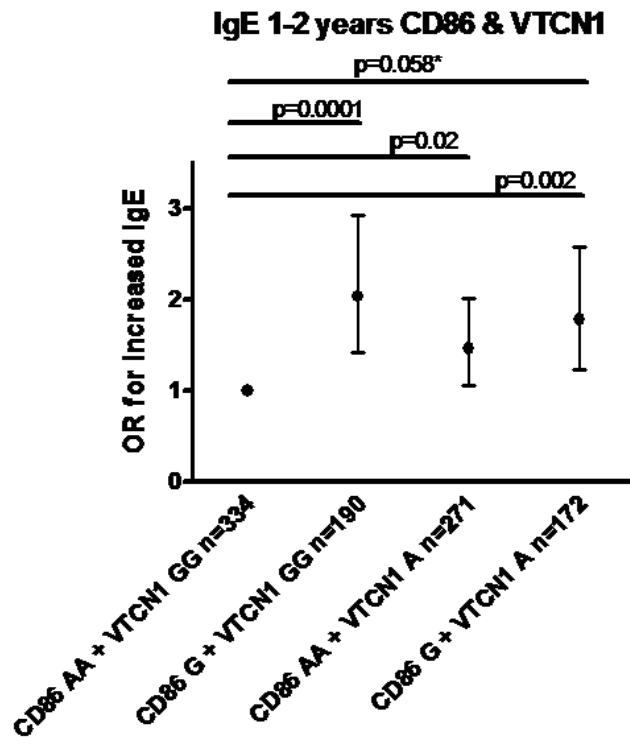


Figure 3 (b)

