ORIGINAL ARTICLE

A functional CD86 polymorphism associated with asthma and related allergic disorders

Thomas Juhl Corydon, Annette Haagerup, Thomas Gryesten Jensen, Helle Glud Binderup, Mikkel Steen Petersen, Keld Kaltoft, Jørgen Vestbo, Torben Arvid Kruse, Anders Dupont Børglum

J Med Genet 2007;**44**:509–515. doi: 10.1136/jmg.2007.049536

See end of article for authors' affiliations

Correspondence to: Anders Dupont Børglum, Institute of Human Genetics, the Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark; anders@humgen.au.dk

Received 31 January 2007
Revised 20 April 2007
Accepted 3 May 2007
Published Online First 18 May 2007

Background: Several studies have documented a substantial genetic component in the aetiology of allergic diseases and a number of atopy susceptibility loci have been suggested. One of these loci is 3q21, at which linkage to multiple atopy phenotypes has been reported. This region harbours the *CD86* gene encoding the costimulatory B7.2 protein. The costimulatory system, consisting of receptor proteins, cytokines and associated factors, activates T cells and regulates the immune response upon allergen challenge.
Methods: We sequenced the *CD86* gene in patients with atopy from 10 families that showed evidence of linkage to 3q21. Identified polymorphisms were analysed in a subsequent family-based association study of two independent Danish samples, respectively comprising 135 and 100 trios of children with atopy and their parents. Functional analysis of the costimulatory effect on cytokine production was performed in an autologous cell-based system based on cells expressing CD86 variants.
Results: Two polymorphisms were identified, encoding the amino acid changes Ile179Val and Ala304Thr, respectively. Significant associations were observed between the Ile179Val polymorphism and allergy phenotypes in both samples (eg, asthma, $p = 4 \times 10^{-3}$ in the two samples combined). The undertransmitted (protective) Val179 allele was found to induce higher production of both Th1 and Th2 cytokines than the overtransmitted (risk) Ile179 allele, suggesting a functional impact of the polymorphism.
Conclusion: The *CD86* gene, and specifically the Ile179Val polymorphism, may be a novel aetiological factor in the development of asthma and related allergic disorders.

Allergic diseases such as asthma, rhinitis and atopic dermatitis affect approximately one-fifth of the general population in the Western world and their frequencies seem to be increasing. Asthma alone affects 155 million people in the world and hay fever is even more common.[1] Atopic dermatitis at present affects 21% of Danish children.[2] Because of their high prevalence and the associated personal morbidity and social costs, the allergic diseases are a major global health problem.

Family, twin and adoption studies have documented a substantial genetic component in the aetiology of allergic diseases.[3–5] Several genome scans of large numbers of families with atopy have been carried out in the past decade, suggesting a number of atopy loci. Relatively few of the identified loci meet the criteria for genomewide significance. One that does is 3q21, at which significant linkage to atopic dermatitis was reported.[6] We and others have reported evidence of linkage to the same region for multiple atopy phenotypes, including asthma, rhinitis and atopy.[7–10] This region harbours the *CD86* gene encoding one of two costimulatory B7 proteins required for T cell activation. Antigen-presenting cells (APCs) express CD86 (B7.2), constituting the ligand for CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) at the T cell surface.[11] Binding of CD86 to CD28 initiates a costimulatory signal for T cell activation, proliferation, differentiation and production of a number of cytokines.[11] In contrast, binding to CTLA4 negatively regulates T cell proliferation and diminishes the immune response.[12–14] CTLA4 has higher affinity for CD86 than CD28 molecules and is predominantly expressed upon activation of T cells. T cell responses are categorised as T helper (Th)1 and Th2 according to the cytokines they produce. Th1 cells produce interferon (IFN)-γ, tumour necrosis factor (TNF)α and interleukins (IL)-2, IL-12 and IL-18, whereas Th2 cells typically produce IL-4, IL-5, IL-9, IL-10 and IL-13. It is generally accepted that a Th1/Th2 imbalance is implicated in the development of asthma and atopy,[15–19] and a role for the costimulatory B7 molecules in the regulation of the Th1/Th2 cytokine balance has been suggested by several investigators.[20–24]

The *CD86* gene is composed of eight exons and spans >22 kb. Jellis *et al* found that alternatively spliced cDNAs result from the use of either exon 1 or 2 giving rise to *CD86* isoform 1 (GenBank accession number NM_175862) and 2 (GenBank accession number NM_006889), respectively.[25] The N-terminus of isoform 2 is six amino acids shorter than isoform 1. Exon 2 does not contain coding sequences. Exon 3 corresponds to the signal peptide, exon 4 to an IgV-like domain, exon 5 to an IgC-like domain and exon 6 to the transmembrane region and part of the cytoplasmic tail. Exons 7 and 8 encode the remainder of the tail. The two *CD86* cDNAs with alternate 5′ untranslated sequences may represent constitutively expressed and inducible forms of *CD86*.[25]

This study investigated the possible role of *CD86* in susceptibility to asthma and related allergic disorders by family-based association analysis of two independent Danish samples. Furthermore, a substitution polymorphism (Ile179Val) showing association in both samples was subjected to functional analysis in an autologous cell-based system.

Abbreviations: APC, antigen-presenting cell; CTLA4, cytotoxic T-lymphocyte-associated protein 4; FEV^1, forced expiratory volume in 1 second; FITC, fluorescein isothiocyanate; HLA, human leucocyte antigen; IFN, interferon; IL, interleukin; LD, linkage disequilibrium; PBS, phosphate-buffered saline; RAST, radioallergosorbent test; TCR, T cell receptor; TDT, transmission disequilibrium test; Th, T helper; TNF, tumour necrosis factor; UTR, untranslated region

METHODS

Participants

Informed consent was obtained from each participant prior to inclusion in the study. Local ethics committee approval was obtained in all regions where families were recruited.

In total, 235 families (964 individuals) were recruited from two studies on atopy. In the first study (sample A), 135 nuclear families with asthmatic sibpairs were enrolled primarily from patient records in four clinical centres in the eastern, western and central areas of Denmark.[26 27] Inclusion criteria were recurrent cough, wheezing and dyspnoea and a positive metacholine challenge using the methods described by Yan *et al*.[28] Both affected siblings were aged 15–45 years. Skin-prick tests using 10 common aeroallergens and lung function measurements including FEV_1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) were performed on all participants. Blood samples were analysed for allergen-specific IgE (CAP Phadiatop; Pharmacia Upjohn, Copenhagen, Denmark); a positive test was defined as IgE $\geqslant$0.35 kUA/l for one or more of the allergens. Hay fever and eczema was self-reported in the questionnaires.

The second study (sample B) included a total of 424 individuals from 100 Danish atopic sibpair families enrolled in the Danish Inheritance of Type I Allergy (ITA) project. Recruitment, examination and clinical information have been described previously.[7 8] In brief, patients were recruited through four paediatric and one adult outpatient allergy clinic in Western Denmark. Inclusion criteria were a minimum of two atopic siblings with doctor-diagnosed asthma, hayfever or atopic dermatitis and reported effect of appropriate medication. Mean age among the offspring was 10.8 years. All subjects were clinically examined and questionnaire tested by the same doctor. Atopic disease was diagnosed by this doctor according to standard criteria in consensus with a second doctor evaluating the questionnaires only. Blood was drawn for serum measurement of specific IgE to 11 common allergens (CAP RAST FEIA test; Pharmacia Upjohn); a positive test was defined as above.

For each phenotype, only one affected offspring from each family (in both samples A and B) was selected for genotype analysis. Where there were $\geqslant$2 affected offspring in a family, the oldest child was selected for the analysis. The family structures and phenotypes of the two family samples are shown in table 1.

Sequencing, genotyping and statistical analysis

From all family members in both sample A and B, genomic DNA was isolated from blood samples according to standard procedures. Exon 1–8 of *CD86*, all intron/exon boundaries of the two isoforms and approximately 1 kb of the promoter region (of isoform 1) were screened for sequence variations in 10 unrelated patients from families (from sample B) showing evidence of linkage to 3q21.[8] Direct sequencing was performed using a commercial kit (Big Dye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) and an automated analyser (ABI-310 Genetic Analyzer; Applied Biosystems). Genotyping of parents and offspring was performed by single base extension technology (SNaP-shot Kit; Applied Biosystems). Standard PCR conditions were used for both sequencing and genotyping. Primer sequences are available upon request.

Pairwise linkage disequilibrium was calculated using Simwalk2[29] and the GOLD software package (http://www.sph.umich.edu/csg/abecasis/GOLD).[30] For association analysis, the transmission disequilibrium test (TDT) was used.[31] As only one affected child per family was analysed, the method is a valid test for association even in the presence of linkage, which is the case for both sample A[10] and sample B.[7 8] The allergy phenotypes investigated are strongly correlated (table 1) and the two SNPs tested are in (moderate) linkage disequilibrium with each other. Consequently, the presented p-values were not corrected for multiple testing. However, if analysis of the two SNPs were considered independent, most of the associations reported remain significant after Bonferroni correction.

Table 1 Family structures and phenotypes of the two samples analysed

	Number of families with affected children			
	Asthma	Positive RAST	Atopic dermatitis	Rhinitis
Sample A (135 families)				
1 affected child	0	35	51	47
2 affected children	135	73	68	74
Total	135	108	119	121
Sample B (100 families)				
1 affected child	31	36	33	36
2 affected children	45	40	34	35
3 affected children	12	7	6	8
Total	88	83	73	79

RAST, radioadioallergosorbent test.

Establishment of T cells and melanoma cell lines

After informed consent was obtained, a cutaneous biopsy specimen was taken from a patient with metastatic malignant melanoma. From this biopsy, T cell and melanoma cell lines were established.[32 33] Both cell lines were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc., Gaithersburg, Maryland, USA), with 10% fetal calf serum (Life Technologies Inc.), 100 U/ml penicillin and 0.1 mg/ml streptomycin (both Leo Pharma, Ballerup, Denmark), in 5% CO_2. In addition, 100 pM IL-2 and 500 pM IL-4 were added to the T cell medium.

Cloning of CD86 variants and expression constructs

Total RNA from human T cells expressing the most frequent CD86 allele, Ile179, was purified (Aurum total RNA kit; Bio-Rad, Hercules, California, USA) and cDNA was synthesised using Omniscript RT-kit (Qiagen, Valencia, California, USA) according to the manufacturers' instructions. Jellis *et al* have previously suggested that translation of both cDNAs (isoforms 1 and 2) begins at the methionine codon in exon 3.[25] Based on this, full-length, isoform 2 human *CD86* cDNA was amplified using a forward primer located in the exon 1–3 boundary and a reverse primer in the 3′ end of exon 8 and cloned into pcDNA3.1 (Invitrogen Corp., Carlsbad, California, USA). The CD86 Ile179Val mutation was introduced into the CD86-Ile179 clone using site-directed mutagenesis (QuickChange; Stratagen. La Jolla, California, USA). To achieve stable expression in melanoma cells, *CD86* variants were inserted into the pBabePuro retroviral vector.[34] All vectors were sequenced.

Transduction of human melanoma cells

pBabePuro vectors containing the *CD86* variants were packaged in packaged PG13 cells (American Type Culture Collection), and supernatants were used to transduce human melanoma cells using methods described previously.[35] Transduced melanoma cells were selected with 5 μg/μl puromycine (Sigma Chemical Co., Minneapolis, Minnesota, USA). Expression of Ile179 or Val179 CD86 receptor proteins was confirmed by sequencing of reverse-transcribed cDNA from the transduced melanoma cells (data not shown).

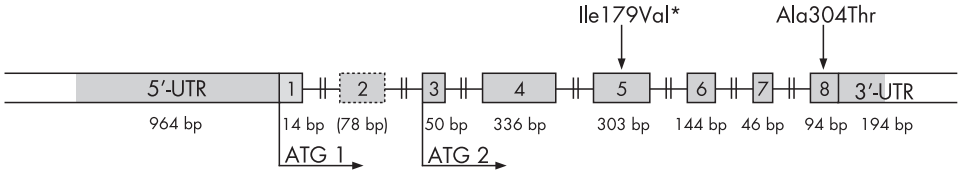

Figure 1 *CD86* genomic organisation, sequenced regions and localisation of the identified single nucleotide polymorphisms. *CD86* is composed of eight exons and spans >22 kb. Jellis *et al* established that alternatively spliced cDNAs result from the use of either exon 1 (ATG 1) or 2 (ATG 2) giving rise to *CD86* isoforms 1 or 2.[25] Exon 2 does not contain coding sequences. The sequenced regions of the *CD86* gene, including the 5′-untranslated region (UTR), proximal promoter region, exon 1–8 and 3′-UTR, are shown in grey. In addition, all intron–exon boundaries were sequenced. Numbers below boxes represent the size in base pairs. In total, around 3.8 kb were sequenced. The position of the two identified SNPs encoding the amino acid changes Ile179Val in exon 5 (rs2681417) and Ala304Thr (rs1129055) in exon 8 (numbering according to *CD86* isoform 2), are indicated. The asterisk denotes the SNP associated with asthma and related allergic disorders.

Analysis of CD86 expression in transduced melanoma cells

For flow cytometry analysis, transduced melanoma cells were harvested by incubation in phosphate-buffered saline (PBS) containing 0.6 mmol/l EDTA followed by incubation in PBS containing 0.6 mmol/l EDTA and 0.005% trypsin (v/v) (Life Technologies Inc). Approximately 2×10^5 cells were stained with either 10 µl primary anti-CD86 monoclonal antibody (Becton Dickinso, Franklin Lakes, NJ, USA) or 10 µl R-phycoerythrin (R-PE)-conjugated anti-HLA-class I (Dako, Glostrup, Denmark) for 30 min at 4℃ in 50 µl PBS containing 5% human serum (obtained from the local blood bank). For visualisation of CD86, 1 µl secondary fluorescein isothiocyanate (FITC)-conjugated Alexa Fluor goat-anti-mouse antibody (Molecular Probes, Breda, The Netherlands) in 50 µl PBS containing 5% human serum was added. Following incubation, the cells were washed in PBS and resuspended in PBS containing 5% human serum. Approximately 2×10^4 live cells were analysed on a FACS Calibur flow cytometer with CELLQuest V.3.3 software (Becton Dickinson). Dead cells were excluded by propidium iodide staining.

Cellular localisation and confocal microscopy was carried out as described.[36] The melanoma cells were fixed in neutral buffered formalin (Mallinckrodt Baker, Deventer, The Netherlands) for 15 min at room temperature, labelled with anti-CD86 monoclonal antibody (Becton Dickinson) and visualised using FITC-conjugated Alexa Fluor goat-anti-mouse antibody (Molecular Probes). Nuclear DNA was counterstained with Hoechst 33258 (1 µg/ml). Labelled cells were analysed by confocal microscopy analysis (Leica TCS microscope; Leica, Heerbrugg, Switzerland).

FACS analysis of T cells

The established T cell line was evaluated by flow cytometry for the expression of the markers T cell receptor (TCR), CD28, CD4 and CD8. In parallel experiments, approximately 2×10^4 T cells were stained with either 20 µl FITC-conjugated mouse anti-human TCR-α/β-1 (IgG_1, κ) antibodies (Becton Dickinson), 10 µl FITC-conjugated mouse anti-human CD4 (IgG_1, κ) antibodies (Dako), 20 µl FITC-conjugated mouse anti-human CD8 (IgG_1, κ) antibodies (Becton Dickinson) or 10 µl primary anti-rat CD28 antibodies (Serotech, Raleigh, North Carolina, USA), followed by 1 µl secondary FITC-conjugated Alexa Fluor goat-anti-mouse antibody (Molecular Probes) in 50 µl PBS containing 5% human serum. Anti-human CD16 (IgG_1, κ) antibodies (Becton Dickinson) were used as isotype control for TCR, CD4 and CD8 staining.

Quantification of the cytokine profile from T cells stimulated with transduced, autologous melanoma cells

Approximately 0.4×10^6 washed T cells were incubated with 0.2×10^6, 0.1×10^6 or 0.05×10^6 autologous, transduced melanoma cells. The cells were incubated for 2, 4 or 24 hours at 37℃. Following incubation medium was collected from the respective wells and centrifuged for 15 seconds at 10 000 *g*. The supernatant (free of cytokine-containing T cells) were analysed for various cytokines (Becton Dickinson human Th1/Th2 CBA kit and a Beckman Coulter FC500 flow cytometer) according to the manufacturer's instructions. Samples were then immediately analysed on the cytophotometer.

RESULTS

Association analysis

To identify potential susceptibility variants, we screened *CD86* (figure 1) for sequence variation in 10 unrelated atopic patients from families that showed evidence of linkage to 3q21 in a previous linkage study.[8] Two SNPs were identified, encoding the amino acid changes Ile179Val in exon 5 (rs2681417) and Ala304Thr (rs1129055) in exon 8 (numbering according to CD86 isoform 2), respectively. No other sequence variations were found in the ~3.8 kb region surveyed.

In a subsequent family-based association study of two independent Danish samples, comprising 135 (sample A) and 100 (sample B) families with members with atopy (table 1), significant associations were observed between the Ile179Val polymorphism and most of the allergy phenotypes investigated (table 2). The most frequent allele encoding Ile179 was overtransmitted to the affected offspring in both samples (whereas the Val179 allele was conversely undertransmitted). The results were most significant in sample A, showing association in all phenotypes except rhinitis. In sample B, there was a general trend towards Ile179 overtransmission, but significant association was observed only for asthma. In the two samples combined, the association was strongest for asthma ($p = 4\times10^{-3}$) but also strong for atopy (positive RAST) and in patients with both positive RAST and asthma or atopic dermatitis. The Ala304Thr polymorphism showed no evidence of association, and haplotype analysis of the two SNPs merely reflected the association of Ile179Val separately (results not shown).

The two SNPs were found to be in Hardy-Weinberg equilibrium in both samples and showed moderate pairwise linkage disequilibrium (LD) with a D′ of 0.70 and δ^2 of 0.02 ($p<0.0001$).

Expression of CD86 Ile179 and Val179 and establishment of an autologous cell-based system

To examine whether a variation in CD86 has any impact on the stimulation of T cells, it is necessary to avoid the costimulatory signal being masked by an alloresponse. Because CD86 present on melanoma cells can elicit effective costimulation of T cells,[37–39] genetically modified melanoma cells can be used to investigate the influence of CD86 polymorphisms on T cell activation. We therefore used autologous T cells and CD86 expressing melanoma cells cultured from the same patient to determine whether the Ile179Val variation in CD86 may influence T cell activation.

Table 2 Family-based association analysis of a *CD86* polymorphism* encoding an Ile179Val substitution in two Danish samples of case/parents trios with allergic diseases

	Asthma, 135/88†		RAST+, 108/83		Asthma and RAST+, 107/70		AD (eczema), 119/73		AD and RAST+, 94/55		Rhinitis, 121/79		Rhinitis and RAST+, 97/69	
Samples	**T/U‡**	**TDT§**	**T/U**	**TDT**	**T/U**	**TDT**	**T/U**	**TDT**	**T/U**	**TDT**	**T/U**	**TDT**	**T/U**	**TDT**
A	26/13 (0.67)	**0.04**	23/10 (0.70)	**0.02**	23/10 (0.70)	**0.02**	24/12 (0.67)	**0.05**	23/9 (0.72)	**0.01**	23/12 (0.66)	0.06	20/11 (0.65)	0.11
B	20/9 (0.69)	**0.04**	19/11 (0.63)	0.14	19/10 (0.66)	0.10	14/9 (0.61)	0.30	12/6 (0.67)	0.16	16/11 (0.59)	0.34	14/11) (0.56)	0.55
A+B	46/22 (0.68)	**4×10^{-3}**	42/21 (0.67)	**8×10^{-3}**	42/20 (0.68)	**5×10^{-3}**	38/21 (0.64)	**0.03**	35/15 (0.70)	**5×10^{-3}**	39/23 (0.63)	**0.04**	34/22 (0.61)	0.11

AD, atopic dermatitis; RAST+, positive radioallergosorbent test; TDT, transmission disequilibrium test. Sample A, 135 families; sample B, 100 families; A+B combined, 235 families.
*Corresponding to SNP rs2681417 in dbSNP (http://www.ncbi.nlm.nih.gov/SNP). The allele frequency among parents in both samples was 0.90 for Ile179 and 0.10 for Val179.
†For each phenotype the number of trio families is shown (sample A/sample B).
‡T/U, number of Ile179 encoding alleles transmitted from heterozygous parents to the affected offspring/number of untransmitted Ile179 encoding alleles. The frequency of transmitted alleles is given in brackets.
§ p Values calculated by the TDT.[30] p Values <0.05 are in bold.

Human CD86 Ile179 and Val179 were expressed in melanoma cells after retroviral transduction as described in Methods. Analysis of the expression levels of the two CD86 variants by means of flow cytometry using a CD86 antibody showed almost identical amounts of CD86 protein on the surface (figure 2A). To further investigate the subcellular localisation of the two CD86 variants, labelling with anti-CD86 followed by confocal microscopy of transduced melanoma cells was performed (figure 2C,D). In all cases, both variants displayed a typical surface-staining pattern and no other subcellular structures were labelled. No surface staining was observed in untransduced cells (data not shown). These data corroborate our findings that transduced CD86 Ile179 and Val179 are transported to the cell surface. To characterise the melanoma cells further, the amount of human leucocyte antigen (HLA) classes I and II was investigated by flow cytometry. We found comparable amounts of HLA class I in melanoma cells expressing CD86 Ile179 and Val179, and no expression of HLA class II.

The autologous T cells isolated from the same biopsy specimen as the melanoma cells were cultured as described above. A functional T cell normally expresses TCR, CD28, CD4 and/or CD8 proteins on the cell surface, which are known to play an important role in the activation and development of the T cell response.[11] To investigate whether these proteins were present at the surface of the isolated T cells, immunostaining using antibodies against TCR, CD28, CD4 and CD8 was performed and aliquots of stained T cells were analysed by flow cytometry. As shown in figure 3, the T cells were found to collectively express TCR, CD28, CD4 and CD8 on the surface.

Functional effect of CD86 Ile179Val on T cell response

To examine the positional effect of the Ile179Val substitution on costimulation, washed T cells (free of IL-2 and IL-4) were incubated with melanoma cells expressing either CD86 Ile179 or CD86 Val179. Flow cytometry analysis of the cell-culture media after 2, 4 and 24 hours of incubation revealed that melanoma cells expressing the Val179 variant induced a higher

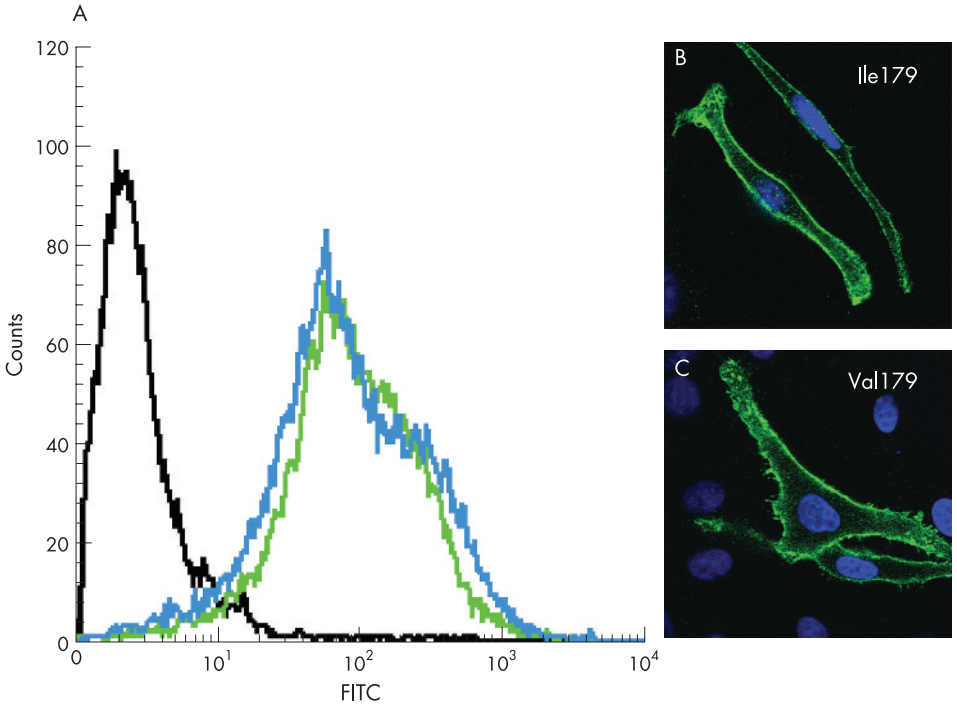

Figure 2 CD86 expression in transduced human melanoma cells localises to the cell surface. (A) Flow cytometry analysis of melanoma cells expressing CD86-Ile179 (blue line) or CD86-Val179 (green line). Transduced melanoma cells were stained with CD86 antibodies. CD86 was visualised with fluorescein isothiocyanate (FITC)-conjugated Alexa secondary antibody. As a negative control for CD86, transduced cells stained only with secondary antibody were used (black line). In each case, 2×10^4 cells were analysed. (B,C) Confocal microscopy of melanoma cells expressing (B) CD86-Ile179 or (C) CD86-Val179. CD86 was visualised by CD86 antibody and FITC-conjugated Alexa antibodies. CD86 expression and nuclear DNA are shown in green and blue, respectively. Original magnification ×100.

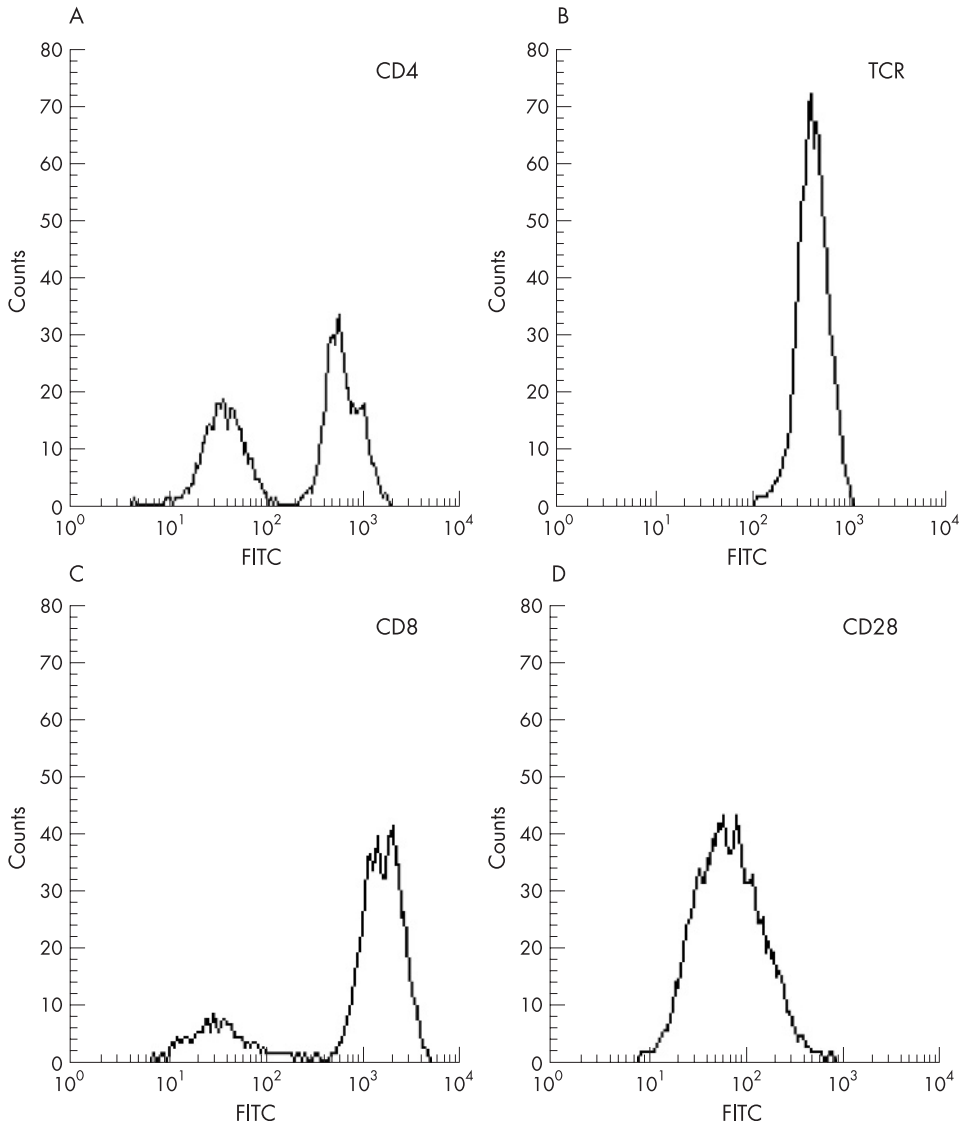

Figure 3 Characterisation of T cells. The established T cell line was evaluated for the expression of the following markers by flow cytometry: (A) CD4, (B) TCR, (C) CD8 and (D) CD28. The markers were visualised by FITC-conjugated CD4 antibody, FITC-conjugated TCR-α/β-1 antibody, FITC-conjugated CD8 or CD28 antibody, followed by secondary FITC-conjugated Alexa antibody, respectively. As an isotype control for TCR, CD4 and CD8 staining, FITC-conjugated anti-human CD16 antibodies were used. As negative control for CD28, the T cells were stained only with secondary antibody. None of the controls was found to overlap with the specific marker profiles (data not shown). In all cases, 2×10^4 cells were analysed. The results were reproduced in three independent experiments.

production of both Th1 cytokines (IL-2, TNFα, IFNγ) and Th2 cytokines (IL-4, IL-5) than melanoma cells expressing the Ile179 variant (figure 4, A–C and D–E, respectively). No difference between the two variants was observed for the production of IL-10 (predominantly an anti-inflammatory cytokine, typically produced by either Th2 or T regulatory (Tr) cells). As the melanoma cells do not express HLA-class II, the cytokine production in the autologous system may thus derive from CD8+ T cells.[40] However, the possibility that CD4+ T cells also contribute to the cytokine production cannot be excluded, as it is known that IFNγ produced early during the antigen response can induce HLA-class II expression on melanoma cells.[41]

The presented data show that the T cells of the autologous system are capable of producing a wide range of cytokines upon stimulation by CD86-expressing melanoma cells and indicate that the two CD86 variants differentially affect the cytokine production.

DISCUSSION

Using family-based association analysis of two independent Danish samples and functional analysis in an autologous cell-based system, we obtained results implicating the Ile179Val substitution polymorphism, located in the costimulatory protein CD86, with the aetiology of allergic disorders.

In this study, the allele encoding Ile179 was overtransmitted to the affected offspring, suggesting that this variant confers increased susceptibility to asthma and atopy, whereas Val179, which was undertransmitted, conversely may act as a protective allele. The association was observed in two independent Danish samples, most prominently in sample A, whereas significant replication was observed only for the asthma phenotype in sample B. The less significant association in sample B may partly be explained by demographic and clinical differences between the two samples: all the individuals in sample A were >15 years old, whereas in sample B the probands were mainly children. Other inclusion criteria and diagnostics were also not completely identical. Thus, even though the diagnoses were the same, the underlying risk alleles might not be identical or of equal importance in the two samples. Even though there was a general trend toward Ile179 overtransmission in all phenotypes in sample B, and the analysis of the combined samples showed association for all the phenotypes investigated, the results regarding the ''non-asthma'' phenotypes in particular should be replicated in additional populations.

The data provided by the HapMap project (http://www.hapmap.org) show that most of the *CD86* gene (from the proximal promoter region to exon 3) resides within a haplotype block of around 37 kb, and the remainder of the gene is located in smaller distinct blocks. Specifically, the Ile179Val polymorphism is

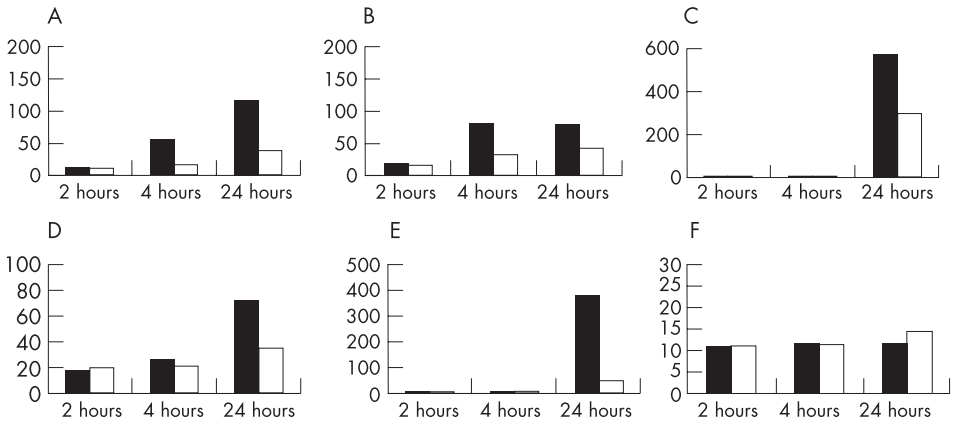

Figure 4 Effect of CD86 variants on cytokine production. Cytokine production by cytokine-dependent T cell lines after incubation for 2, 4 or 24 hours with transduced, autologous melanoma cells expressing CD86-Ile179 (white bars) or CD86-Val179 (black bars) proteins: (A) IL-2, (B) TNFα, (C) INF-γ, (D) IL-4, (E) IL-5 and (F) IL-10. Approximately 2.0×10^6 washed T cells were incubated with 0.2×10^6, 0.1×10^6, or 0.05×10^6 transduced melanoma cells, and the cytokine profile was quantified at the indicated time points by flow cytometry (see Methods). In all three dilution series, the cytokine profiles were similar. The figure illustrates the results using 0.2×10^6 melanoma cells, where the differences generally were most pronounced. The results were reproduced in two independent experiments.

located in a small block encompassing exon 5 and 6, and only very limited LD seems to extend from this block to the surrounding regions. This implies that it is not likely that the association reported is due to LD to an SNP residing outside this region. This is in accordance with the observation that no evidence of association was seen for the Ala304Thr in exon 8. However, it is possible that other *CD86* susceptibility variants, such as rare coding variants or intronic SNPs, might exist, which were not targeted in our screening procedure. Moreover, the region showing positive linkage is broad, and the maximum peaks vary between studies and between the specific atopy phenotypes investigated,[7–10] indicating that there might be two or more susceptibility genes located in this region. In particular, the maximum rhinitis peak recently reported by our group is located closer to the centromere at 3q13.31,[10] and rhinitis was the phenotype showing the least evidence of association in the present study, again suggesting that there might be another susceptibility gene in a more centromeric location in the region.

The three-dimensional structure of the CD86 protein has not been resolved. To investigate the structural consequences of the Ile179Val substitution, we therefore turned to the known structure of the homologous CD80.[42] However, it is difficult to predict the effect of Ile179Val on the structure/function relationships, as comparison of the amino acid sequences of CD86 and CD80 revealed low homology in the region harbouring the Ile179Val codon. On the other hand, as CD86 Ile179 and Val179 proteins were expressed in equal amounts on the cell surface (figure 2), we hypothesise that the Ile179Val SNP may influence the interaction with CD28 or CTLA-4 by affecting the conformation or assembly of CD86 rather than protein synthesis, trafficking or membrane embedding.

It is known that Th1 cells secrete IFNγ, TNFα, IL-2, IL-12 and IL-18. These cytokines help to clear invading bacteria and viruses by activating macrophages and cytotoxic T cells, and Th1 cells may dampen allergic inflammation.[11] In contrast, Th2 cells produce IL-4, IL-5, IL-9 and IL-13, which promote allergic inflammation and stimulate B cells to generate antibodies.[43] The two subgroups of T cells develop in response to various stimuli, including cytokines. Interestingly, a Th1/Th2 counter-regulation between these groups has recently been described, with each cell population able to influence the development induced by the other.[19] Although the possible role of costimulatory B7 molecules in the regulation of Th1/Th2 development is not clear, accumulating evidence suggest a role of CD86 in regulation of the Th1/Th2 cytokine balance *in vivo*.[20–24 44] Furthermore, it has been found that the Th1/Th2 regulation seems to depend on the nature of the APC, emphasising the role of CD86 for Th2 priming by both macrophages and dendritic cells.[45] The second finding of this study is that the production of both Th1 and Th2 cytokines triggered by melanoma cells expressing the overtransmitted CD86 Ile179 variant was lower than that of melanoma cells expressing the undertransmitted Val179 variant (figure 4). Having established that the clear difference in the evoked cytokine production between the two variants is mediated by equal expression of CD86 Ile179 and Val179, we suggest that the Ile179Val polymorphism has a functional effect on cytokine production. However, from this study we cannot predict whether the alleles predominantly lead to a Th1 or Th2 polarised response *in vivo*. As the Th1/Th2 balance is a pivotal factor in the development and aetiology of asthma and atopy, we hypothesise that the increased disease risk associated with the Ile179 variant and the protective effect of the Val179 variant might be mediated through a differential effect on the Th1/Th2 balance.

In conclusion, we suggest that the *CD86* gene and specifically the Ile179Val polymorphism may influence susceptibility to asthma and related allergic disorders.

ACKNOWLEDGEMENTS

The work was supported by grants from the Hørslev Foundation, The Lundbeck Foundation, The Danish Lung Association, Aarhus University Research Foundation, The Augustinus Foundation and The Danish Medical Research Council. We thank Tina F. Hindkjær and Hanne Jacobsen for excellent laboratory work. Professor Poul Nissen is thanked for fruitful scientific discussions regarding the structural considerations.

Authors' affiliations

Thomas Juhl Corydon, Annette Haagerup, Thomas Gryesten Jensen, Helle Glud Binderup, Keld Kaltoft, Anders Dupont Børglum, Institute of Human Genetics, the Bartholin Building, University of Aarhus, Aarhus C, Denmark
Annette Haagerup, Department of Paediatrics, Viborg Sygehus, Viborg, Denmark
Thomas Gryesten Jensen, The Kennedy Institute, Glostrup, Denmark
Mikkel Steen Petersen, Institute of Medical Microbiology and Immunology, University of Aarhus, Aarhus C, Denmark
Jørgen Vestbo, Institute of Preventive Medicine, Kommunehospitalet, Copenhagen, Denmark and North West Lung Centre, Wythenshawe Hospital, Manchester, UK
Torben Arvid Kruse, Department of Clinical Biochemistry and Genetics, Odense University Hospital, University of Southern Denmark, Odense C, Denmark

Competing interests: None declared.

REFERENCES

1 **Cookson WO**, Moffatt MF. Genetics of asthma and allergic disease. *Hum Mol Genet* 2000;**9**:2359–64.

2 **Mortz CG**, Lauritsen JM, Bindslev-Jensen C andersen KE. Prevalence of atopic dermatitis, asthma, allergic rhinitis and hand and contact dermatitis in adolescents. The Odense Adolescence Cohort Study on Atopic Diseases and Dermatitis. *Br J Dermatol* 2001;**144**:523–32.
3 **Skadhauge LR**, Christensen K, Kyvik KO, Sigsgaard T. Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. *Eur Respir J* 1999;**13**:8–14.
4 **Palmer LJ**, Burton PR, James AL, Musk AW, Cookson WO. Familial aggregation and heritability of asthma-associated quantitative traits in a population-based sample of nuclear families. *Eur J Hum Genet* 2000;**8**:853–60.
5 **Hanson B**, McGue M, Roitman-Johnson B, Segal NL, Bouchard TJ Jr, Blumenthal MN. Atopic disease and immunoglobulin E in twins reared apart and together. *Am J Hum Genet* 1991;**48**:873–9.
6 **Lee YA**, Wahn U, Kehrt R, Tarani L, Businco L, Gustafsson D andersson F, Oranje AP, Wolkertstorfer A, v Berg A, Hoffmann U, Kuster W, Wienker T, Ruschendorf F, Reis A. A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. *Nat Genet* 2000;**26**:470–3.
7 **Haagerup A**, Bjerke T, Schiotz PO, Binderup HG, Dahl R, Kruse TA. Asthma and atopy - a total genome scan for susceptibility genes. *Allergy* 2002;**57**:680–6.
8 **Haagerup A**, Borglum AD, Binderup HG, Kruse TA. Fine-scale mapping of type I allergy candidate loci suggests central susceptibility genes on chromosomes 3q, 4q and Xp. *Allergy* 2004;**59**:88–94.
9 **Kurz T**, Altmueller J, Strauch K, Ruschendorf F, Heinzmann A, Moffatt MF, Cookson WO, Inacio F, Nurnberg P, Stassen HH, Deichmann KA. A genome-wide screen on the genetics of atopy in a multiethnic European population reveals a major atopy locus on chromosome 3q21.3. *Allergy* 2005;**60**:192–9.
10 **Brasch-Andersen C**, Haagerup A, Borglum AD, Vestbo J, Kruse TA. Highly significant linkage to chromosome 3q13.31 for rhinitis and related allergic diseases. *J Med Genet* 2006;**43**:e10.
11 **Roitt I**, Brostoff J, Male D. *Immunology*, 6th ed. London: Mosby, 2001.
12 **Chambers CA**, Allison JP. Co-stimulation in T cell responses. *Curr Opin Immunol* 1997;**9**:396–404.
13 **Krummel MF**, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 1996;**183**:2533–40.
14 **Waterhouse P**, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 1995;**270**:985–8.
15 **Umetsu DT**, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002;**3**:715–20.
16 **Horak E**, Murr C, Streif W, Schroecksnadel K, Schennach H, Fuchs D. Association between neopterin in cord blood, urinary neopterin in early childhood and the development of atopic dermatitis, asthma and hay fever. *Pediatr Allergy Immunol* 2006;**17**:11–16.
17 **Romagnani S**. The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000;**105**:399–408.
18 **Chen YQ**, Shi HZ. CD28/CTLA-4--CD80/CD86 and ICOS--B7RP-1 costimulatory pathway in bronchial asthma. *Allergy* 2006;**61**:15–26.
19 **Elias JA**, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;**111**:291–7.
20 **Natesan M**, Razi-Wolf Z, Reiser H. Costimulation of IL-4 production by murine B7-1 and B7-2 molecules. *J Immunol* 1996;**156**:2783–91.
21 **Kuchroo VK**, Das MP, Brown JA, Ranger AM, Zamvil SS, Sobel RA, Weiner HL, Nabavi N, Glimcher LH. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 1995;**80**:707–18.
22 **Jirapongsananuruk O**, Hofer MF, Trumble AE, Norris DA, Leung DY. Enhanced expression of B7. 2 (CD86) in patients with atopic dermatitis: a potential role in the modulation of IgE synthesis, *J Immunol* 1998;**160**:4622–7.
23 **Cazzola M**, Polosa R. Anti-TNF-alpha and Th1 cytokine-directed therapies for the treatment of asthma. *Curr Opin Allergy Clin Immunol* 2006;**6**:43–50.
24 **Bashian GG**, Braun CM, Huang SK, Kagey-Sobotka A, Lichtenstein LM, Essayan DM. Differential regulation of human, antigen-specific Th1 and Th2 responses by the B-7 homologues, CD80 and CD86. *Am J Respir Cell Mol Biol* 1997;**17**:235–42.
25 **Jellis CL**, Wang SS, Rennert P, Borriello F, Sharpe AH, Green NR, Gray GS. Genomic organization of the gene coding for the costimulatory human B-lymphocyte antigen B7-2 (CD86). *Immunogenetics* 1995;**42**:85–9.
26 **Vestbo J**, Thomas W, the ASTHMAGEN group. Asthma and genes in a Danish population: Outline of an on-going study. *Eur Respir Rev* 2000;**10**:396–9.
27 **Christensen U**, Haagerup A, Binderup HG, Vestbo J, Kruse TA, Borglum AD. Family-based association analysis of the IL2 and IL15 genes in allergic disorders. *Eur J Hum Genet* 2006;**14**:227–35.
28 **Yan K**, Salome C, Woolcock AJ. Rapid method for measurement of bronchial responsiveness. *Thorax* 1983;**38**:760–5.
29 **Sobel E**, Lange K. Descent graphs in pedigree analysis: applications to haplotyping, location scores and marker-sharing statistics. *Am J Hum Genet* 1996;**58**:1323–37.
30 **Abecasis GR**, Cookson WO. GOLD—graphical overview of linkage disequilibrium. *Bioinformatics* 2000;**16**:182–3.
31 **Spielman RS**, Ewens WJ. A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test. *Am J Hum Genet* 1998;**62**:450–8.
32 **Kaltoft K**, Hansen BH, Pedersen CB, Pedersen S, Thestrup-Pedersen K. Common clonal chromosome aberrations in cytokine-dependent continuous human T-lymphocyte cell lines. *Cancer Genet Cytogenet* 1995;**85**:68–71.
33 **Friedrich U**, Houman M, Hansen BH, Kaltoft K. Microdissection and reverse painting in a melanoma cell line: a detailed description of structurally abnormal chromosomes. *Cancer Genet Cytogenet* 2001;**125**:5–9.
34 **Morgenstern JP**, Land H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* 1990;**18**:3587–96.
35 **Jensen TG**, Sullivan DM, Morgan RA, Taichman LB, Nussenblatt RB, Blaese RM, Csaky KG. Retrovirus-mediated gene transfer of ornithine-delta-aminotransferase into keratinocytes from gyrate atrophy patients. *Hum Gene Ther* 1997;**8**:2125–32.
36 **Corydon TJ**, Bross P, Holst HU, Neve S, Kristiansen K, Gregersen N, Bolund L. A human homologue of Escherichia coli ClpP caseinolytic protease: recombinant expression, intracellular processing and subcellular localization. *Biochem J* 1998;**331**:309–16.
37 **Townsend SE**, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* 1993;**259**:368–70.
38 **Chamberlain RS**, Carroll MW, Bronte V, Hwu P, Warren S, Yang JC, Nishimura M, Moss B, Rosenberg SA, Restifo NP. Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. *Cancer Res* 1996;**56**:2832–6.
39 **Marti WR**, Zajac P, Spagnoli G, Heberer M, Oertli D. Nonreplicating recombinant vaccinia virus encoding human B-7 molecules elicits effective costimulation of naive and memory CD4+ T lymphocytes in vitro. *Cell Immunol* 1997;**179**:146–52.
40 **Sad S**, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity* 1995;**2**:271–9.
41 **Muczynski KA anderson SK**, Pious D. Discoordinate surface expression of IFN-gamma-induced HLA class II proteins in nonprofessional antigen-presenting cells with absence of DM and class II colocalization. *J Immunol* 1998;**160**:3207–16.
42 **Stamper CC**, Zhang Y, Tobin JF, Erbe DV, Ikemizu S, Davis SJ, Stahl ML, Seehra J, Somers WS, Mosyak L. Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. *Nature* 2001;**410**:608–11.
43 **Mosmann TR**, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;**7**:145–73.
44 **Ip WK**, Wong CK, Leung TF, Lam CW. Plasma concentrations of soluble CTLA-4, CD28, CD80 and CD86 costimulatory molecules reflect disease severity of acute asthma in children. *Pediatr Pulmonol* 2006;**41**:674–82.
45 **Moser M**. Regulation of Th1/Th2 development by antigen-presenting cells in vivo. *Immunobiology* 2001;**204**:551–7.