



IL-4 and IL-13 mRNA real-time PCR quantification on whole blood to assess allergic response[☆]

Annick Ocmant^a, Alain Michils^b, Liliane Schandené^a, Yannick Peignois^a,
Michel Goldman^{a,c}, Patrick Stordeur^{a,*}

^a Département d'Immunologie—Hématologie—Transfusion, Hôpital Erasme, Université Libre de Bruxelles,
Route de Lennik, 808, Brussels B-1070, Belgium

^b Département de Pneumologie, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium

^c Institute for Medical Immunology, Université Libre de Bruxelles, Aérople, 8 rue Adrienne Bolland, B-6041 Charleroi, Belgium

Received 23 March 2005; received in revised form 6 June 2005; accepted 13 June 2005

Abstract

Although routinely used in clinical practice, skin prick tests and serum specific IgE often fail to distinguish between IgE-sensitization and symptomatic IgE-mediated allergy. There is therefore a need for new laboratory tests relating allergic symptoms to the offending agent. In this way, we evaluated the diagnostic reliability of a new whole blood quantitative real-time PCR assay for IL-4 and IL-13 mRNAs. We compared the response of cat allergic patients and non-cat allergic controls upon anti-IgE and cat allergen (*Fel d1*) stimulation of whole blood. Allergen addition led to a significant increase of IL-4 and IL-13 mRNAs in allergic patients compared to non-allergic controls ($p < 0.0001$). Both cytokine mRNA levels were strongly correlated and peaked within 2 h after *Fel d1* or anti-IgE addition. This rapid increase as well as purification experiments led us to the conclusion that basophils represent an important if not the main source of both transcripts in this setting. The effect was allergen-specific since not observed when stimulating blood from cat allergic patients with birch pollen. Interestingly, we found that IFN- γ mRNA, contrary to IL-4 mRNA, reached higher levels in response to *Fel d1* with control individuals than with patients allergic to cat.

This study shows that whole blood real-time PCR is a valuable method for IL-4 and IL-13 mRNA measurement after in vitro allergen challenge. We suggest that it might be useful, complementing conventional markers, for the diagnosis and the follow-up of allergic diseases. Further assessments are required to evaluate the clinical potential of this technique.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Allergy; IgE; Real-time PCR; IL-4; IL-13

1. Introduction

The prevalence of atopic disease has been increasing for the last decades in the Western World, becoming a major problem of public health. However, the diagnosis of allergic status remains a challenging area.

Indeed, the procedures routinely used in clinical practice (i.e. skin testing and detection of serum IgE) do not allow a clear distinction between non-symptomatic IgE-sensitization and symptomatic IgE-mediated allergy although cut-off values for specific IgE levels have been determined for a few allergens [1]. Therefore new laboratory based diagnostic tests allowing the clear identification of the offending allergen are still required. Several functional tests focusing on basophils, which play a key role in IgE-mediated reactions, have been developed. The detection of mediator release (histamine,

[☆] This study was supported by a grant from “Nutripôle-Région Bruxelles-Capitale”.

* Corresponding author. Tel.: +32 2 5553862; fax: +32 2 5554499.
E-mail address: patrick.stordeur@ulb.ac.be (P. Stordeur).

leukotrienes) and cell activation markers (expression of CD63, up-regulation of CD203c) upon stimulation with allergens has been proved to be useful in selected cases [2]. As Th2 cytokines are potent actors of the atopic process, the quantification of their synthesis could be another valuable approach to investigate the response to an allergen. However, most experiments focusing on Th2 cytokine production have been performed on purified cells. To avoid such a time-consuming procedure, we developed a convenient quantitative real-time PCR test performed on whole blood. In the present study, we evaluated the diagnostic reliability of this quantitative real-time PCR assay for two Th2 cytokine mRNAs, IL-4 and IL-13, in patients selected on a clinical basis and characterized by skin tests and specific IgE measurement. IL-4 and IL-13 were analyzed because they can also be produced by the basophils, and the cat allergy model was selected because the relationship between symptoms and allergen exposure is clearly identified.

2. Results

2.1. Real-time PCR on whole blood

Preliminary experiments showed that *Fel d1* induced a rapid and transient increase of IL-4 mRNA levels, peaking 1 h after allergen addition and coming back to

basal levels 8 h after (Fig. 1). This effect was maximal at the 10 $\mu\text{g/ml}$ *Fel d1* concentration. We thus compared IL-4 mRNA levels obtained 1 h after 10 $\mu\text{g/ml}$ *Fel d1* addition with blood samples from cat allergic patients to blood samples from non-cat allergic controls. A basal production of IL-4 mRNA was found at time 0 in the same proportion between atopic and controls ($p = 0.4119$). IL-4 mRNA levels upon *Fel d1* stimulation, expressed as mRNA copy numbers normalized against β -actin and related to spontaneous levels, were significantly higher in cat allergic patients (CN of 91.2 ± 43.3) compared to controls (4.1 ± 0.8) ($p < 0.0001$). This effect was allergen dependent, as no significant difference was observed following anti-IgE stimulation (Fig. 2).

2.2. Reproducibility and specificity of the real-time PCR assay

The reproducibility of this real-time PCR technique was evaluated by repeating the complete procedure 15 times. More precisely, 15 aliquots of whole blood from one cat allergic patient were cultured in the presence of 10 $\mu\text{g/ml}$ *Fel d1* for 1 h; two additional aliquots being directly subjected to lysis to allow the CN calculation. After correction according to β -actin mRNA, the CN were calculated relatively to the non-stimulated whole blood at time 0 (mean of the two aliquots), giving an intra-run coefficient of variation (CV) of 11.7%. In order to further evaluate the intra-individual variability,

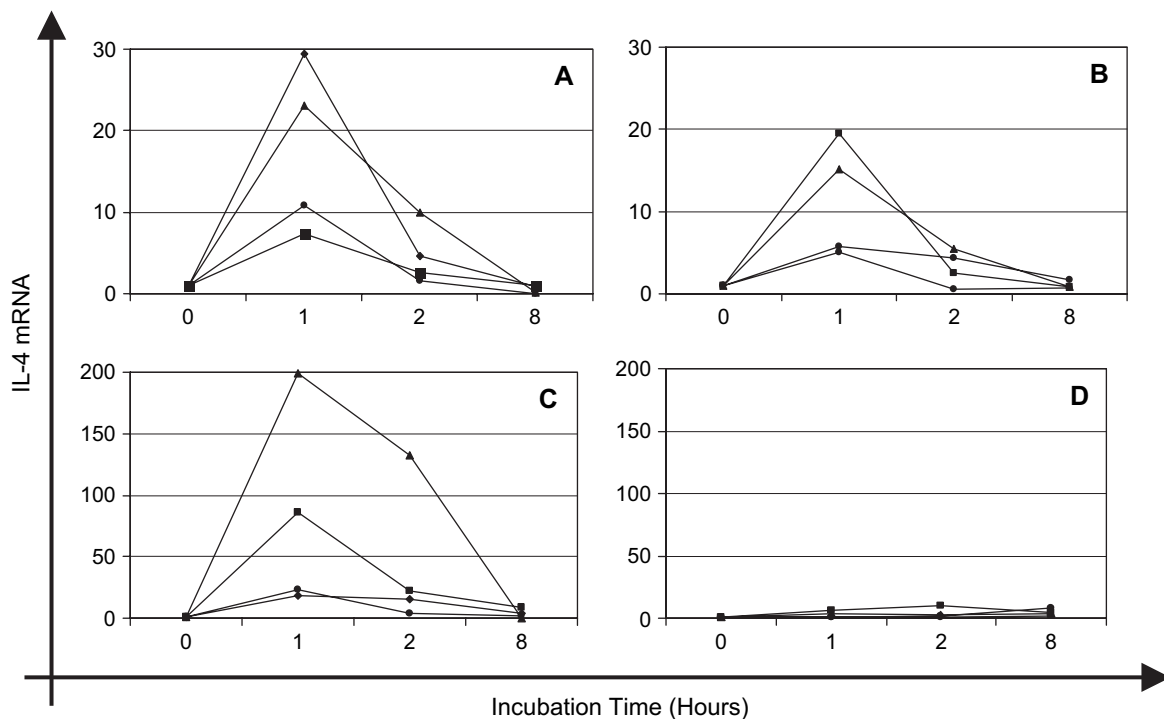


Fig. 1. Kinetics of IL-4 mRNA. Anti-IgE stimulated whole blood from (A) four cat allergic patients and (B) four controls; *Fel d1* stimulated whole blood from (C) four cat allergic patients and (D) four controls. IL-4 mRNA levels are expressed in relative copy numbers normalized against β -actin mRNA.

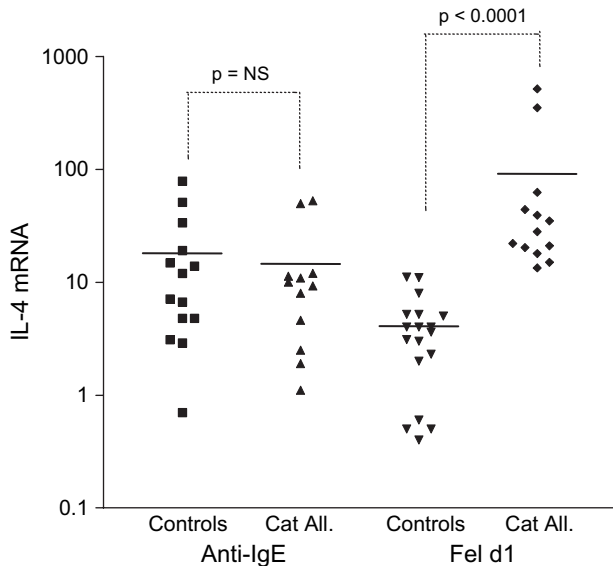


Fig. 2. Real-time PCR analysis for IL-4 mRNA after anti-IgE or *Fel d1* (10 µg/ml) stimulation of whole blood from cat allergic patients and controls. IL-4 mRNA are expressed in relative copy numbers normalized against β -actin mRNA. Bars indicate the mean.

two blood samples from six cat allergic patients were collected approximately at one year of interval. Whole blood stimulation by *Fel d1* revealed variable levels of IL-4 mRNA (Table 2), probably depending on the allergic status of the patient at the time of blood collection, but always higher than the levels found with the controls.

The antigen specificity of the cytokine response was evaluated by repeating the experiments with birch extract. Adequate experimental conditions to analyze birch allergy were preliminarily determined using blood from patients allergic to birch (data not shown). Blood from five patients allergic to cat but not to birch were challenged with this “non-relevant” allergen. The induction of IL-4 mRNA was clearly observed after allergen-specific activation (*Fel d1*) but not after birch extract challenge (Fig. 3).

2.3. IL-4 mRNA is increased in basophils

The rapid induction of IL-4 mRNA (i.e. peaking at 1 h) after allergen addition and the similar kinetic observed after *Fel d1* and anti-IgE addition (Fig. 1) suggested that the transcript was enhanced in basophils. Basophil purification and depletion experiments indeed confirmed that these cells represent an important source of IL-4 mRNA in this setting (Fig. 4).

2.4. Correlation between IL-4 and IL-13 mRNA levels on whole blood

Similar experiments performed for IL-13 mRNA quantification revealed similar kinetic pattern compared

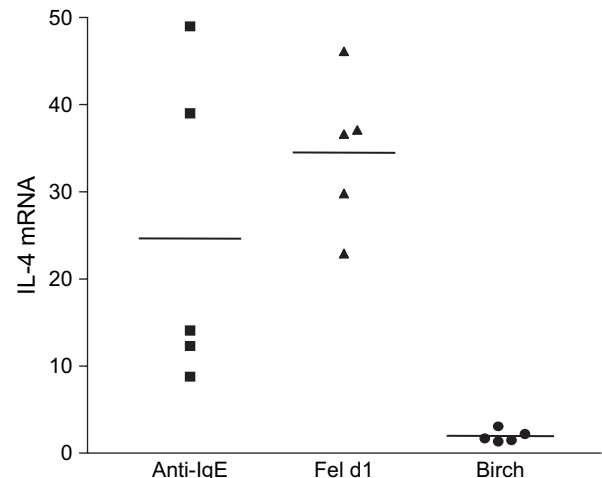


Fig. 3. Comparison of IL-4 mRNA response after anti-IgE, *Fel d1* or Birch extract stimulation in five patients allergic to cat but not allergic to birch. IL-4 mRNA is expressed in relative copy numbers normalized against β -actin mRNA. Bars indicate the mean.

to IL-4 mRNA, with a slightly delayed maximal increase (peak was at 2 h) and more sustained levels after 8 h of stimulation (Fig. 5). Moreover, IL-13 mRNA levels in response to *Fel d1* were also significantly higher with allergic patients compared to controls (CN of 3157 ± 990 and 265 ± 68 respectively, $p < 0.0001$). In fact, IL-13 and IL-4 mRNAs increased together as shown by a significant correlation analysis between the two transcript levels ($r = 0.91$, $p < 0.0001$) (Fig. 6).

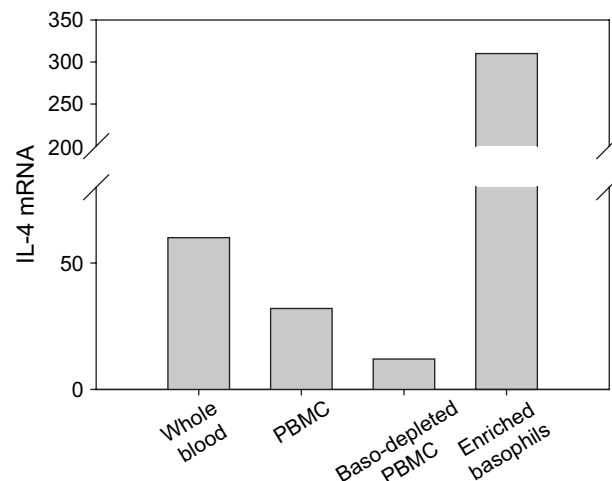


Fig. 4. IL-4 mRNA is increased mainly in basophils after *Fel d1* stimulation. IL-4 mRNA quantification was performed, after *Fel d1* stimulation, on different fractions (whole blood, PBMC, basophil-depleted PBMC and enriched basophils) obtained from cat allergic patients. A representative experiment out of three is shown. Results are expressed as mRNA CN corrected to non-stimulated whole blood at time 0.

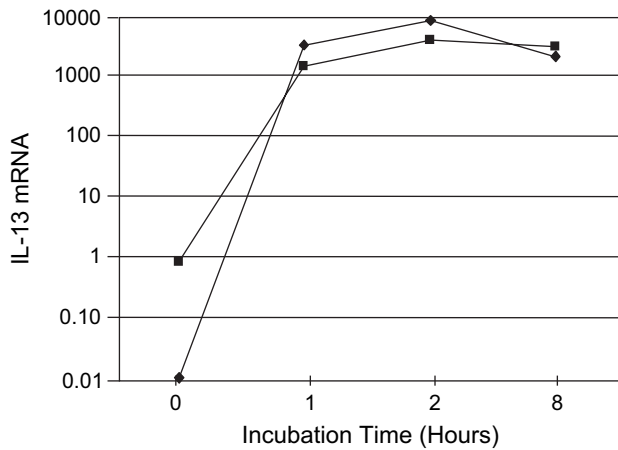


Fig. 5. Kinetics of IL-13 mRNA after anti *Fel d1*-stimulated whole blood from two cat allergic patients. IL-13 mRNA is expressed in absolute copy numbers per million of β -actin mRNA copies.

2.5. Real-time PCR for IFN- γ mRNA

We further evaluated the cytokines response by analyzing IFN- γ mRNA levels in similar conditions. Time course experiments revealed that maximal response to *Fel d1* was delayed, compared to the response observed for IL-4 and IL-13 mRNAs. Peak of IFN- γ mRNA indeed appeared after 8 h of stimulation. Furthermore, anti-IgE stimulation had no effect on IFN- γ production, while specific allergen stimulation led to higher IFN- γ mRNA levels with the healthy controls (456.2 ± 190.5) compared to patients allergic to cat (56.2 ± 18.9) ($p = 0.005$) (Fig. 7).

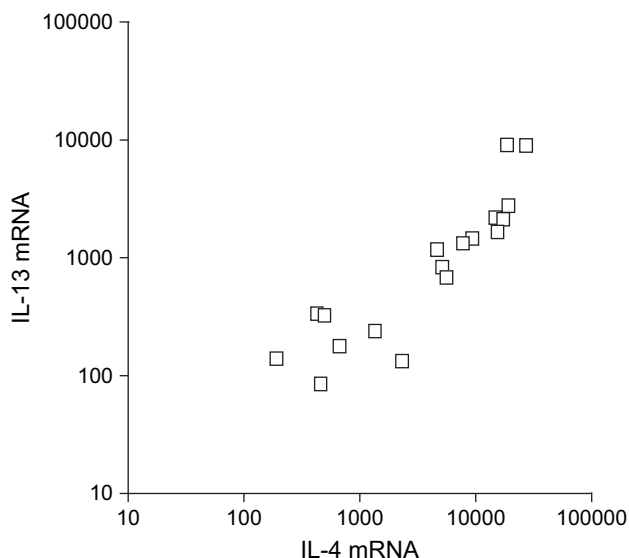


Fig. 6. Relationship between IL-4 and IL-13 mRNA levels following, respectively, 1 h and 2 h *Fel d1* (10 μ g/ml) stimulation. mRNAs are expressed in absolute copy numbers per million of β -actin mRNA copies.

3. Discussion

Current procedures used to support the clinical diagnosis of allergy (i.e. skin testing and serum IgE measurement) document IgE-sensitization rather than actual allergic status. Since basophils and mastocytes are the main effector cells responsible for release of mediators that ultimately trigger allergic symptoms, newer techniques have focused on in vitro basophil activation upon allergen challenge [2]. Basophil activation also lead to synthesis of Th2 cytokines that play a key role in the regulation of the allergic response. In the present study we evaluated the diagnostic reliability of a new real-time PCR for IL-4 and IL-13 mRNAs quantification after whole blood allergen challenge. Because of its ability to accurately quantify low levels of mRNA, real-time PCR represents a sensitive technique to monitor immune response. Notably, it has successfully been applied to evaluate cancer immunotherapy [4] and to monitor graft acceptance/rejection and GVHD in transplantation [5,6]. The technique used in this study, i.e. in vitro monitoring of immune response on whole blood using real-time PCR, was first reported for bacterial antigens [7]. Recently, Kruger et al. used a similar approach to demonstrate, between atopic and non-atopic individuals, different steady state levels of IL-1 β and IL-8 mRNAs after mould extracts stimulation of blood [8]. Performing real-time PCR on whole blood provides several advantages: first, it avoids time-consuming cell cultures; second, there is no need to purify PBMC or basophils, this step being able to either non-specifically activate the cells or modulate cytokine

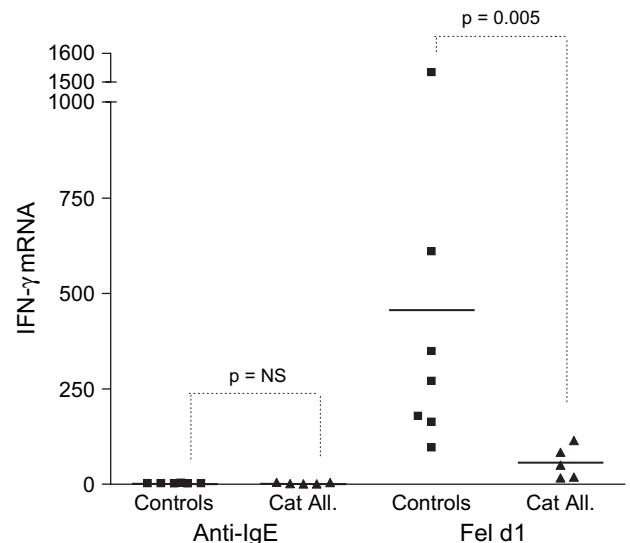


Fig. 7. Real-time PCR analysis for IFN- γ mRNA after anti-IgE or *Fel d1* stimulation of whole blood from cat allergic patients and controls. IFN- γ mRNA is expressed as copy numbers after stimulation referred to non-stimulated conditions at time 0, all normalized against β -actin mRNA. Bars indicate the mean.

mRNA levels [9,10] and finally, it allows the use of very small blood volumes.

By using this method, we demonstrated that challenging blood from cat allergic patients with the relevant antigen (i.e. *Fel d1*) led to a rapid and strong induction of IL-4 and IL-13 mRNAs. These two transcripts were indeed detected at significantly higher levels in cat allergic patients compared to healthy controls. This up-regulation was antigen-specific, since no up-regulation was observed when stimulating blood from cat allergic patients with birch pollen extract.

Despite the presence of T lymphocytes, basophils did appear to be also an important source of IL-4 mRNA detected in the *Fel d1* stimulated blood from cat allergic patients. Indeed, maximal IL-4 mRNA levels occurred as early as 1 h after allergen or anti-IgE challenge, which fits with the kinetic of antigen-induced IL-4 release by basophils (30 min to 4 h) and not with that of T cells (i.e. 12–16 h) [11,12]. Furthermore, despite a 30:1 ratio between T cells and basophils among PBMC, basophils have been shown to be responsible for at least 80% of IL-4 production [13]. Finally, the important contribution of basophils to IL-4 mRNA up-regulation following *Fel d1* addition is confirmed by our experiments on purified basophils and basophil-depleted PBMC. We thus describe here a technique that analyzes the ability of circulating basophils to respond specifically to an allergen which is therefore probably more representative of the *in vivo* status than classical specific serum IgE detection.

Real-time PCR quantification of IL-4 mRNA after allergen stimulation has yet been described [14,15], also demonstrating basal levels and allergen up-regulation of IL-4 mRNA, but in a lesser extent. This difference may result from the use of PBMC for stimulation experiments rather than whole blood and from a longer incubation time (24–96 h), suggesting a substantial contribution of T lymphocytes in IL-4 mRNA production. As far as T cell response is concerned, we also demonstrated an increase of IFN- γ mRNA after *Fel d1* addition to blood. Interestingly, the amplitude of this response was dramatically higher with healthy controls compared to cat allergic patients. In effect, the ratio [(IL-4 mRNA/IFN- γ mRNA) $\times$ 100] ranged from 16 to 243 for allergic patients versus 0.06–2.4 for the controls. These results suggest that allergen specific response is in favor of IL-4 mRNA production in cat allergic patients and of IFN- γ in controls, what is in accordance with previously published data. Indeed, Teramoto et al. demonstrated a defection of IFN- γ production by Th1-like cells from atopic patients upon treatment with mitogens, which was associated with elevated serum IgE [16]. In the same vein, in a model of rhinitis to grass pollen, non-atopic individuals were shown to exhibit high levels of IFN- γ after allergen stimulation, in contrast to what was observed among grass pollen sensitive individuals [17].

In conclusion, real-time PCR represents a new powerful tool to monitor immune responses, and provides new laboratory potential in the field of allergic diseases. The technique takes advantage of PCR sensitivity and high specificity. Indeed, we demonstrate here that allergic patients respond only to the offending allergen whereas non-allergic controls do not. This specificity is confirmed by other experiments that are currently performed in our laboratory with other allergens (peanuts, egg, and birch). Furthermore, the PCR results are in accordance with serum specific IgE, skin tests and the clinical history of our patients. These observations suggest that the real-time PCR assay could lead to a discrimination between clinically allergic and non-allergic individuals. We suggest that cytokine mRNA measurement by real-time PCR could help the diagnosis of allergic diseases, in addition to conventional parameters like specific IgE, CD63 or leukotrienes measurements. This is of particular interest in case of discrepancies between clinical history, serology and skin tests or in the field of food allergy for which serology and skin tests offer only low predictive value. Obviously, clinical additional studies are required to further confirm the ability of real-time PCR to differentiate sensitization from allergic status, and to determine its possible application in the monitoring of patients undergoing tolerance.

4. Methods

4.1. Patients and controls

Blood samples were collected from two groups of volunteers:

- Fifteen patients allergic to cat dander (mean age 31 years). All had a history of asthma or rhinitis when exposed to cat and gave positive response for cat extract (Stallergènes, Fresne, France) in the skin prick test. They were proved to be non-allergic to birch pollen (no history of symptoms during birch pollen season, negative skin test and absence of serum specific IgE towards birch pollen).
- Fifteen non-cat allergic individuals (mean age 22 years) with no history of cat allergic disease and a negative prick test to cat extract.

Cat specific IgE (CAP-fluoroenzyme immune assay, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) were positive for all allergic patients, ranging from 0.5 to 73.3 IU/ml (mean = 16.5 IU/ml) and negative (<0.35 IU/ml) for all controls.

The institutional scientific and ethics committees approved this study.

4.2. Allergens

The major purified cat dander allergen of *Felis domesticus* (*Fel d1*) and birch extract were purchased from ALK-Abello A/S (Horsholm, Denmark) and Stallergènes (Fresne, France), respectively.

4.3. Quantitative real-time PCR

Real-time PCR on whole blood has been previously described in detail [3]. Briefly, in vitro whole blood stimulation was performed on 100 µl of heparinized venous blood with allergen (*Fel d1* or birch extract) or anti-IgE antibody (1/800 dilution, Sigma Aldrich Chemic GmbH, Steinheim, Germany) at 37 °C in a 5% CO₂ atmosphere incubator. Stimulation was started within 4 h after blood collection, and stopped by adding 250 µl of the reagent contained in PAXgene™ tubes (Qiagen, Westburg, The Netherlands). This reagent induced total cell lysis and mRNA stabilization by nucleic acids precipitation. The nucleic acids pellet was then dissolved in the lysis buffer contained in the MagNA Pure™ mRNA extraction kit (Roche Applied Science, Roche Diagnostics Belgium, Vilvorde, Belgium). mRNA was extracted from 300 µl of this solution, using this kit on the MagNA Pure™ instrument (Roche Applied Science) following manufacturer's instructions ("mRNA I cells" Roche's protocol, final elution volume 100 µl). Reverse transcription and real-time PCR were performed in one step on a LightCycler instrument, following the standard procedure described in the "LightCycler – RNA Master Hybridization Probes" Kit (Roche Applied Science).

Table 1 describes PCR conditions specific to the measured mRNA cytokine, i.e. the final concentration of the primers, all other parameters being the same for all mRNA targets. After an incubation period of 20 min at 61 °C to allow mRNA reverse transcription, and then an

initial denaturation step at 95 °C for 30 s, temperature cycling was initiated. Each cycle consisted of 95 °C for 0 s and 60 °C for 20 s, the fluorescence being read at the end of this second step (F1/F2 channels, no color compensation). Forty cycles were performed, in total. For each sample, the mRNA copy number was calculated by the instrument software using the Ct value ("Arithmetic Fit point analysis") from a standard curve. This later was constructed for each PCR run from serial dilutions of a purified DNA. mRNA levels were expressed either in absolute copy numbers normalized against β-actin mRNA (cytokine mRNA copies per million of β-actin mRNA copies) or in relative copy numbers normalized against β-actin mRNA. In this latter case, a corrected cytokine mRNA copy number (CN) was calculated comparatively to the non-stimulated whole blood at time 0, all normalized against the transcripts of the housekeeping β-actin gene [corrected cytokine mRNA CN = (cytokine mRNA CN of the sample/β-actin mRNA CN of the sample) × (β-actin mRNA CN of the non-stimulated sample/cytokine mRNA CN of the non-stimulated sample)] (ΔΔCt method).

4.4. Basophil purification and depletion from PBMC

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized whole blood after Ficoll gradient. This was followed by negative selection using a MACS Basophil Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells preincubated with paramagnetic beads (anti-CD2, CD7, CD14, CD15, CD16, CD36, CD45RO and anti-HLA-DR) were applied to an LS cell-separation column mounted on a Midi MACS magnet (Miltenyi Corp). Basophils were directly collected as they passed through the column, washed and resuspended in RPMI. On the other hand, basophil-depleted PBMC were flushed out using the

Table 1
Experimental conditions for IL-4 and IL-13 mRNAs real-time PCR assay

Forward (F) and reverse (R) primers and probes (P) for RT-PCR mRNA target oligonucleotides (5' → 3') ^a		Product size (bp)	Final concentration (nM) ^b
IL-13	F155: TGAGGAGCTGGTCAACATCA R230: CAGGTTGATGCTCCATACCAT P187: 6Fam-AGGCTCCGCTCTGCAATGGC-Tamra-p	76	F900 R900
IL-4	F174: ACTTTGAACAGCCTCACAGAG R247: TTGGAGGCAGCAAAGATGTC P204: 6Fam-CTGTGCACCGAGTTGACCGTA-Tamra-p	74	F300 R900
IFN-γ	F464: CTAATTATTCGGTAACTGACTTGA R538: ACAGTTCAGCCATCACTTGGA P491: 6Fam-TCCAACGCAAAGCAATACATGAAC-Tamra-p	75	F600 R900

^a F, R and P indicate forward and reverse primers and probes, respectively; numbers indicate the sequence position from Genebank accession numbers NM_000589 for IL-4, NM_002188 for IL-13 and X13274 for IFN-γ.

^b Final concentration of forward (F) and reverse (R) primers.

Table 2

Intra-individual variability: IL-4 mRNA results (CN) after *Fel d1* stimulation obtained for two blood samples distant of approximately one year

	Sample 1	Sample 2
Patient 1	18.1	13.6
Patient 2	89.2	37.1
Patient 3	515.5	36.6
Patient 4	17.5	45.9
Patient 5	22.0	29.8
Patient 6	350.9	46.1

pluger provided with the column. Basophil content of PBMC, basophil-depleted PBMC and enriched basophil fractions were analyzed by flow cytometry after staining with anti-IgE FITC (Caltag Laboratories, Burlingame, California), anti-CD3 APC and anti-CD45 PerCP (Becton Dickinson). Basophils were defined as CD45^{dim}, CD3[−] and IgE^{high}. We found 1–1.5% of basophils in the PBMC suspension, 0.2% in the basophil-depleted fraction and 40–60% after basophil enrichment.

4.5. Statistical analysis

Results are expressed as mean $\pm$ SEM. The non-parametric Mann–Whitney test was used for comparisons, and correlations were analyzed with Spearman's rank order correlation coefficient test.

References

- [1] Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* 2001;107:891–6.
- [2] Hamilton RG, Franklin Adkinson Jr N. In vitro assays for the diagnosis of IgE-mediated disorders. *J Allergy Clin Immunol* 2004;114:213–25.
- [3] Stordeur P, Poulin LF, Craciun L, Zhou L, Schandene L, de Lavarelle A, et al. Cytokine mRNA quantification by real-time PCR. *J Immunol Methods* 2002;259:55–64. Erratum, In: *J Immunol Methods* 2002;262:229.
- [4] Mocellin S, Rossi CR, Pilati P, Nitti D, Marincola FM. Quantitative real-time PCR: a powerful ally in cancer research. *Trends Mol Med* 2003;9:189–95.
- [5] Sabek O, Dorak MT, Kotb M, Gaber AO, Gaber L. Quantitative detection of T-cell activation markers by real-time PCR in renal transplant rejection and correlation with histopathologic evaluation. *Transplantation* 2002;74:701–7.
- [6] Michalek J, Collins RH, Hill BJ, Brenchley JM, Douek DC. Identification and monitoring of graft-versus-host specific T-cell clone in stem cell transplantation. *Lancet* 2003;361:1183–5.
- [7] Stordeur P, Zhou L, Byl B, Brohet F, Burny W, de Groote D, et al. Immune monitoring in whole blood using real-time PCR. *J Immunol Methods* 2003;276:69–77.
- [8] Kruger T, Sigsgaard T, Bonefeld-Jorgensen EC. Ex vivo induction of cytokines by mould components in whole blood of atopic and non-atopic volunteers. *Cytokine* 2004;25:73–84.
- [9] Hartel C, Bein G, Muller-Steinhardt M, Kluter H. Ex vivo induction of cytokine mRNA expression in human blood samples. *J Immunol Methods* 2001;249:63–71.
- [10] Stordeur P, Zhou L, Goldman M. Analysis of spontaneous mRNA cytokine production in peripheral blood. *J Immunol Methods* 2002;261:195–7.
- [11] Gibbs BF, Haas H, Falcone FH, Albrecht C, Vollrath IB, Noll T, et al. Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur J Immunol* 1996;26:2493–8.
- [12] MacGlashan Jr D, White JM, Huang SK, Ono SJ, Schroeder JT, Lichtenstein LM. Secretion of IL-4 from human basophils. The relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J Immunol* 1994;152:3006–16.
- [13] Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J Allergy Clin Immunol* 1999;104:811–9.
- [14] Ekerfelt C, Ernerudh J, Jenmalm MC. Detection of spontaneous and antigen-induced human interleukin-4 responses in vitro: comparison of ELISPOT, a novel ELISA and real-time RT-PCR. *J Immunol Methods* 2002;260:55–67.
- [15] Laaksonen K, Waris M, Makela MJ, Terho EO, Savolainen J. In vitro kinetics of allergen- and microbe-induced IL-4 and IFN-gamma mRNA expression in PBMC of pollen-allergic patients. *Allergy* 2003;58:62–6.
- [16] Teramoto T, Fukao T, Tashita H, Inoue R, Kaneko H, Takemura M, et al. Serum IgE level is negatively correlated with the ability of peripheral mononuclear cells to produce interferon gamma (IFN-gamma): evidence of reduced expression of IFN-gamma mRNA in atopic patients. *Clin Exp Allergy* 1998;28(1): 74–82.
- [17] Imada M, Simons FE, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon-gamma production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunology* 1995;85(3):373–80.