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Full Length Article

Increased IL-5 and IL-13 cytokine level in *ex vivo* stimulated whole blood cells from grass pollen allergic donors correlate with seasonal exposure

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ABSTRACT

There is a need for simple and physiological assays to characterize the immune status of allergic individuals. Whole blood samples from 15 adult subjects (10 with positive clinical history to grass pollen and 5 with negative clinical history) were obtained before the start (April 2010) and during the middle of the grass pollen season (June 2010). The investigators were blinded to the allergic status of the subjects. A skin prick test (SPT) to grass pollen was carried out at the end of the study. Cytokines (IL-5, IL-13, IL-10 and IFNγ) and activation of T-lymphocytes were determined after *ex vivo* culture of whole blood cells. IL-5, IL-10 and IL-13 cytokines were significantly elevated in allergic individuals during the middle of the season ($p \leq 0.02$) compared to the start. This assay can be a valuable tool in clinical trials especially in pediatric population where limited quantities of blood are available to study immune responses.

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1. Introduction

Allergies are now a global health problem and affect 1 in every 5 individuals in the developed nations [11,12,23]. Depending on the site of exposure and the sensitizing allergen the symptoms can manifest in different anatomical locations and lead to skin, food or respiratory allergy (rhinitis and asthma). Allergic reactions are mediated by an immune bias towards a Th-2 phenotype. This bias in the immune system is brought about by an increase in Th-2 cytokines (Interleukin-4, -5, -13) levels in allergy prone individuals [5,22]. However, it is not well understood how the levels of these Th-2 cytokines fluctuate during seasonal exposure to the allergen [1] and correlate to allergic symptoms. Among the different forms of allergies, respiratory allergies (allergic rhinitis and allergic asthma) are the most common and account for considerable morbidity both in children and in adults [7,11,13,14]. Allergic rhinitis affects the upper airways and common symptoms associated with the disorder include runny and blocked nose, sneezing and nasal itching. Most subjects also present with ocular symptoms (allergic rhino-conjunctivitis) [2,3,27].

Allergic rhinitis can be classified into seasonal and perennial. This classification depends on the causative environmental allergen. Seasonal allergies (hay-fever) typically occur in the spring and summer season and the main causative allergens are pollen from grass and trees [2,6]. The majority of clinical trials conducted in seasonal allergy are performed in artificial settings (nasal provocation tests and aerosol chambers) because of the limited duration of the pollen season to a few months in a year. These studies have been designed to assess the efficacy of anti-allergy medications such as anti-histaminic drugs and corticosteroids. More often than not, a relief in symptomatic parameters was not paralleled by a reduction in the levels of Th-2 cytokines in these well designed clinical trials [8,15,21]. We have sought to study the levels of different immunological parameters in a “field” trial setting, i.e. within the pollen season (at the start and middle of pollen season). Our hypothesis was that the levels of Th-2 cytokines fluctuate in grass pollen allergic individuals with the severity of the grass pollen exposure during the course of the season and that this could be visualized via the whole blood assay. Based on the cytokine profile we could then predict the allergic status of the participating subjects.

2. Patients and methods

2.1. Subjects and design of study

The protocol was submitted to and accepted by the Ethics Commission of the Hospital of Lausanne, Switzerland (“Commission Cantonale d’Ethique pour la Recherché sur l’Etre Humain”,

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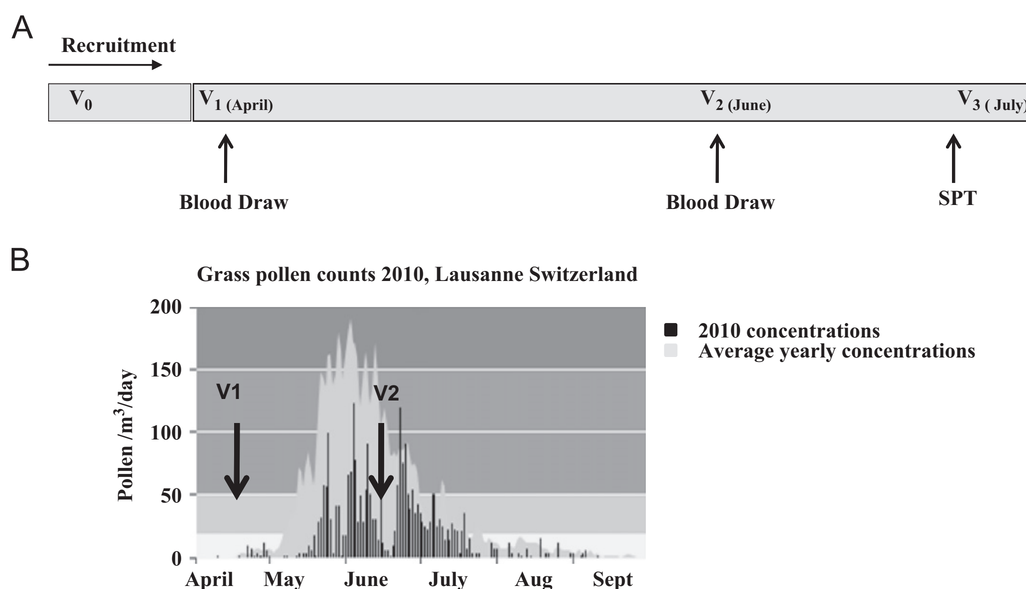

Fig. 1. Protocol for the clinical study with whole blood assay in grass pollen allergics. (A) 2 visits for blood drawing once subjects were included in the protocol. First visit (V1) was scheduled at the start of the grass pollen season (April 2010) and second visit (V2) in the middle of the pollen season (June 2010). (B) The pollen count in the city of Lausanne coincided with the 2 visits, i.e. in April little pollen exposure (outside season) and in June the middle of the pollen exposure (inside season) was observed. SPT to a grass pollen mix was carried out at the end of the study (V3).

Lausanne, Switzerland) under the reference 63/10. The study was performed at the Metabolic Unit facility at Nestlé Research Center (Study no. 10.02.MET), Lausanne, Switzerland between March and August 2010. Fifteen adult subjects aged 20–50 years who gave informed consent were recruited based on their clinical history (10 with positive clinical history and 5 with negative clinical history) of having allergic symptoms to grass pollen in the season (baseline visit, V0). Exclusion criteria included subjects with a history of anemia, regularly taking anti-allergy medications, pregnancy, common cold (flu-like) symptoms in the last month and subjects currently participating or having participated in another clinical trial. Two visits for 10 mL blood draws were scheduled (Fig. 1A), first visit at the start of the grass pollen season (V1, April 2010) and second visit at the middle of the season (V2, June 2010). The pollen counts in the city of Lausanne coincided with the 2 visits, i.e. in April little pollen exposure, and in June consistent pollen exposure were observed (Fig. 1B). A skin prick test (SPT) to a mix of grass pollen (SOLUPRICK SQ, 6-grass mix, ALK-Albello AG, Horsholm, Denmark) was conducted at the end of the clinical study (V3, July–August 2010). Saline and histamine were used as negative and positive controls, respectively. Wheal and flare responses were observed for 15 min and a final recording was done. Individuals with a mean wheal diameter >3 mm were considered positive to the grass pollen allergen. For all the analysis of the results, we considered subjects with a positive SPT as true allergic subjects and those with a negative SPT as non-allergic subjects. Thirteen subjects who had a positive SPT to grass pollen at the end of the study were included in the final analysis.

2.2. Whole blood assay

Heparinized, venous blood (10 mL) from male and female adults (20–50 years old) with a positive clinical history to grass pollen or no allergy (no clinical history) was collected both at the start and middle of the pollen season. Whole blood cells were cultured for 120 h (5 days) in a 1:5 dilution (100 μL of whole blood + 400 μL of culture media) in triplicate wells with culture medium RPMI (Sigma) complemented with 1% L-glutamine, 1% Penicillin/Streptomycin, 1% of non-essential amino acids (Invitrogen, Lucerne, Switzerland), and 0.1% Gentamycin (Sigma). The cultures were carried out in 48 wells plate (Milian, Meyrin, Switzerland) either with no stimulation (medium alone) or in the presence of different stimuli, mainly anti-CD2 at a concentration of 2 μg/mL of each clone (Sanquin, Amsterdam, Netherlands; Clones: CLB-T11.1/1, CLB-T11.1/2) and anti-CD28 at a concentration of 4 μg/mL (Sanquin, Amsterdam, Netherlands; Clone: CLB-CD28/1). For allergen-specific stimulation, a 6-grass pollen mix extract (ALK-Albello AG, Horsholm, Denmark) was used where the lyophilized extract (450 000 SQ/vial) was re-suspended in RPMI and used at 100 μg/mL final concentration. Cell supernatants were collected from the triplicate wells and stored at −20 °C until analysis. For cytokine kinetics in the whole blood culture supernatants in both un-stimulated and stimulated culture conditions, the cultures were carried out for 48, 72 and 96 h.

2.3. Flow cytometry and cytokine analysis

The centrifuged whole blood cell pellets were collected after the 5-day culture from the triplicate wells, subjected to lysis of red blood cells (RBC's) and stained first with cell surface and then with intracellular fluorochrome conjugated monoclonal antibodies (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The samples were analyzed via a 4 color FACS Calibur flow cytometer (BD, San Jose, CA, USA) for immune markers (CD3, CD4 and CD25). Cytokines in the supernatant (IL-5, IL-10, IFNγ and IL-13) were measured by a human MESOSCALE kit (MesoScale Discovery®, Gaithersburg, MD, USA).

2.4. Statistical analyses

Data is expressed as arithmetic mean ± standard error of the mean (SEM). Paired *t*-test values of different cytokine levels at the two visits were compared with Wilcoxon signed rank test for paired observations, and the Mann–Whitney test, respectively, using the GraphPad Prism 5 software (La Jolla, CA, USA). A difference of $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Comparison of whole blood assay with SPT

Level of allergy related Th-2 cytokines (IL-5 and IL-13) were measured in *ex vivo* stimulated whole blood cells both before the start (V1, April 2010) and during the middle of the pollen season (V2, June 2010). By comparing the level of Th-2 cytokines in the whole blood assay of the 15 subjects, we identified 11 subjects in the study as allergic subjects (subjects with >30% increase in IL-5 and IL-13 level at V2 when compared to V1 visit). SPT to a grass pollen mix was carried out at the end of this single blinded study (V3, July 2010), which revealed 13 subjects to be sensitized to grass pollen. When compared to SPT, the whole blood assay had positive predictive value (PPV)=100% (Table 1). This means that subjects which had high level of the Th-2 cytokines ($n=11$) also tested positive for SPT to grass pollen. However, the whole blood assay had a negative predictive value (NPV) of 50%, which indicates that out of the subjects identified as negative by the whole blood assay ($n=4$), 2 subjects still tested as sensitized to grass pollen.

3.2. Kinetics of Th-2 cytokines IL-5 and IL-13 in ex vivo stimulated whole blood cells from allergic subjects

To obtain the best conditions for stimulation in the whole blood assay for our clinical study, we performed before the start of the clinical study a kinetic response of allergy related cytokines (IL-5 and IL-13) in the whole blood assay for different time points, i.e. 48, 72, 96 h. Compared to un-stimulated conditions, IL-5 levels started peaking in stimulated conditions after 72 h of culture of whole blood cells (Fig. 2A). For IL-13 levels, 96 h of culture of whole blood cells in stimulated conditions was necessary to differentiate from un-stimulated conditions (Fig. 2B). For all donors in the study, we chose to set whole blood assays for 120 h (5 days) in un-stimulated and stimulated conditions to get a comprehensive view of their cytokine response and also to be able to account for donor to donor variability.

Table 1

	SPT+	SPT−	Total
Whole blood+	**11 (TP)**	**0 (FP)**	**11**
Whole blood−	**2 (FN)**	**2 (TN)**	**4**
Total	**13**	**2**	**15**

PPV=100%.
NPV=50%.
Subjects with high levels of IL-5 and IL-13 cytokines in the whole blood assay (subjects with >30% increase in IL-5 and IL-13 levels at V2 when compared to V1 visit) were considered as whole blood positive (+) and those with no increase between the visits as whole blood negative (−). Subjects with wheal diameter >3 mm on SPT with a grass pollen mix were considered SPT+ and those with no wheal response or wheal diameter <3 mm were taken as SPT−.
Positive predictive value (PPV) was calculated by the formula True Positive (TP)/True Positive (TP)+False Positive (FP).
Negative predictive value (NPV) was calculated by the formula True Negative (TN)/True Negative (TN)+False Negative (FN).

3.3. Increased level of Th-2 cytokines, IL-5 and IL-13 in ex vivo stimulated whole blood cells between V1 and V2 visits in allergic subjects

Whole blood cells obtained at V1 and V2 were stimulated *ex vivo* with or without T-cell mitogens (anti-CD2+anti-CD28). The level of Th-2 cytokines IL-5 and IL-13 were significantly increased ($p=0.001$) in the supernatant of stimulated whole blood cells (Fig. 3A and B) at V2 (middle of the grass pollen season, June 2010) when compared to V1 (start of the grass pollen season, April 2010). No changes in un-stimulated conditions were detected. There were also, no differences in the level of the Th-1 cytokine IFNγ (Fig. 3C) between the two visits. Interestingly, levels of the immune-regulatory cytokine IL-10 (Fig. 3D) were also increased ($p=0.03$). These results suggest that with continued exposure towards the peak of the pollen season there is a shift in the immune system towards a Th-2 bias that can be detected in *ex vivo* stimulated whole blood cells.

3.4. Whole blood assay as a tool to study allergen-specific ex vivo immune responses

We stimulated whole blood cells collected at visit V2 with a mixture of 6-grass pollen extracts *ex vivo*. Grass pollen stimulation alone was not sufficient to induce IL-5, IL-13 and IFNγ levels, but it stimulated IL-10 production. Stimulation with a combination of grass pollen and IL-2 led to secretion of IL-5, IL-13, IFNγ and IL-10 cytokines in the whole blood assay. However, IL-2 alone was sufficient to stimulate IL-5 and IL-13 while the induction of IL-10 and IFNγ was not modulated (Fig. 4A–D).

4. Discussion

Previous studies have typically relied on measuring immune responses in allergic individuals from isolated peripheral blood

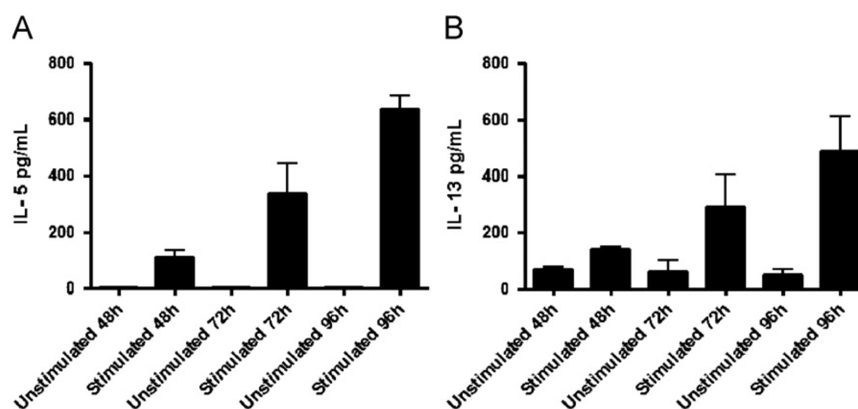

Fig. 2. Kinetics of Th-2 cytokines IL-5 and IL-13 in *ex vivo* whole blood assay. To optimize culture conditions of whole blood cells before the start of the study, levels of Th-2 cytokines IL-5 (A) and IL-13 (B) were compared in whole blood cells from 2 grass pollen allergic donors between un-stimulated and stimulated conditions at different time points (48, 72, 96 h). Under stimulated conditions, the levels of the cytokines peaked in the whole blood assay typically at 96 h of culture, whereas no changes in un-stimulated conditions were detected.

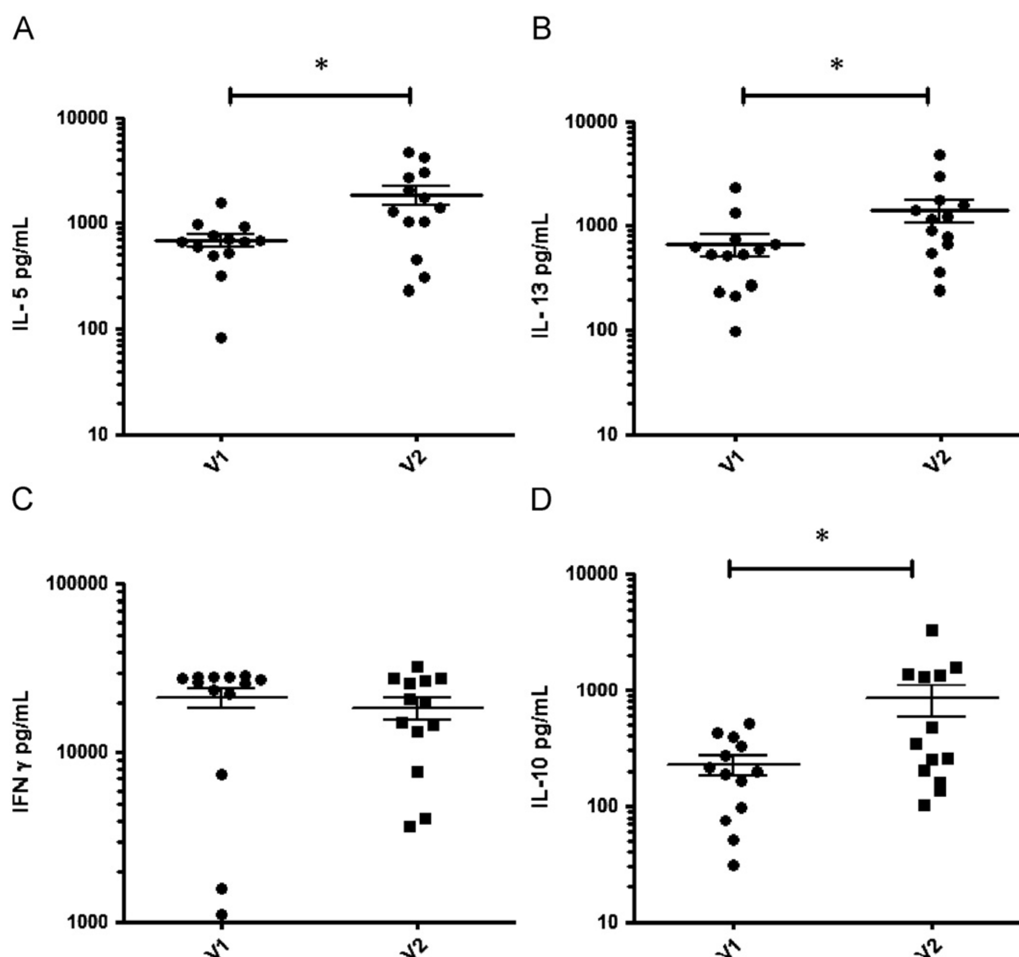

Fig. 3. Increased Th-2 cytokine level (IL-5 and IL-13) in *ex vivo* stimulated whole blood cells at V2 compared to V1 in allergic subjects. Levels of allergy related Th-2 cytokines (IL-5 and IL-13), Th-1 cytokine (IFNγ) and the immune-regulatory cytokine (IL-10) were measured in *ex vivo* stimulated whole blood cells both before the start (April 2010) and during the middle of the pollen season (June 2010). The levels of Th-2 cytokines IL-5 and IL-13 were significantly increased ($p=0.001$) in the supernatant of stimulated whole blood cells during V2 (middle of grass pollen season) when compared to the V1 (start of the season). There were no differences in the levels of the Th-1 cytokine IFNγ between the visits. Levels of the immune-regulatory cytokine IL-10 were also increased ($p=0.03$).

mononuclear cells (PBMCs). In these studies, the levels of Th-2 cytokines (IL-4, IL-5 and IL-13) following polyclonal and allergen-specific stimulation of PBMCs *ex vivo* has been measured [10,16–18,29]. Isolating PBMCs and their subsequent *ex vivo* culture is long and tedious and requires working with large quantities of blood samples in order to obtain sufficient PBMCs to study immune responses. In the current study, we have investigated the feasibility of setting up a fast and practical assay that allows studying Th-2 specific immune responses in allergic individuals in a field setting via small amounts of whole blood.

Fifteen subjects with ($n=10$) or without ($n=5$) a known clinical history to grass pollen allergy were recruited. During seasonal exposure it is well known that fluctuations exist in pollen counts in the environment (depending on other environmental factors such as humidity and temperature) and the goal was to schedule the two visits to coincide with baseline (V1, before the start of the pollen season) and V2 (in the middle of the season) for the study (Fig. 1A). An important point to consider for the whole blood study is that it was single blinded in order to enable the investigators to identify allergic subjects. Before the start of the study, we identified optimal conditions for the whole blood assay for analyzing Th-2 cytokines (Fig. 2). Comparing Th-2 cytokines under stimulated conditions between the visits V1 (start of the grass pollen season, April 2010) and V2 (middle of the grass pollen season, June 2010) we identified 11 out of the 15 subjects recruited as potential allergic subjects (subjects with $>30\%$ increase in IL-5 and IL-13 levels at V2 when compared to V1 visit). These subjects were considered as whole blood positive. When whole blood positive subjects were compared to SPT positive subjects at the end of the study (V3, July 2010), the whole blood assay had a positive predictive value (PPV)=100% (Table 1). This signifies that subjects in the study who had high levels of Th-2 cytokines (IL-5 and IL-13) at V2 compared to V1, also tested positive for SPT to grass pollen ($n=11$). Interestingly, three out of the five subjects in our study who were recruited with no previous clinical history to grass pollen were SPT positive to grass pollen and 1 was whole blood positive. These findings are intriguing and suggest that a substantial number of sensitized or atopic subjects could be unaware of their allergic status and remain undiagnosed in the general population. However, these findings need to be further verified in a larger cohort of non-allergic subjects.

While diagnostic tests such as SPT and measurement of specific IgE levels (RAST, UniCAP) exist in the clinic today, these tests only predict the levels of sensitization specific to the allergen [21,23,24] and do not evaluate Th-2 cytokine level in allergic subjects. We propose that an easy to implement assay such as the whole blood assay that is both immunologically and physiologically relevant could routinely be employed in a clinical

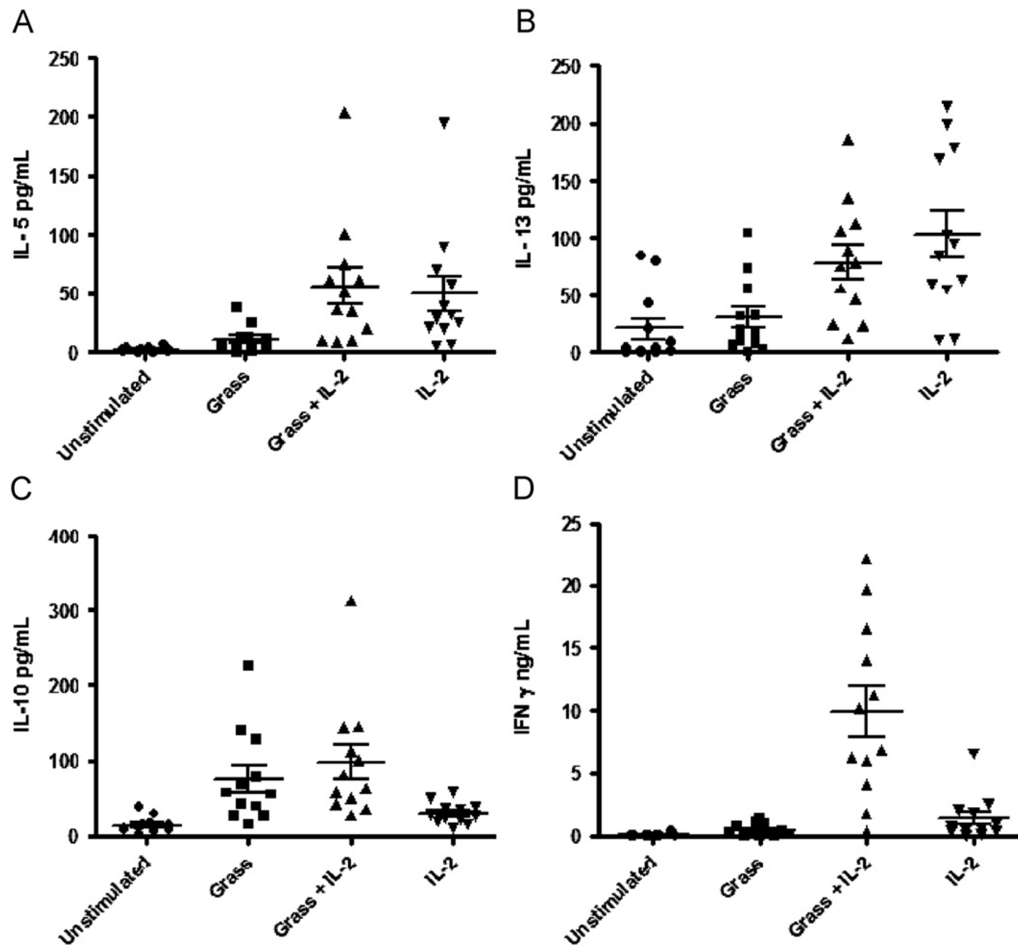

Fig. 4. Determination of allergen-specific responses via the whole blood assay. Whole blood cells were stimulated with a 6-grass pollen mix extract *ex vivo* (at V2, June 2010). Addition of IL-2 with or without the grass mix to the cultures is sufficient to significantly increase the secretion of Th-2 cytokines ($p=0.04$). Detectable levels of IFNγ and IL-10 were obtained in the presence of both the allergen (grass mix) and IL-2 while IL-10 production could be induced with allergen stimulation alone.

setting to confirm and track the allergic status of subjects. The whole blood study was conducted in a "field" trial like setting, i.e. during the actual pollen season. We stimulated whole blood cells *ex vivo* (with anti-CD2 and anti-CD28) rather than directly look at serum levels of the Th-2 cytokines in order to get a comprehensive view of the immune status of the subjects. This approach has previously been described in allergy related trials focusing on atopic eczema [19].

A significant increase in the level of Th-2 cytokines (IL-5 and IL-13) in allergic subjects at V2 when compared to V1 visit (Fig. 3A and B) was observed using the whole blood assay. While the finding itself that Th-2 cytokines are elevated in the pollen season when compared to out of the season is not new, the fact that we could observe these changes (Fig. 1) simply by stimulating small quantities of whole blood provides validation to this assay as a tool to study immune changes in allergics. We evaluated two other cytokines, IFNγ and IL-10 and compared their level at both the visits (Fig. 3C and D). IFNγ is a predominantly Th-1 cytokine and is known to shift the bias from Th-2 allergic responses. IL-10 is described as an immune-regulatory cytokine that has the potential to inhibit Th-2 responses. Our initial thinking was that there would be defects in secreting either IFNγ or IL-10 within the allergic population [4,22,24–26]. However, we did not observe any significant changes in the levels of IFNγ during the two visits in allergic subjects. We also determined the percentages of activated T-cells in stimulated whole blood cells of the subjects. No differences were evident in the activation status of T-cell populations at both the visits (data not shown).

Allergen-specific stimulation with a mix of grass pollen extracts was also investigated, to examine cytokine levels during both the visits (Fig. 4A–D). A combination of grass pollen and the T-cell growth factor IL-2, stimulated production of IL-5, IL-13, IL-10 and IFNγ production. IL-2 alone was sufficient to stimulate IL-5 and IL-13 production. Grass stimulation alone did not increase any cytokine other than IL-10. This possibly suggests that continued allergen exposure is necessary to maintain immune-regulatory (IL-10) responses but possibly not required to sustain optimal allergen-specific Th-2 responses. However, compared to the PBMC assay in which known cell numbers are cultured under different conditions, the whole blood assay is meant to be a quick and easy tool to get an immunological profile to known stimuli. In this assay there certainly are limitations such as the number of cells plated per well, as this varies from donor to donor. We continue to adapt this assay to better study allergen-specific responses [9,20,28]. In summary, the whole blood cell assay is a tool that can help to identify the allergic status of subjects in parallel to SPT and measurement of specific IgE levels. The assay allows studying of immune responses in grass pollen allergic individuals at different time points of the allergic season. Future studies are needed to study the effect of therapeutic interventions on Th-2 cytokine levels in whole blood and evaluate cytokine changes in whole blood in perennial (house dust mite) allergic subjects.

Conflict of interest statement

SH, MB, AM, AS are employees of Nestec S.A., which funded the work.

Acknowledgments

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