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Carry-over effect on IFN-gamma production induced by allergen-specific immunotherapy

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ABSTRACT

Background: Allergic rhinitis is characterized by Th2 polarization and defective IFN- γ production. Specific immunotherapy determines an allergen-specific clinical improvement.

Aim of the study: It was to evaluate whether a course of pre-seasonal SLIT with pollen allergen extract might affect the *in vitro* IFN- γ production using *Dermatophagoides farinae* (Df) allergen as stimulus, in patients presenting with rhinitis due to pollen allergy.

Methods: Thirty-nine AR patients with pollen allergy were included in the study. They assumed pre-seasonal SLIT for 3 months. IFN- γ -specific producing cells were assessed by cytokine ELISPOT before and 3 months after the SLIT course end.

Results: SLIT provided a significant increase of both pollen-induced and Df-induced IFN-gamma production ($p < 0.001$).

Conclusions: The present study provides evidence that though the defective IFN- γ production is allergen-specific in allergic subjects, the SLIT increasing effects on IFN- γ may be non-specific.

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1. Introduction

Allergic rhinitis (AR) is sustained by a mucosal inflammation orchestrated by T helper2 (Th2) cells [1]. In addition, peripheral blood mononuclear cells (PBMC) of AR patients display a predominant interleukin-4 (IL-4) production by Th2 cells over interferon-gamma (IFN- γ) expression by Th1 cells, which constitutes the so-called “Th2 polarization” [2]. This Th2 polarization is typically characterized by a reduced IFN- γ production.

Moreover, the therapeutic administration of subcutaneous increasing doses of the causal allergen, such as the specific immunotherapy (SIT), reduces allergic symptoms and the need for medications in patients with AR and mild forms of asthma, as stated in a World Health Organization position paper [3]. The SIT effectiveness depends on achieving clinical tolerance towards the causal allergen. Even though the exact mechanism of action of SIT is not completely understood, there is evidence that it induces hyposensitization by modifying peripheral and mucosal predominant Th2-responses into a prevalent Th1-polarization, as evidenced by an increased production of IFN- γ [4,5]. This issue reinforces the concept concerning the functional dichotomy between Th1 and Th2 response. Recently,

sublingual immunotherapy (SLIT) has been developed and introduced to minimize possible serious adverse events as it is very safe and effective.

About the SLIT mechanisms of action on immune response, two studies have demonstrated that also SLIT was able to increase allergen-specific-driven IFN- γ production [6,7], another one showed that SLIT may also prevent the onset of new sensitisations [8]. Moreover, it is well known that the clinical effects of SIT are actually allergen-specific [8]. However, all published studies investigated the IFN- γ production induced only by the causal allergen. Therefore, the aim of the present study was to evaluate whether a course of pre-seasonal SLIT with pollen allergen extract might affect *in vitro* IFN- γ production induced by a non-causal allergen, such as *Dermatophagoides farinae*, in patients monosensitized to pollens.

2. Materials and methods

Thirty-nine AR patients with pollen allergy (17 females and 22 males, mean age 37.5 years, range 14–75) were included in the study. All of them were monosensitized to pollen: 16 to *Betula alba*, 16 to *Parietaria*, and 7 to Graminaceae. The relevant causality of the pollen was demonstrated by a positive allergen-specific conjunctival challenge. Mite allergy was excluded both by a negative skin prick test and negative history for perennial symptoms. Patients suffered from seasonal allergic rhinitis alone and with moderate nasal symptoms during the previous pollen seasons. The study was conducted with the

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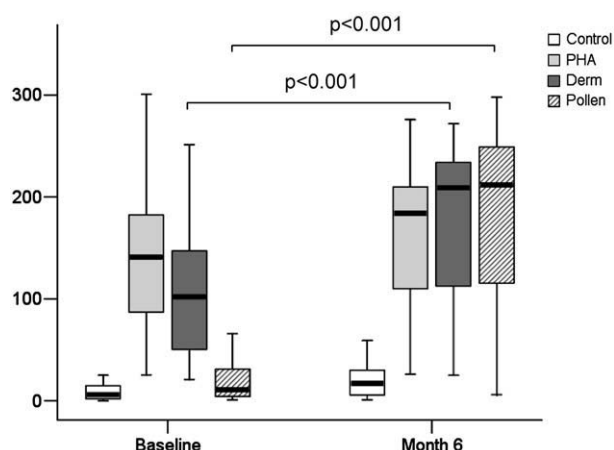


Fig. 1. IFN- γ number of spots at baseline and month 6 for the four stimuli. Plots are based on the median, quartile and extreme values. The box represents the inter-quartile range, which contains 50% of individual patients' values. The whiskers are lines that extend from the box to the highest and lowest values.

approval of the local Ethics Committee and performed with the written informed consent of all participants. The Ethics Committee approved this study as open study for three main reasons: i) the aim of the study was to investigate the effect of SLIT on the production of IFN- γ , thus the control may be considered the *per se* patient as a placebo group could not provide useful data; ii) this was a spontaneous study without sponsor; iii) patients, knowing the aim of this study, would not like to assume no SLIT.

The blood samples of patients were collected before SLIT, and after 3 months since the end of the treatment, such as 6 months after starting SLIT.

Patients undergoing SLIT had received a pollen allergen extract (Anallergo, Florence, Italy) of *B. alba* (16 cases, the accumulate dose was 30 mcg), *Parietaria judaica* (16 cases, the accumulate dose was 12 mcg), and a mix of 7 Graminaceae (7 cases, the accumulate dose was 12 mcg) for at least 3 months before the pollen season (such as pre-seasonal immunotherapy). The dose in the maintenance phase was: five drops 3 times per week. The study started during the late autumn–winter, such as outside the pollen season.

Allergy symptoms were scored 6 months after SLIT starting. The patient questionnaire included a visual analogue scale (VAS) of nasal symptoms [9]. Patients were considered SLIT responders when VAS was <5 as previously defined [10].

Enumeration of IFN- γ -producing cells was performed by cytokine ELISPOT. PBMC were isolated from heparinized blood by Ficoll-Hypaque (Lymphoflot, Biotest, Germany) density gradient centrifugation. The PBMC were washed with PBS and resuspended in complete medium (RPMI-1640 with 2 mmol/l L-glutamine, Invitrogen, Italy) with 10% autologous plasma at 2×10^6 /ml. ELISPOT was performed according with manufacturer's instructions. Briefly, polyvinylidene difluoride plates (Diaclone, France) were coated overnight with IFN- γ capture mAb. After saturation with RPMI-10% fetal calf serum (FCS, Invitrogen), 100 μ l of PBMC suspension was added per well. The cells were stimulated with phytoemagglutinine (PHA; 1 μ g/ml; Sigma Aldrich, Italy) as positive control, with allergen extracts for 20 h at 37 $^{\circ}$ C and 5% CO $_2$ in humidified air. The allergen concentration of the whole extract of birch, *Parietaria*, or grasses (used for SLIT), or *D. farinae*, kindly provided by Anallergo, Florence, Italy, in the final volume of culture medium were respectively 2.5, 1, 1, and 2 mcg/ml, based on assessment in preliminary experiments. After the incubation, the plates were washed with PBS-1% bovine serum albumine (BSA) and incubated with biotinylated detection antibody for 1 h and 30 min at room temperature (RT). After the washing, streptavidin-alkaline phosphatase conjugate was added for 1 h at RT. Finally, BCIP/

NBT was distributed in wells. Elispot assays were set in duplicates, and detection of spots representing cytokine-producing cells was performed by bioreader (BIO-SYS, Germany). The ELISPOT assay is the “ex-vivo” assay with the lowest limit of detection of IFN- γ producing T cells, allowing the reliable detection of as few as 10 specific T cells per 1 million PBMC [11,12]. The production of IFN- γ by not-stimulated cells was also evaluated and considered as negative control.

Descriptive statistics were reported as median and inter-quartile (IQR) ranges. Statistical analysis was performed using the Wilcoxon test for paired data when comparing two repeated measurements, the Mann Whitney U test when comparing two independent groups and the Kruskal Wallis test when comparing more than two independent groups. Differences in IFN-gamma changes between baseline and follow up between groups were evaluated using a repeated measures analysis of variance with an interaction term.

3. Results

At baseline, the median number of spots was 6 (IQR=14) for the negative control, 141 (IQR=102) for PHA, 102 (IQR=106) for *D. farinae* (Df), and 11 (IQR=35.5) for pollens. The difference between Df-induced and pollen-induced IFN- γ production as well as between PHA-induced and pollen-induced IFN- γ production was statistically significant ($p < 0.001$). PHA-induced and Df-induced IFN- γ production were also significantly different ($p = 0.01$).

After 6 months, the median number of spots was 17 (IQR=25.5) for negative control, 184 (IQR=105) for PHA, 209 (IQR=123) for Df, and 212 for pollens (IQR=134) (Fig. 1). The increase in the number of spots between baseline and 6 months is significant for both allergens ($p < 0.001$), but not for PHA ($p = 0.08$). Moreover, the ratio between PHA-induced and Df-induced IFN- γ production (median=57%, IQR=214%) is significantly higher than the ratio between PHA- and pollen-induced IFN- γ production at baseline (median=6.8%, IQR=30%) ($p = 0.003$) (Fig. 2); after 6 months, the two ratios (median=123%, IQR=170% vs median=114%, IQR=98%) are more similar and the difference is no longer statistically significant ($p = 0.15$).

The change in the IFN- γ level between baseline and month 6 after SLIT was evaluated in four conditions: no stimulus (negative control), PHA, Dermatophagoides and pollen stimuli. The 6 month value was significantly higher both for Dermatophagoides- and pollen-stimulated cells ($p < 0.001$ for both conditions), while the difference did not reach significance for the PHA and not-stimulated cells (Fig. 3).

The IFN- γ change after SLIT for the pollen- and for Dermatophagoides-stimulated cells was not heterogeneous between groups of patients with different specific allergens (p for interaction=0.12 and $p = 0.43$ respectively, Fig. 4).

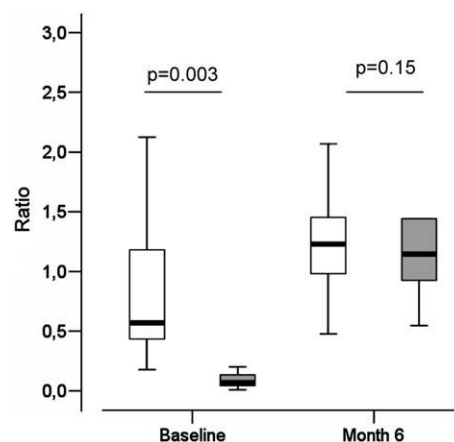


Fig. 2. Ratio between PHA- and *Dermatophagoides farinae*-induced IFN- γ production (white boxes) and between PHA- and pollen-induced IFN- γ production (grey boxes) at baseline and after 6 months.

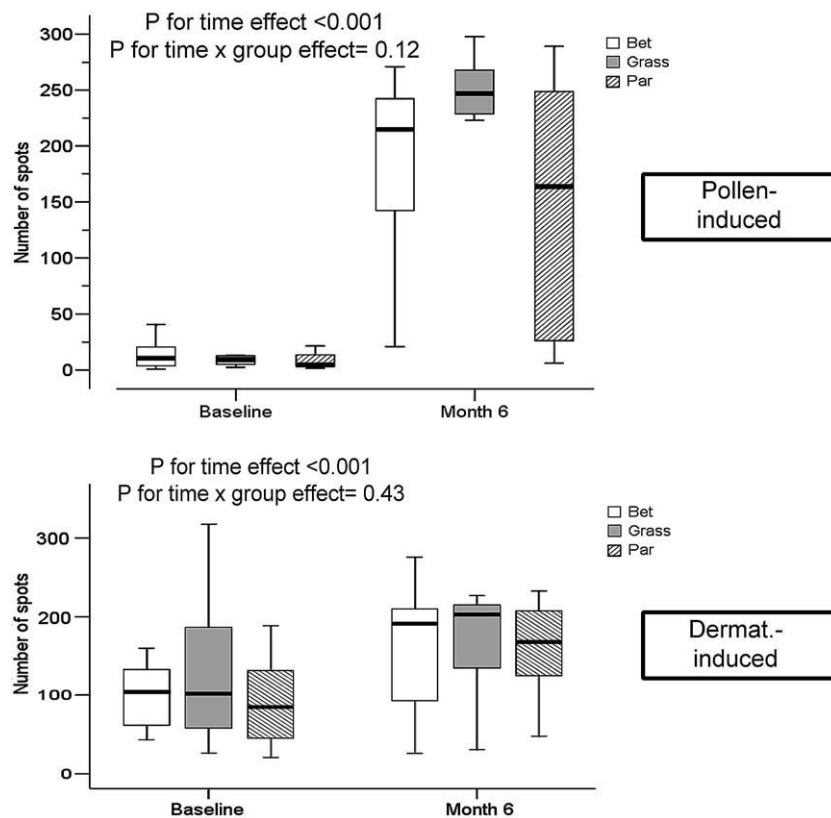


Fig. 3. IFN- γ number of spots at baseline and month 6 according to the specific causal allergen after stimulus with pollen (upper panel) and Dermatophagoides (lower panel) in the 3 sub-groups of patients according to the causal pollen allergen.

The change in the IFN- γ level between baseline and month 6 after SLIT was significantly higher (median change = +209.5, IQR = 169) in responders than in non-responder patients (median change = -5, IQR = 339.5) ($p = 0.027$).

4. Discussion

Allergic rhinitis is characterized by a Th2-polarized inflammation. Th2-derived cytokines, such as IL-4 and IL-13, are the main pathogenic factors able to induce, maintain and amplify the allergic inflammation. IL-4 and IL-13 orchestrate this inflammation by promoting IgE synthesis, by up-regulating adhesion molecules, selective for eosinophil recruitment, and by causing an increased production of mucus and hyperreactivity of the airways. It has been evidenced that specific immunotherapy increases IFN- γ production and reduces Th2-dependent cytokines, thus inducing a shift from a Th2 towards a Th1 response [4–7]. Therefore, Th1 polarization represents an important change in the immune response after SLIT.

This study was aimed at evaluating the production of IFN- γ induced by different stimuli in a group of patients with rhinitis due to pollen allergy and treated with a course of pre-seasonal SLIT. To this end, PBMC were stimulated by two different allergens: the causal allergen, such as the causal pollen extract used for SLIT, and an ubiquitous allergen, such as *D. farinae*, which was not cause of allergy in such patients. The purpose of our study derived from the acknowledgement that the clinical effects of SIT are typically allergen-specific. Thus, the primary goal of the present study was to evaluate whether pollen SLIT could affect IFN- γ production induced by *D. farinae*.

As previously observed, IFN- γ production induced by the causal allergen, such as the pollen, was significantly defective in comparison with that induced by PHA before SLIT. This finding confirms the paradigm of the Th2 polarization in allergic patients and that the

defect of IFN- γ production is typically allergen-specific. Indeed, using another allergen, that is not causal, the defect is significantly lower in comparison with the pollen-induced production. As expected, SLIT induced a significant increase of allergen-specific IFN- γ production. This phenomenon may be dependent on the shift from Th2-polarized toward Th1-oriented immune response consequent the SLIT effect as well known in literature [4–7].

However, the new insight is represented by the demonstration that pollen SLIT also increased the *Dermatophagoides*-induced IFN- γ production.

This finding represents the first evidence that SLIT mechanisms on immune response carry over an allergen-specific response. Therefore, though it is well recognised that clinical response to SLIT is strictly allergen-specific, immunotherapy may induce a generalised shift from Th2 to Th1 polarization beyond the allergenic restriction. This phenomenon might be not surprising as it has been evidenced that

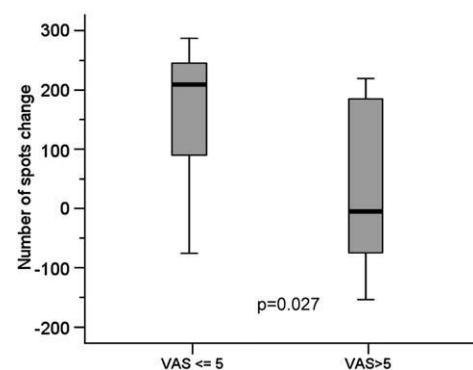


Fig. 4. IFN- γ number of spots change between baseline and month 6 in responders (VAS ≤ 5) and non-responder patients.

antigen re-challenge promoted by vaccinations generate antigen-dependent as well as antigen-independent memory B cells response [13–15]. However, this study has some limitations concerning the lack of a control group, the inclusion of patients with different allergies, the evaluation of a single parameter. In addition, even if there was no natural exposure to pollens, it is not possible to exclude that the patients were exposed to greater amount of mites during the treatment period, such as autumn–winter, and this fact could influence, at least partially, the results.

In conclusion, the present study has evidenced that though the defective IFN- γ production in allergic subjects is allergen specific, the SLIT effects on immune response may be non-specific. These preliminary findings need to be confirmed by further controlled studies.

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