

Original article

Allergen-induced *in vitro* expression of IL-18, SLAM and GATA-3 mRNA in PBMC during sublingual immunotherapy

Background: Signalling lymphocytic activation molecule (SLAM) and interleukin (IL)-18 induce interferon (IFN)- γ production from Th1 cells. The allergen-induced SLAM and IL-18 mRNA expressions are increased during subcutaneous immunotherapy (SCIT), but nothing is known about their role during sublingual immunotherapy (SLIT). Transcription factor GATA-3 is associated with Th2 cells but its role in SCIT and SLIT is yet unexplored. This study was undertaken to analyse the allergen induced *in vitro* mRNA expression of IL-18, SLAM and GATA-3 in peripheral blood mononuclear cells (PBMC) of children with allergic rhinitis (AR) during SLIT.

Methods: Ten patients with AR undergoing pollen SLIT with a weekly dose of 200 000 SQ-U, 10 with 24 000 SQ-U of mixture of *Betula verrucosa*, *Corylus avellana* and *Ahus glutinosa* and 10 with placebo were included. Peripheral blood mononuclear cell were stimulated with birch extract prior to, after 1 and 2 years of the treatment. The mRNA expression was assessed using kinetic real-time RT-PCR (TaqMan[®]; Applied Biosystems, Foster City, CA, USA).

Results: The expression of IL-18 mRNA was increased in the high-dose group in comparison to the placebo group after 1 year of therapy ($P = 0.028$) and had an inverse correlation with the late phase skin reaction after the second study year ($r = -0.41$, $P = 0.041$). SLAM mRNA expression increased in the high-dose group from baseline to 1 year ($P = 0.028$) and correlated with IL-10 ($r = 0.96$, $P < 0.0001$) and transforming growth factor- β ($r = 0.80$, $P = 0.0037$) mRNA expression. No significant changes were seen in GATA-3 mRNA expression.

Conclusions: During SLIT, IL-18 and SLAM are upregulated, suggesting that the Th2 type inflammatory response is downregulated during SLIT by increased Th1 type response.

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In pollen allergy and allergic rhinitis (AR), there is a seasonal mucosal inflammation characterized by mast cell and eosinophil activation. The inflammation displays a predominant interleukin (IL)-4 expression (Th2 cells) in CD4⁺ T cells over interferon (IFN)- γ expression (Th1 cells). Specific immunotherapy (SIT) or allergen vaccination using subcutaneous injections (SCIT) is a widely used treatment of AR. Sublingual immunotherapy (SLIT) is the most effective noninjection route and found effective in AR and asthma (1–3). During subcutaneous immunotherapy (SCIT), there is a local mucosal shift from Th2 to Th1 type cytokine predominance and downregulation of IL-5 and eosinophilia (4, 5). According to recent studies, IL-10 and transforming growth factor (TGF)- β -induced tolerance is another key phenomenon in SCIT (6–8). Interleukin-10 is also upregulated and IL-5 downregulated during SLIT (9).

Interleukin-18 is a macrophage-derived cytokine originally found as a factor inducing IFN- γ production from Th1 cells (10). The effects of IL-18 *in vitro* depend on

co-stimulatory factors. When administrated with IL-12, it induces IFN- γ production from Th1 cells and B cells, inhibition of immunoglobulin (Ig) E-production and antigen-specific Th2-like cell development (11). However, in the absence of IL-12, IL-18 induces mast cells and basophils that express IL-18R- α to produce IL-4 and IL-13, resulting in increased IgE levels (12).

Signalling lymphocytic activation molecule (SLAM; CD150) is a self-ligand, transmembrane, cell surface glycoprotein that belongs to the CD2 subset of the immunoglobulin superfamily (13). Its activation promotes T-cell proliferation, IFN- γ production and it is preferentially expressed in Th1 cells over Th2 cells (14, 15). The allergen-induced SLAM and IL-18 mRNA expressions are increased during SCIT, but nothing is known about their role during SLIT (8, 16).

The development and differentiation of T cells are influenced by many factors including the transcription factor GATA-3, which is associated with the induction of Th2 cells (17, 18). And, cells expressing GATA-3 develop

the profile of Th2 cells, secreting IL-4, IL-5 and IL-13 (217). Binding of GATA-3 in the regulatory regions of these genes alters chromatin structure, increasing accessibility to other transcription factors. GATA-3 is activated by IL-4 or autoactivated and one of its basic functions is T-cell antigen regulation-inducing Th2 profile expression of Th cells (17, 18). Although the transcription factor GATA-3 is associated with the induction of Th2 cells its role in SIT is so far totally unexplored.

This study was undertaken to investigate the allergen-induced *in vitro* mRNA expression of the Th1 type effector cytokine IL-18, the signalling molecule SLAM and the transcription factor GATA-3 in peripheral blood mononuclear cell (PBMC) during SLIT among allergic children suffering from rhinoconjunctivitis caused by allergy to tree pollens.

Material and methods

Patients

Thirty children aged 5–15 years with a clinical history of tree pollen-induced allergic rhinoconjunctivitis with/without seasonal asthma for at least 2 years were randomly selected from a single centre, randomized, double-blind, placebo-controlled dose-response phase II trial including tree pollen-allergic children as earlier described (3). The study was conducted with the approval of the local ethics committee and with the written informed consent by the parents. The children were randomized into three groups (10 in each group) receiving SLIT with a tree pollen extract of SQ-standardized *Betula verrucosa* (birch), *Corylus avellana* (hazel) and *Alnus glutinosa* (alder) up to 18 months as described earlier (3). In the *Dose group 1* the daily dose in the maintenance phase was 4800 SQ-U, there were four boys and six girls with the mean age of 7.8 (SD 2.2) years and with a symptom score of 3.2 (SD 2.7) after treatment. In the *Dose group 2*, the daily dose in the maintenance phase was 40 000 SQ-U; there were five boys and five girls with the mean age of 10.2 (SD 2.3) years and with a symptom score of 1.9 (SD 1.6) after treatment. In the *Placebo group* receiving diluent containing 50% glycerol and 50% saline buffer, there were five boys and five girls with the mean age of 11.1 (SD 3.4) years and with symptom score of 3.6 (SD 3.4) after treatment. Symptom and medication data were collected using a patient's diary as previously described, and the late phase skin reaction (LPSR) was performed with an allergen solution (0.05 ml corresponding to 50 SQ-U Tree Mix, Aquagen SQ[®]; ALK-Abelló, Hørsholm, Denmark) injected intracutaneously as described earlier (3). The early skin reaction was measured after 15 min and the LPSR after 24 h, and recorded. The LPSR sizes were determined and analysed by using a computerized scanner. The blood samples of the 30 patients were collected, during the pollen-free season, before SLIT was commenced and after 1 or 2 years from the start of the treatment.

Peripheral blood mononuclear cell cultures

The stimulation of PBMC with allergen *in vitro* was performed as described earlier (9). Briefly, PBMC were isolated from heparinized whole blood by Ficoll gradient centrifugation and the washed mononuclear cells were cultured in RPMI-based medium at +37°C. Prior to allergen stimulation, the cells rested for 3 days and were then stimulated with whole extract of birch (*B. verrucosa*, ALK-Abelló),

50 µg/ml, for 3 days. After harvesting and centrifugation, the cell pellets were collected, lysed and frozen at –70°C in Trizol (Gibco Life Technologies, Paisley, UK) solution for RNA extraction.

mRNA detection by TaqMan[®] RT-PCR

The RNA isolation and the TaqMan[®] RT-PCR were performed as previously described (16). Briefly, RNA was isolated according to Trizol instructions and the extracted RNA was stored in 75% ethanol at –20°C. The RT reaction was performed with First-strand cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden) using oligo (dT) primers, and cDNA was stored at –70°C. The amplification of β-actin, IL-18, SLAM, GATA-3, IL-10 and TGF-β cDNA was performed in MicroAmp[®] optical 96-well reaction plate (PE Applied Biosystems, Foster City, CA, USA). A negative PCR control without template and a positive PCR control with a template of known amplification were included in each assay. The reaction was performed in ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). For analysis of IL-10, IL-18 and TGF-β Predeveloped Assay Reagents (PDAR) kits were purchased from PE Applied Biosystems. The primer and probe sequences of β-actin, SLAM and GATA-3 were designed to be cDNA-specific and to work under equivalent reaction conditions using PRIMER EXPRESS software (PE Applied Biosystems) and were labelled with FAM (6-carboxyfluorescein) at the 5'-end and with TAMRA (6-carboxytetramethylrhodamine) at the 3'-end, as previously described (19). During PCR the Ct values (the cycle number at which the detected fluorescence exceeds the threshold) for each amplification product were determined using a threshold value of 0.03. The specific signals were normalized by constitutively expressed β-actin signals using the formula $2^{-\Delta Ct} = 2^{-(Ct - \text{actin} - Ct, \text{Specific})}$. The stimulation indices were thereafter calculated by the formula $2^{-\Delta \Delta Ct} = 2^{-(\Delta Ct \text{ for stimulated culture} - \Delta Ct \text{ for unstimulated culture})}$ as described in ABI PRISM 7700 SDS relative quantitation of gene expression protocol by PE Applied Biosystems.

Statistics

The Wilcoxon signed rank test was used for paired comparisons within the individuals and the Spearman rank order correlation test for paired comparison of groups. The Mann–Whitney *U*-test was used for unpaired comparison of groups.

Results

Figure 1 presents the *in vitro* allergen-induced mRNA expression of IL-18, SLAM and GATA-3 by PBMC of pollen-allergic children undergoing SLIT at three time points. The expression of IL-18 mRNA was clearly increased in the dose group 2 (daily dose 40 000 SQ-U) in comparison to placebo group after 1 year of therapy ($P = 0.028$, Mann–Whitney *U*-test). There were no significant differences between the study groups at any time points in SLAM mRNA expression, but there was a clear increase in the dose group 2 from baseline to 1 year ($P = 0.028$, Wilcoxon signed rank test). No significant changes were seen in GATA-3 mRNA expression.

The correlations (Spearman rank order correlation test) of the SLAM mRNA expression with those of the regulatory cytokines TGF-β and IL-10 are illustrated in

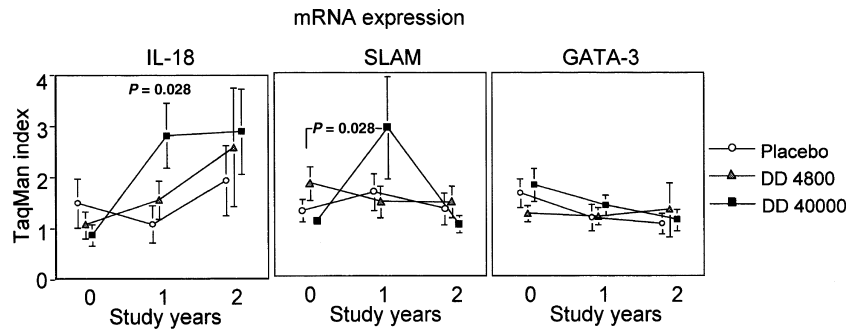


Figure 1. *In vitro* allergen-induced interleukin-18, signalling lymphocytic activation molecule (SLAM) and GATA-3 mRNA expression in peripheral blood mononuclear cell cultures of the study patients assessed with real-time kinetic RT-PCR (TaqMan®). The expression is shown as TaqMan index, indicating fold increase in comparison to no stimulus. The mean and SEM are shown separately for patients treated in the dose group 2 (black square), the dose group 1 (grey triangle) and placebo (white circle) before the treatment and after 1 and 2 years of therapy. The *P*-values refer to nonparametric unpaired comparison to placebo group at each time point (Mann–Whitney *U*-test). There was a significant increase in SLAM mRNA expression from commencement to 1 year of therapy in the dose group 2 ($P = 0.028$, Wilcoxon signed rank test).

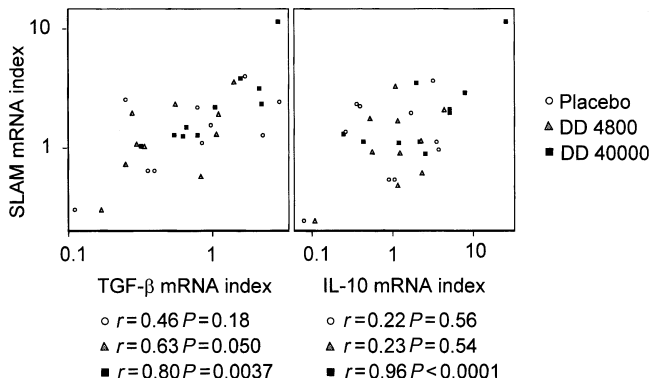


Figure 2. The correlations between signalling lymphocytic activation molecule and transforming growth factor-β and interleukin-10 mRNA expression after the second study year. The expression is shown as TaqMan index, indicating fold increase in comparison to no stimulus. The plot symbols and correlations are shown separately for patients treated in the dose group 2 (black square), the dose group 1 (grey triangle) and placebo (white circle).

Fig. 2. While the whole study population ($n = 30$) displayed significant correlations with both TGF-β ($r = 0.88$, $P < 0.0001$) and IL-10 ($r = 0.65$, $P < 0.001$), a detailed analysis of the treatment groups showed less correlation in the dose group 1 (daily dose 4800 SQ-U) and placebo group. In the dose group 2, the SLAM mRNA expression correlated with TGF-β ($r = 0.80$, $P = 0.0037$) and IL-10 ($r = 0.96$, $P < 0.0001$) mRNA expression. No correlations between the studied genes (IL-18, SLAM and GATA-3) and cytokines IFN-γ, IL-4 and IL-5 were found.

The correlations of the IL-18 mRNA expression and the LPSR at commencement and after 1 and 2 years are illustrated in Fig. 3. Interleukin-18 mRNA expression had an inverse correlation with the LPSR after the second study year ($r = -0.41$, $P = 0.041$). There is a clear trend of widely distributed plot developing by time to a more

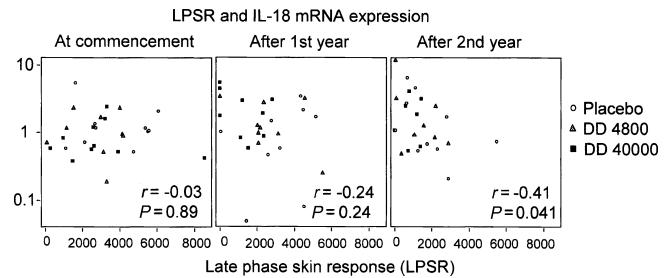


Figure 3. The correlations of the late phase skin response and interleukin-18 at commencement, after the first study year and after the second study year. Interleukin-18 mRNA expression is expressed as TaqMan index, indicating fold increase in comparison to no stimulus. The correlations are shown separately for patients treated in the dose group 2 (black square), the dose group 1 (grey triangle) and placebo (white circle).

densely distributed plot in the upper left quarter mainly because of increased IL-18 and decreased LPSR in the dose group 2. No correlations between the studied genes (IL-18, SLAM and GATA-3) and symptom or medication scores were detected.

Discussion

Our study demonstrated Th1 enhancement as a potential mechanism in SLIT, suggested by increased IL-18 and SLAM mRNA expression in the group receiving the highest daily dose. We previously observed the augmented expression of IL-10 mRNA and the inhibited expression of IL-5 mRNA in PBMC stimulated with allergen in pollen-allergic children undergoing SLIT (9). The levels of GATA-3 did not decrease accordingly with the levels of Th1 type genes.

According to our findings, IL-18 mRNA appears to be upregulated during SLIT, being associated with decreased sizes of LPSRs. Increased expression of IL-18 in periph-

eral blood or nasal exudate of patients with asthma or AR have been demonstrated, although contrary observations, decreased IL-18 in atopic disease, have also been made (20–23). However, there is strong evidence that in the presence of IL-12, IL-18 is clearly enhancing Th1 type immune response, including IFN- γ production. It is in contrast antagonizing Th2 type allergic immune response, such as IgE production and airway hyperresponsiveness, IL-12 having an essential role as a co-stimulatory cytokine (10–12). Our findings of IL-18 mRNA upregulated during SCIT and SLIT are in agreement with these and previous findings of upregulation of IL-12 and IFN- γ mRNA during SCIT as in the presence of IL-12, IL-18 induces IFN- γ production and inhibits IgE synthesis (4, 8, 11, 24).

The role of SLAM in allergy and SIT has acquired very little interest even though, as a molecule expressed at higher levels in Th1 cells than in Th2 cells, it is likely to be involved in the Th2 to Th1 shift observed during SIT (4, 15, 24, 25). We recently showed that the *in vitro* allergen-induced SLAM, mRNA expression in peripheral blood lymphocytes is lower in AR patients than in healthy control subjects, but is enhanced by SIT (16). An early and transient increase in SLAM mRNA expression is associated with the clinical symptom improvement. In this study, we demonstrated that after 1 year of therapy in the high-dose group SLAM, mRNA was increased. During SCIT an IL-10 response by the maintenance dose is associated with good therapeutic outcome, whereas in SLIT no systemic changes can be seen in cytokine responses during the dose build-up phase or the early maintenance period (8, 26). Subcutaneous immunotherapy and SLIT may differ in timing of the observed responses, in SCIT, early responses are essential, whereas in SLIT, responses may be seen after a long period of maintenance dose.

Enhanced SLAM expression is seen in autoimmune diseases with Th1 dominance. In multiple sclerosis SLAM-expressing lymphocytes and the IL-12 production of PBMC are increased (27). In patients with rheumatoid arthritis, the expression of SLAM is significantly upregulated on synovial fluid and synovial tissue T cells

compared with peripheral blood T cells or with cells from healthy volunteers (28). Furthermore, SLAM expression is downregulated by IL-10 in T cells, and SLAM activation increases the production of IL-10, IFN- γ and TNF- α in synovial fluid mononuclear cells. Low levels of SLAM expression may be associated with Th2 responses, as the expression of SLAM is diminished in atopics with elevated serum IgE levels (29). Our findings suggest that Th1 cell activation by SLAM may be one of the key phenomena in SLIT.

Earlier findings on mainly humoral immunological effects of local routes differ from those of SCIT. An effect of oral immunotherapy and SLIT on serum immunoglobulins was observed only in a minority of studies local routes do not induce a decrease of IgE and an increase of IgG1 and IgG4, or that these changes are not constant and reproducible as they are in SCIT (30). Decreased serum levels of IL-13, another Th2 type cytokine, were observed during SLIT and recently an increase in PHA-induced IL-10 production was demonstrated in house dust mite SLIT in young adults (31, 32). During high-dose SLIT, there were concurrent increased expressions of both regulatory cytokines, such as IL-10 and TGF- β mRNA, and Th1 effector cytokine IL-18 and signalling molecule SLAM mRNA, suggesting that the Th2 type inflammatory response was downregulated by increased response of both Treg and Th1 cells. In our earlier study, we showed that a high-dose SLIT induced an inhibitory effect on IL-5 mRNA expression increase that was associated with TGF- β mRNA expression (9).

The importance of the regulatory cytokine IL-10 in venom immunotherapy (VIT) and SCIT was demonstrated in several studies (6–8, 33, 34). We earlier demonstrated that IL-10 had a role in SLIT and here demonstrated an increase in IL-18 and SLAM mRNA expression. Data taken together with earlier studies suggest that in SLIT for pollen allergy, as in VIT and SCIT, upregulation of both regulatory cytokines, especially IL-10, and Th1 type cytokines is essential, and the basic immunological phenomena in peripheral blood may be close to each other in SLIT, VIT and SCIT, but differ in timing (4, 24–26, 33–36).

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