

Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants

Cezmi A. Akdis, Thorsten Blesken, Daniel Wymann, Mübeccel Akdis and Kurt Blaser

Swiss Institute of Allergy and Asthma Research, Davos, Switzerland

Bee venom phospholipase A₂ (PLA) represents the major allergen and antigen in allergic and non-allergic individuals sensitized to bee sting. We have studied specific activation of peripheral T cells by different structural and conformational variants of PLA and secretion of cytokines regulating IgE and IgG4 antibody (Ab) formation. PLA molecules expressing the correctly folded tertiary structure, which show high affinity to membrane phospholipids and were recognized by Ab from bee sting allergic patients, induced high IL-4, IL-5 and IL-13 production in peripheral blood mononuclear cell cultures. In contrast, non-refolded recombinant PLA (rPLA) and reduced and alkylated native PLA (nPLA) induced more IFN- γ and IL-2 and higher proliferative responses. Differences in proliferation and cytokine patterns among correctly folded and non-refolded PLA resulted from conformation-dependent involvement of different antigen-presenting cell (APC) types. Antigen (Ag)-presenting B cells recognized PLA only in its natural conformation, stimulated Th2 type cytokines and induced IgE Ab. Non-refolded PLA was recognized, processed and presented exclusively by monocytes and induced a Th1 dominant cytokine profile leading to IgG4 production by B cells. The possibility that production of particular cytokine patterns and Ig isotype was influenced by the enzymatic activity of PLA was excluded by using enzymatically inactive H34Q point-mutated, refolded rPLA. These findings demonstrate the decisive role of specific Ag recognition by different APC, depending on structural features, membrane phospholipid binding property and the existence of conformational B cell epitopes, in the differential regulation of memory IgE and IgG4 Ab. Furthermore, they show that a change from IgE-mediated allergy to normal immunity against a major allergen can be induced by rPLA variants that are not recognized by specific Ab and B cells but still carry the T cell epitopes. These features may enable new applications for safer immunotherapy.

Key words: IgE regulation / Antigen conformation / Antigen presentation / Th1/Th2 / Recombinant allergen

Received	5/9/97
Revised	15/12/97
Accepted	15/12/97

1 Introduction

Development of specific immune responses requires the encounter of antigens with the cells of the immune system and interaction between T and B lymphocytes and other APC [1]. The presentation of soluble antigens to T cells by APC involves a complex process including Ag capture, processing and presentation of Ag fragments by MHC molecules [1–3]. Ag presentation by different types of APC can promote the development of distinct

cytokine-secreting T cell subsets [4, 5]. Th1 cytokine profiles, characterized by IL-2, IFN- γ and TNF- β , are implicated in cell-mediated defense against some intracellular microorganisms and in promotion of memory IgG responses [6–8]. Th2 cells secrete IL-4, IL-5 and IL-13 and predominantly mediate IgE responses and allergic inflammation [6–8]. Mutually exclusive, polarized Th1 or Th2 responses may emerge at least in part by counter-inhibitory effects of cytokines secreted by the reciprocal T cell subsets [9–13]. In particular, IFN- γ can promote its own production [12] and IL-12 is a dominant factor inducing Th1 cell differentiation [10, 11, 13]. In contrast, the presence of IL-4 during initial Ag priming of naive T cells results in generation of Th2 cells, inhibiting Th1 cytokine development [9, 14]. Beside specific MHC-TCR interaction, both monocytes/macrophages and B cells are able to deliver co-stimulatory signals to T cells, such

[17557]

Abbreviations: BV: Bee venom PLA: Bee venom phospholipase A₂ nPLA: Native PLA rPLA: Recombinant PLA raPLA: Reduced and alkylated PLA wtPLA: Refolded wild-type recombinant PLA wtPLA un: Unrefolded recombinant PLA sCD40L: Soluble CD40 ligand

as ligands for CD28, CTLA-4 and CD2 [1, 15–17]. Ag-presenting monocytes secrete the pro-inflammatory cytokines IL-1, TNF- α , and/or cytokines and factors like TGF- β , IL-10, IL-12 and prostaglandin E₂, which are suppressive for IgE. B cells produce these cytokines only at low levels after activation [10, 15–18]. Allergic diseases are clinical manifestations of IgE Ab responses to environmental antigens in atopic individuals. However, classical allergens are also potent inducers of IgG Ab [19]. Whereas the Th2 cytokines IL-4 and IL-13 are potent inducers of both human IgE and IgG4 synthesis, IFN- γ acts as an IgE inhibitor [20–22] and induces IgG4 Ab to allergen in activated memory B cells [23, 24].

Bee venom (BV) phospholipase A₂ (PLA) is the major allergen and immunogen in both allergic and non-allergic individuals sensitized to bee sting [25, 26]. It is a biochemically well-defined glycoprotein of 14–16 kDa, consisting of 134 amino acids and a single carbohydrate side chain [27]. The three-dimensional structure, which has been resolved by x-ray crystallography, is stabilized by five intra-chain cysteine disulfide bridges [28]. The protein has been cloned and produced in *E. coli*. Generation of enzymatically active recombinant PLA (rPLA), with full Ab and lipid affinities, requires an additional step of refolding *in vitro* [28, 29]. Crucial for PLA activity is a His residue at position 34 (H34). An enzymatically inactive H34Q point-mutated PLA (H34Q-PLA) has been developed [30]. Bee stings generally elicit anti-PLA Ab recognizing conformational epitopes [31]. Normally, low-affinity IgG1 anti-PLA is generated during the initial response [19]; high-affinity IgG4 anti-PLA Ab develop after repeated stings [19, 32] and specific immunotherapy of allergic patients [32, 33]. In contrast, individuals allergic to bee sting develop IgE anti-PLA Ab [25, 32, 33].

We investigated T cell activation, cytokine production and their regulatory effects on specific and total IgE and IgG4 synthesis, in response to PLA molecules expressing the native structure and PLA preparations that are not recognized by specific Ab or B cells from bee sting-allergic patients. We demonstrate that PLA variants lack-

ing the original tertiary structure are presented by monocytes and induce a Th1 dominant cytokine profile and IgG4 formation in PBMC from BV-allergic individuals. The same allergen expressing the native conformation was presented by B cells as well and induced Th2 type cytokines and IgE formation. Thus, our observations point to the pivotal role of antigen structure resulting in presentation by different APC and subsequent induction of distinct cytokine profiles in T cells, which differentially regulate IgE and IgG4 responses.

2 Results

2.1 Requirement of native PLA conformation for specific binding of serum IgE and IgG4 Ab from bee sting-sensitized subjects

To study immunoregulatory properties of different conformational PLA variants and their recognition by specific B cells, we determined the IgE and IgG4 Ab binding capacity of the different PLA preparations. PLA-specific IgE and IgG4 binding to native PLA (nPLA), and correctly folded recombinant wild-type PLA (wtPLA) and enzymatically inactive, point-mutated H34Q-PLA was determined in pooled serum from five bee sting-sensitized allergic patients and compared to binding of non-refolded wtPLA, non-refolded H34Q-PLA and reduced and alkylated PLA (raPLA). The different PLA preparations used in the study are listed in Table 1. Fig. 1 shows that nPLA and refolded wtPLA, as well as correctly folded H34Q-PLA bind to the same extent to anti-PLA IgE and IgG4 Ab. Non-refolded rPLA preparations or raPLA did not bind to the specific isotypes, suggesting an essential requirement of the intact PLA structure for specific Ab binding and recognition by specific B cells.

2.2 Antigen conformation-dependent T cell proliferation

To elucidate whether conformational differences of Ag may influence the specific proliferative response, PLA

Table 1. PLA preparations used in the study

PLA	Abbreviation	Cell Membrane Binding	Enzymatic Activity	Specific IgE/IgG4 Binding
Native	nPLA	+	+	+
Recombinant wild-type refolded	wtPLA	+	+	+
Recombinant point-mutated refolded	H34Q-PLA	+	–	+
Reduced and alkylated native	raPLA	–	–	–
Recombinant wild-type non-refolded	wtPLA un	–	–	–
Recombinant point-mutated non-refolded	H34Q-PLA un	–	–	–

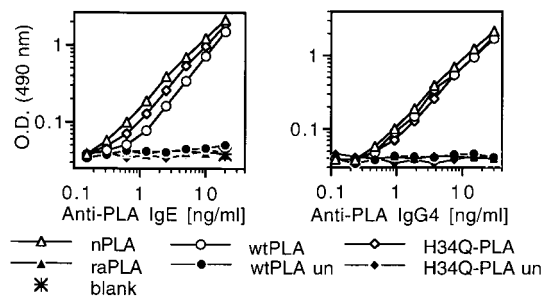


Figure 1. Binding of serum anti-PLA IgE and IgG4 Ab from pooled serum of five bee sting-allergic patients to different refolded and non-refolded PLA preparations as determined by solid-phase ELISA. Only nPLA and refolded rPLA showed specific IgE and IgG4 binding.

preparations of different molecular conformation were used to stimulate PBMC from allergic patients. The non-refolded wtPLA, H34Q-PLA and the chemically modified raPLA, lacking the correct structure, induced two to three times higher proliferation, compared to the nPLA expressing the natural conformation, refolded wtPLA and H34Q-PLA. In all cases a plateau of proliferation was obtained at 1–3 μ M Ag (Fig. 2A). The differences in proliferative T cell response at 1 μ M of refolded and non-refolded wtPLA among 14 bee sting-allergic patients is demonstrated in Fig. 2B. The mean proliferative response to non-refolded wtPLA was $31\,394 \pm 5727$ cpm (mean $\pm$ SEM), compared to $11\,398 \pm 2636$ cpm of its refolded analogue ($p < 0.001$). The same findings were observed using refolded and non-refolded H34Q-PLA in PBMC of 18 bee sting-allergic patients (Fig. 2C). A two times higher proliferative response was induced by non-refolded H34Q-PLA ($37\,758 \pm 8315$ cpm) compared to refolded H34Q-PLA ($18\,358 \pm 4991$ cpm) ($p < 0.001$). There were no significant differences in proliferative responses to wtPLA and H34Q-PLA, indicating that the enzymatic activity has no effect on the proliferation of T cells.

2.3 Differential induction of Th1 and Th2 cytokine profiles by antigen conformation

Distinct cytokine profiles produced by Th1 or Th2 cells direct immune responses towards normal or allergic responses. In Fig. 3 allergen-induced cytokine production in response to refolded and non-refolded wtPLA is shown. PBMC from BV-allergic patients were specifically stimulated and expanded with either form of wtPLA in the presence of IL-2 and IL-4. Cytokines were measured after washing and restimulation of cultures with the same PLA preparation as used for primary stimulation, in the presence of irradiated autologous PBMC. The refolded

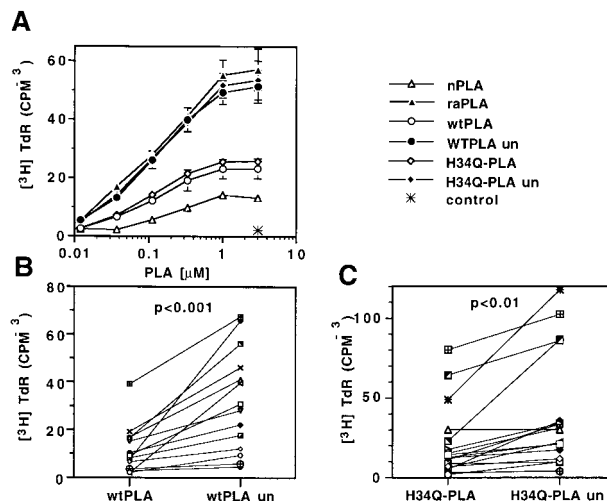


Figure 2. Proliferative response to different conformational PLA variants. (A) PBMC from a BV-allergic patient stimulated with increasing doses of different PLA preparations. (B, C) Refolded rPLA (wtPLA, H34Q-PLA) and non-refolded rPLA (wtPLA un, H34Q-PLA un) at 1 μ M were used to stimulate PBMC of BV-allergic patients. [^{3} H]Thymidine incorporation was measured at day 6. Background proliferation in unstimulated cultures ranged between 258–1212 cpm and stimulation index ranged between 4.54 and 32.6. Non-refolded PLA and raPLA with altered tertiary structure induced significantly higher proliferation in comparison to PLA which express the natural structure.

wtPLA elicited increased Th2 type cytokines IL-4, IL-5 and IL-13, compared to its unrefolded variant. IL-2 and IFN- γ secretion were significantly lower in response to the refolded wtPLA. The ratios of IL-4:IFN- γ among different patients further demonstrated a shift in Ag conformation-associated cytokine profile from Th2 to Th1 by non-refolded allergen (mean IL-4:IFN- γ ratios: 0.680 for refolded and 0.003 for non-refolded wtPLA). The proliferative PBMC response of each patient correlated positively with IL-2 ($r = 0.981$, $p < 0.001$) and IFN- γ ($r = 0.956$, $p < 0.001$) production, as detected after restimulation. There was no correlation of proliferation with IL-4, IL-5 or IL-13 production.

2.4 The Ag conformation determines the APC type directing the formation of particular T cell cytokine profiles

We investigated whether Ag conformation-related differences in specific cytokine patterns result from the selective involvement of APC, in particular of B cells and monocytes. For this purpose, staining of APC with FITC-labeled refolded and non-refolded PLA was studied in mixtures of purified B cells and monocytes. Monocytes

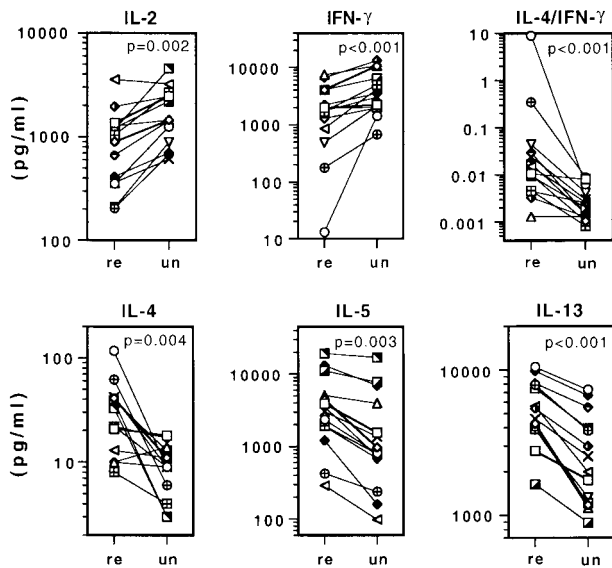


Figure 3. Changes in IL-2, IFN- γ , IL-4, IL-5 and IL-13 secretion in response to refolded or non-refolded wtPLA. PBMC from 14 patients were stimulated with 1 μ M of refolded wtPLA (re) or non-refolded wtPLA (un). Cultures were supplemented with IL-2 and IL-4 and expanded. Cells were washed and restimulated with the initial antigens in the presence of autologous irradiated PBMC. Supernatants were harvested 16 h later for IL-2 and IL-4, and 48 h later for IL-5, IL-13 and IFN- γ determination. IL-2 and IFN- γ were significantly higher by non-refolded wtPLA stimulation, whereas IL-4, IL-5, IL-13 and the IL-4:IFN- γ ratio were significantly higher by stimulation with refolded wtPLA. Background cytokine production was 0–425 pg/ml for IL-2, 0–720 pg/ml for IFN- γ , 0–21 pg/ml for IL-4, 0–823 pg/ml for IL-5 and 301–1974 pg/ml for IL-13. These values were < 22 % of the depicted values of the PLA-stimulated cultures of each patient.

were counter-stained by PE-labeled anti-CD14 and B cells with PE-labeled anti-CD20. As shown in Fig. 4, both B cells and monocytes were increasingly stained with time of incubation by FITC-labeled refolded wtPLA. The mean fluorescence intensity increased from 0.129 to 0.586 in 15 min and to 2.09 in 2 h. With FITC-labeled wtPLA, monocytes showed an early staining at 15 min and 59 % of B cells were stained after 2 h. This suggests that both cell types may act as APC for correctly folded PLA. In contrast, non-refolded wtPLA exclusively stained monocytes, with an increase in mean fluorescence intensity from 0.129 to 0.435 in 15 min and 0.476 in 2 h. The staining of monocytes was significant after 15 min, but B cells were not stained with non-refolded wtPLA even after 2 h. Therefore, it appeared that monocytes, but not B cells, can serve as APC for non-refolded PLA. Binding of the whole molecule to the surface of cells may not reflect the antigen presenting capacity. Although

refolded wtPLA induces less proliferation, it binds to B cells and monocytes more than non-refolded wtPLA. The question whether PLA variants are internalized is answered by simultaneous fluorescent microscopy after 2 h. In these experiments, both surface and cytoplasmic staining of all cells was detected by refolded wtPLA. Non-refolded PLA gave only cytoplasmic staining of the cells, suggesting an internalization by phagocytosis or fluid phase pinocytosis. Cells stained with FITC-labeled H34Q-PLA and wtPLA showed the same pattern. The same PLA binding and internalization patterns were observed in PBMC and mixtures of purified B cells and monocyte from individuals who were not sensitized to BV (data not shown).

The functional relevance of Ag conformation and selective presentation by different APC was further investigated in co-cultures of T cells with monocytes or B cells. After Ag-specific PBMC stimulation, the responding T cells were expanded with IL-2 and IL-4 [32] and co-cultured with either freshly purified autologous B cells or monocytes in the presence of 1 μ M of the same PLA preparation that was used for initial stimulation. As shown in Fig. 5, non-refolded wtPLA induced higher proliferative T cell responses with monocytes than with B cells. The T cell response to refolded wtPLA was similar in cultures containing B cells or monocytes. IL-1 β secretion by activated monocytes was high and IL-12 was detectable only in co-cultures of monocytes but not with B cells. The addition of Ag did not change the IL-1 β and IL-12 production. It appears that IL-1 β and IL-12 are secreted because of an autologous mixed lymphocyte reaction leading to a non-specific T cell-monocyte ligand-receptor interaction and monocyte activation. Higher IFN- γ and lower IL-13 levels were detected in monocyte-dependent Ag presentation, while high IL-13 in co-cultures of T and B cells which were stimulated with refolded wtPLA. Background levels of unstimulated cultures for IL-13 and IFN- γ were < 15 % (data not shown). IL-4 was not detectable under these culture conditions.

2.5 Differential regulation of IgE and IgG4 by antigen conformation *in vitro*

Th1 and Th2 cytokines are effective in Ig isotype switching and differential regulation of specific IgE and IgG4 Ab [23, 24]. Therefore, counter-regulatory properties of different PLA conformations on IgE and IgG4 synthesis were analyzed. PBMC were stimulated with 1 μ M refolded and non-refolded rPLA in the presence of soluble CD40 ligand (sCD40L) and IL-4 as previously described [24]. As shown in Fig. 6A, correctly folded wtPLA induced higher IgE, whereas the non-refolded

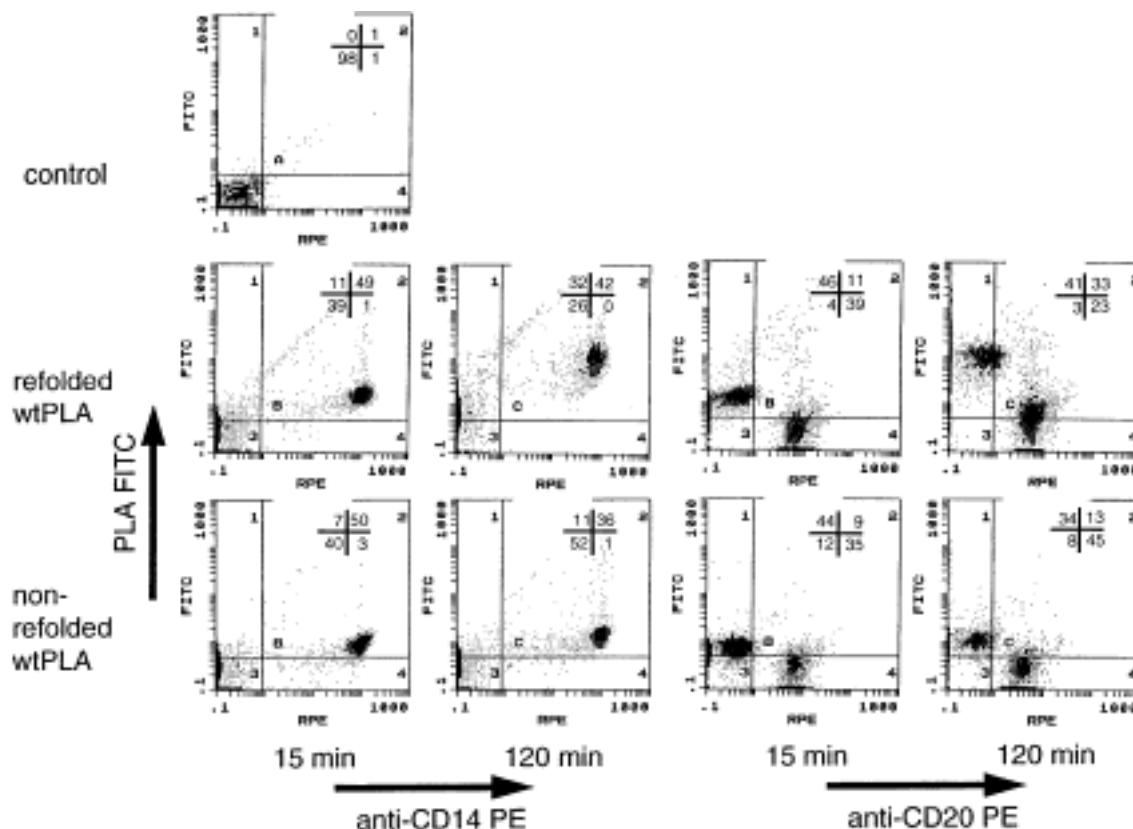


Figure 4. The Ag conformation determines the differential usage of APC. Monocytes and B cells purified from PBMC were mixed in equal amounts. The cells were incubated with FITC-labeled refolded or non-refolded wtPLA for 15 and 120 min. After washing, monocytes were counterstained with PE-labeled anti-CD14 and B cells with PE-labeled anti-CD20. Non-refolded FITC-labeled wtPLA preferentially stained monocytes within 15 min, whereas B cells were stained only with FITC-labeled refolded wtPLA after 2 h. As controls FITC- and PE-labeled mouse IgG1 was used. The same staining patterns were obtained with labeled H34Q-PLA and in PBMC and monocyte B cell mixture of BV non-sensitized individuals in four other experiments.

wtPLA induced higher IgG4. The IgE:IgG4 ratio was 2.14 ± 0.49 in refolded PLA and decreased to 0.43 ± 0.08 in non-refolded PLA in the same culture system.

The effect of Ag conformation on differential PLA-specific IgE and IgG4 production was studied at low Ag doses in freshly purified autologous B cells co-cultured with specifically activated T cells. The requirement of low Ag doses for *in vitro* induction of allergen-specific IgE has recently been demonstrated [24]. As shown in Fig. 6B, the correctly folded PLA induced both IgE and IgG4 anti-PLA synthesis in B cells. In contrast, cultures stimulated with non-refolded PLA induced IgG4 anti-PLA and only little or no specific IgE. In this case the PLA-specific IgE:IgG4 ratio was 3.43 ± 0.25 for refolded PLA and decreased to 0.039 ± 0.002 with non-refolded PLA. There was no significant difference between wtPLA and H34Q-PLA in the regulating IgE and IgG4.

2.6 The enzymatic activity of PLA is not involved in cytokine synthesis and isotype regulation

It can be suggested that the enzymatic activity of PLA on lipid substrate may influence the cytokine and isotype responses of certain cells. As shown in Fig. 1, enzymatically active nPLA and wtPLA show the same Ab binding capacity as non-active H34Q-PLA. In addition, the proliferative responses were not significantly different (Fig. 2). This indicates that differences in Ag-specific recognition and proliferation are not related to the PLA enzymatic activity. Similar to wtPLA (Fig. 3), refolded H34Q-PLA induced higher levels of Th2 cytokines and lower levels of IL-2 and IFN- γ , compared to non-refolded H34Q-PLA (Fig. 7). Again, the IL-4:IFN- γ ratio changed significantly towards IFN- γ by non-refolded H34Q-PLA. The 14 patients in Fig. 7 are the same as in Fig. 3. There was no difference between cytokine patterns induced by wtPLA or H34Q-PLA in these patients. Moreover, Fig. 6 shows

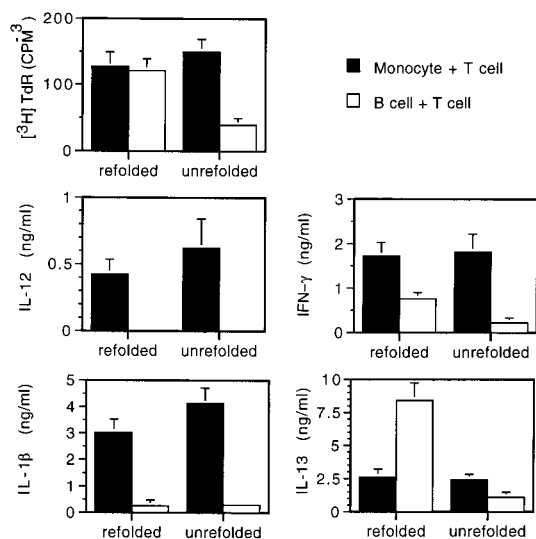


Figure 5. The APC type selected by the Ag conformation determines the T cell cytokine profile. After 12 days of specific stimulation, T cells were co-cultured with freshly purified autologous monocytes or B cells and re-stimulated with 1 μ M refolded or non-refolded wtPLA. Cytokines were measured after 2 days and [³H]thymidine incorporation after 3 days. Monocytes induced similar proliferation and cytokine responses to both refolded and non-refolded wtPLA. Co-cultures of B and T cells preferentially proliferated to refolded wtPLA and induced IL-13. High IL-12, IL-1 β and IFN- γ production was detected in monocyte-containing T cell co-cultures. IL-1 β and IL-12 did not change by the addition of Ag to cultures. Background [³H]thymidine incorporation of unstimulated cultures was 1023 ± 279 cpm. Background of unstimulated cultures for IL-13 and IFN- γ production and proliferation was less than 15 %. Results are mean $\pm$ SD of triplicate cultures. The same results were obtained in two other experiments.

that the IgE and IgG4 production was not influenced by the enzymatic activity of PLA.

3 Discussion

Whereas allergy to bee sting is largely related to excessive IgE Ab production, development of IgG4 Ab against PLA reflects normal immunity [19, 25, 32, 33]. In the present study we demonstrate the Ag conformation-dependent differential regulation of PLA-specific IgE and IgG4 Ab responses in PBMC of bee sting-sensitized allergic patients. The three-dimensional structure and recognition of the Ag by different APC is pivotal in the development of a distinct T cell cytokine profile. Enhancement of specific IgG4 response and simultaneous IgE suppression was achieved by non-refolded rPLA and selective involvement of APC from the mono-

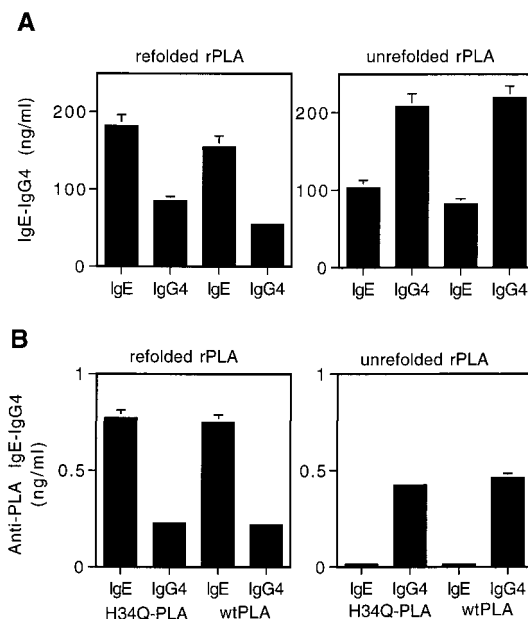


Figure 6. Differential regulation of total and PLA-specific IgE and IgG4. (A) PBMC were stimulated with 1 μ M refolded and non-refolded wtPLA and H34Q-PLA in the presence of IL-4 and sCD40L. Total and anti-PLA IgE and IgG4 were measured after 12 days. Non-refolded PLA predominantly induced IgG4, whereas refolded PLA induced IgE in B cells. (B) PBMC were specifically stimulated, expanded with IL-2 and IL-4, washed and reconstituted after 12 days with freshly purified autologous B cells and stimulated with 0.6×10^{-4} μ M of the same PLA preparation for another 12 days.

cyte/macrophage line, instead of B cells. This isotype change results from a shift in cytokine profile with enhanced IL-2 and IFN- γ and decreased IL-4, IL-5 and IL-13. These findings are supported by studies in mice showing that glutaraldehyde-polymerized Ag elicits Th1 cytokine patterns and inhibits specific IgE responses [34]. Furthermore, reduced and alkylated *Fel d 1* [35] and chemically altered PLA allergen preparations are not recognized by specific Ab [28, 29] and are internalized only by phagocytic APC. Staining of a B cell/monocyte mixture with FITC-labeled refolded wtPLA demonstrated cell surface binding to phospholipids of B cells and monocytes, whereas non-refolded wtPLA was internalized by phagocytosis or fluid phase pinocytosis only by monocytes. After activation monocytes secrete the Th2 regulating cytokines IL-12 and IFN- α [10, 11, 36]. In addition, high IFN- γ production by T cells increases the Ag presenting capacity of monocytes [37]. Therefore, increased IFN- γ levels in monocyte-containing T cell cultures may have been induced by IL-12. In addition, low concentrations of PLA were shown to preferentially induce IL-4, whereas IFN- γ production was increased at

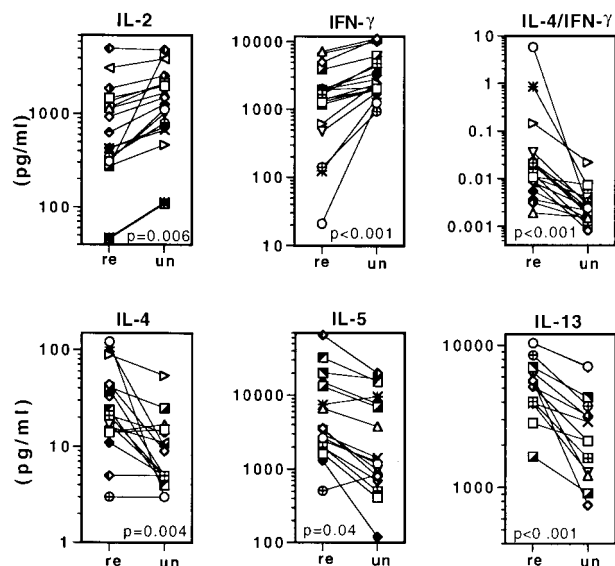


Figure 7. Specifically induced cytokine responses are not influenced by the enzymatic activity of PLA. Changes in cytokine secretion, as analyzed in 18 BV-allergic patients, in response to enzymatically inactive refolded and non-refolded H34Q-PLA was parallel to refolded and non-refolded wtPLA. In response to non-refolded H34Q-PLA, IL-2 and IFN- γ were significantly higher, whereas IL-4, IL-5, IL-13 and the IL-4:IFN- γ ratio were higher after stimulation with refolded H34Q-PLA. Background cytokine production was between 0–313 pg/ml for IL-2, 0–562 pg/ml for IFN- γ , 0–19 pg/ml for IL-4, 0–451 pg/ml for IL-5 and 0–1218 pg/ml for IL-13. These values were <26 % of the depicted values of PLA-specific stimulations of each patient.

high Ag amounts [38]. Higher Th1 cytokine production was observed when the Ag was presented by monocyte/macrophages [5, 39]. Interestingly, non-refolded PLA induced higher proliferative responses compared to native structured PLA. This may result from high IL-2 production by non-refolded PLA and can be explained by the fact that T cell proliferation requires production and autocrine action of IL-2 [7, 8]. A direct correlation between IL-2 production and T cell proliferation was found, whereas IL-4, IL-5 and IL-13 production did not correlate with proliferation. In agreement with our findings, dissociation of Th2 cytokine secretion from T cell proliferation has been observed in human [5] and mouse [40, 41] studies.

PLA was shown to directly trigger rat and mouse mast cell degranulation [42, 43]. Although it does not induce histamine release from basophils of BV-allergic patients [42], it could be argued that immunoregulatory differences of refolded and non-refolded rPLA might result from loss in enzymatic activity of PLA. This possibility was clearly excluded by the usage of refolded H34Q-PLA, which is still recognized and presented by B cells.

Specific B cells most efficiently present antigen at low concentrations [5, 24]. In spite of the low frequency of specific B cells, due to its high affinity to lipids (K_a : 10^{-13} M), PLA with correctly folded structure can directly bind to non-PLA-specific cells, which then are also able to present PLA to T cells with the same potency as PLA-specific B cells. This was recently shown using PLA-specific and non-specific EBV-transformed B cell lines [38]. It is obvious that experimental conditions in the present study with monocytes or B cells as APC do not fully represent the situation *in vivo*. Mainly dendritic cells but also mucosal mast cells, T cells, eosinophils, in particular any cells that express functional MHC class II molecules, may act as APC *in vivo*. The action of APC other than B cells and monocytes against conformational and nonconformational Ag need to be assessed in further studies. Binding of an antigen to soluble antibodies and interaction with Fc receptors may play a decisive role for the selection of APC and control internalization, processing, activation of APC, leading to increased co-stimulatory signals. For example B cells, which display CD23 (Fc ϵ RII)-bound IgE Ab, are specifically able to concentrate allergen [44] and preferentially stimulate Th2 cytokines in T cells [5].

From a practical point of view, non-refolded rPLA application might be useful in specific immunotherapy (SIT). Although whole BV-SIT is effective in most cases, allergic side effects occur frequently [45]. Different strategies of BV-SIT were investigated [46, 47], but there remains a need for less dangerous and effective SIT. Recently, induction of specific anergy against PLA in peripheral T cells was demonstrated in BV-SIT [33]. The capacity of B cells to produce IgE and IgG4 anti-PLA remained intact and showed a decrease in PLA-specific IgE:IgG4 ratio in favor of IgG4 [33]. Moreover, specific T cell anergy could be induced to the entire PLA by injecting short epitope-containing peptides to allergic patients [48]. Nevertheless, BV-SIT is restrained by the risk of anaphylactic shock and peptide-SIT by the risk of escape from entire tolerance induction due to missing epitopes in the peptide mixture applied. Non-refolded rPLA and raPLA display all T cell epitopes required for anergy induction and modulate cytokine and isotype production towards normal immunity. It could be expected that high amounts of these PLA variants are tolerated and could safely be applied in SIT without risk of anaphylactic reactions.

In conclusion, our results demonstrate the importance of conformational Ag variants in establishment of distinct cytokine profiles generating IgE or memory IgG4 Ab. Such knowledge could be applied in allergy treatment skewing IgE-inducing cytokine patterns towards normal immunity and IgG4 formation to allergen.

4 Materials and methods

4.1 Study population

Eighteen BV-allergic patients (mean age: 33 years) with a history of severe systemic allergic reactions of grade III–IV [49] after a bee sting were included in the study. All patients demonstrated positive intracutaneous reactions to honey BV (ALK, Horsholm, Denmark) at $< 10^{-4}$ g/l and BV-specific serum IgE Ab ≥ 2 kU/l, as estimated by CAP assay (Pharmacia Diagnostics Co., Uppsala, Sweden).

4.2 Reagents

Native PLA was purchased from Boehringer AG (Mannheim, Germany). raPLA was prepared by reacting 2 mg of nPLA with 1 mg dithiotreitol in 1 ml 0.2 M Tris-HCl pH 8.5, containing 8 M urea, for 2 h at room temperature in the dark. The reduced PLA was alkylated with 1.2 mg iodoacetamide and the reaction was stopped after 30 min by adding 1.75 ml of 2-ME. Recombinant wtPLA and the enzymatically inactive H34Q-PLA mutant were produced and purified as described [29, 30]. Non-refolded rPLA was correctly refolded by sulfonation of cysteines followed by extensive dialysis against urea buffer containing excessive amounts of cysteine and cystine [29]. rPLA was purified on a SP-Sephadex C-25 column (Pharmacia Diagnostics Co.) and tested for enzymatic activity as described [29]. The K_m of wt-PLA was $> 75\%$ of nPLA. H34Q-PLA, raPLA and all non-refolded PLA were enzymatically inactive. Physicochemical properties of six different PLA variants are listed in Table 1. IgE and IgG binding of PLA preparations to pooled serum of allergic subjects is shown in Fig. 1. IL-2 and IL-4 were from Novartis (Basel, Switzerland). sCD40L was produced from the transfected cell line 8-40-1 originally generated by Dr. P. Lane [50] (Institute for Immunology, Basel, Switzerland) and cultured for 3 days in CG medium (Vitromex, Vilshofen, Germany). It was standardized according to maximal IgE-inducing capacity after 12 days of PBMC culture in the presence of 25 ng/ml IL-4 [24]. Supernatants from the corresponding untransfected cell line J558L (kindly provided by Dr. M. Reth, University of Freiburg, Germany) were used as control.

4.3 T cell cultures

PBMC were isolated by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed and resuspended in RPMI 1640 medium supplemented as previously described [33]. The induction of PLA-specific T cell cultures and restimulation for determination of Ag-induced cytokines was previously reported [33]. Briefly, 10^6 cells/ml were stimulated in a 24-well tissue culture plate (Costar Corp., Cambridge, MA) with $1 \mu\text{M}$ of different PLA preparations. Cultures were expanded

in medium supplemented at day 7 with a mixture of IL-2 (25 U/ml) and IL-4 (25 ng/ml) and expanded with the same cytokines when necessary. After 12 days, 10^6 cells were washed with PBS and restimulated with $1 \mu\text{M}$ of the same antigen as before in the presence of 10^6 autologous 3000 rad irradiated PBMC in a 24-well plate in duplicates. Supernatants were harvested 16 h later for determination of IL-2 and IL-4 and 48 h later for IL-5, IL-13 and IFN- γ . Previously we demonstrated that expansion of antigen-specific T cells by IL-2 and IL-4 and rebooting with the same antigen allows the detection of antigen-induced cytokines IL-2, IL-4, and gives the same cytokine pattern as in the primary response for IL-5, IL-13 and IFN- γ [33]. T cell proliferation was determined in triplicates by stimulating 2×10^5 PBMC with various concentrations of PLA in 200 μl medium in 96-well flat-bottom tissue culture plates for 5 days. They were pulsed with $1 \mu\text{Ci/well}$ [^3H]thymidine (DuPont/New England Nuclear, Boston, MA), and incorporation of labeled nucleotide was determined after 20 h in a LKB beta plate reader (Wallac; Pharmacia, Turku, Finland).

Monocytes were purified by magnetic activated cell sorter (MACS®) and microbead-conjugated anti-CD14 (Miltenyi, Bergisch Gladbach, Germany). B cells were purified by MACS®-conjugated anti-CD19 in the sequential step procedure following depletion of monocytes [24]. The purity of B cells and monocytes was $> 96\%$, as assessed by flow cytometric analysis (Epics Profile, Coulter Corp.) of cells stained with FITC-labeled anti-CD14 or anti-CD20, respectively (data not shown). Triplicate cultures of 10^5 T cells from PBMC cultures which had been specifically stimulated with refolded or non-refolded wtPLA and expanded by IL-2 and IL-4 were restimulated with $1 \mu\text{M}$ of the same PLA preparation in the presence of 10^5 autologous monocytes or 10^5 autologous B cells in 200 μl medium in 96-well flat-bottom plates. Supernatants were harvested 48 h later for the detection of IFN- γ , IL-12, IL-13 and IL-1 β . Proliferation was measured in parallel cultures at day 3 as described above.

4.4 Quantitation of cytokines

The solid-phase ELISA for IFN- γ , IL-5, IL-12, IL-13 and IL-1 β were described before [24, 33, 51]. Briefly, microtiter plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with mAb 43-11 to human IFN- γ and developed with biotinylated mAb 45-15 and peroxidase-labeled ExtrAvidine (Sigma Chem. Co.). The sensitivity for IFN- γ was ≤ 10 pg/ml. The mAb and IFN- γ were gifts from Novartis, Basel.

IL-5 was determined by using mAb TRFK5 for coating and biotinylated mAb JES15A10 for detection (mAb and IL-5 were from PharMingen, St. Louis, MO). The detection limit for IL-5 was 50 pg/ml.

IL-12 was determined using a combination of mAb G 161-566 and G 161-159 to p40 and p70 of IL-12 for coating and biotinylated mAb C8.6 to IL-12 p40/70 for detection (mAb

and IL-12 p40 and p70 were from PharMingen). The sensitivity for IL-12 was ≤ 50 pg/ml.

IL-1 β was detected by anti-IL-1 β mAb 2D8 used for coating and peroxidase-labeled mAb 1437.96.15 for detection (mAb and IL-1 β were a gift from Dr. H. Towbin, Novartis, Basel). The detection limit for IL-1 β was 10 pg/ml. The chromogenic substrate for IFN- γ , IL-5, IL-12 and IL-1 β detection was o-nitro-phenyldiamine (Sigma Chem. Co.) in citrate buffer pH 5.

For the detection of IL-13 the mAb JES10-2F9 (kindly provided by DNAX, Palo Alto, CA) was used for coating. Recombinant IL-13 from PeproTech Inc. (Rocky Hill, NJ) was used as a standard. Rabbit anti-IL-13 (PeproTech Inc.) and alkaline phosphatase-labeled goat anti-rabbit Ab (Zymed Labs. Inc., San Francisco, CA) were used for detection. The detection limit was 300 pg/ml IL-13. In this case the chromogenic substrate was 4-nitrophenyl-phosphate-disodium-hexahydrate (E. Merck, Darmstadt, Germany) in diethanolamine buffer pH 9.8.

IL-4 was measured by chemiluminescent immunoassay as described [33, 51]. Briefly, mAb 3H4 (Novartis, Basel) was used for coating 96-well white plates (Microlite AG, Dynatech, Chantilly, VA) and dimethyl acridinium ester-labeled mAb 8F12 (Novartis, Basel) for detection [51]. Chemiluminescence was measured by a LB 96 luminometer (Berthold AG, Wildbad, Germany), immediately after addition of 0.5 % H₂O₂ in 0.1 N HNO₃ and 0.25 N NaOH. The detection limit was ≤ 5 pg/ml IL-4. IL-2 activity was measured by [³H]thymidine uptake of CTLL cells as described [52].

4.5 Induction of PLA-specific IgE and IgG4

For PLA-specific IgE and IgG4 induction, Ag-specific T cell cultures were generated by stimulation and expansion with 1 μ M of refolded or non-refolded wtPLA or with H34Q-PLA, in the presence of IL-2 and IL-4. At day 12 washed cells (2.5×10^5) were reconstituted with 2.5×10^5 freshly purified autologous B cells in 48-well plates in 500 μ l insulin- and transferrin-supplemented medium and cultured for additional 12 days with 0.6×10^{-4} μ M of the same PLA preparation that was used for initial T cell stimulation at the beginning of culture. Supernatants were harvested after 12 days for determination of anti-PLA IgE and IgG4. Total IgE and IgG4 production was analyzed in 0.5×10^6 PBMC, stimulated with 25 ng/ml IL-4 and 25 % sCD40L supernatant in 48-well tissue culture plates, in the presence of 1 μ M of refolded or non-refolded wtPLA or H34Q-PLA, in 0.5 ml/well supplemented RPMI 1640 medium, with additional 40 μ g/ml human transferrin and 4 μ g/ml bovine insulin (both from Sigma Chem. Co.) [24]. Supernatants were collected after 12 days.

4.6 Quantification of total and PLA-specific IgE and IgG4

Anti-PLA IgE and IgG4 contents in culture supernatants were measured in duplicates by solid-phase ELISA [23, 24, 33]. In brief, nPLA-coated ELISA plates (Maxisorb, Nunc) were incubated with culture supernatants. Biotinylated anti-IgE mAb 6-7 (Novartis, Basel) and peroxidase-labeled Extr-Avidine were used to develop anti-PLA IgE. Anti- γ 4 mAb RJ4 (Oxoid Ltd., Basingstoke, GB) and peroxidase-labeled anti-mouse Ig antibody (Tago Inc., Burlingame, CA) were used in anti-PLA IgG4 ELISA. Bee venom RAST (Pharmacia Diagnostics AB, Uppsala, Sweden) calibrated sera from allergic patients were used as anti-PLA IgE standard [24]. Human PLA-specific IgG4 mAb BVA2 was used as anti-PLA IgG4 standard [31]. The sensitivity of these ELISA were < 0.15 ng/ml of anti-PLA IgE and < 0.2 ng/ml of anti-PLA IgG4.

Total IgE was determined using anti- ϵ mAb 14-41 (Novartis, Basel) for coating and biotinylated mAb 6-7 for detection. The detection limit was 0.2 ng/ml of human IgE standard (Behringwerke AG, Marburg, Germany) [24]. Mouse anti- γ 4 mAb RJ4 and peroxidase-conjugated anti-human IgG (Sigma Chemical Co.) were used to quantify IgG4. The detection limit of total IgG4 was 0.6 ng/ml of WHO reference serum 67-97 [24].

4.7 Staining of APC with FITC-labeled PLA

FITC-labeled refolded and non-refolded H34Q-PLA and wtPLA were used for cell staining. B cells and monocytes were purified from PBMC of BV-allergic patients by MACS® separation. The cells were reconstituted in equal amounts ($10^5/50$ μ l) and incubated with FITC-labeled PLA preparations for 15 and 120 min at 37 °C. Cells were washed and monocytes were counter-stained with PE-labeled anti-CD14 and B cells with anti-CD20 at 4 °C. FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG1 were used as controls and incubated for 2 h with the cells. Stained cells were fixed in 2 % paraformaldehyde and fluorescence analysis was performed on an Epics Profile flow cytometer (Coulter Corp.). Cells were simultaneously analyzed by fluorescent microscopy (Zeiss, Axiovert 405 m, Oberkochen, Germany) to differentiate surface or cytoplasmic staining.

4.8 Statistical analysis

Student's *t*-test for paired samples was used for statistical analysis. Pearson's correlation analysis was applied to determine correlation factors.

Acknowledgments: This work was sponsored by the Swiss National Science Foundation, grant nr. 31.39.177.93. The authors thank Dr. C. H. Heusser, Dr. S. S. Alkan, Dr. H.

Towbin, Novartis, Basel, Switzerland for reagents and are grateful to Dr. R. H. Zubler University of Geneva, Switzerland for careful reading and discussion of the manuscript.

5 References

- 1 **Lanzavecchia, A.**, Mechanisms of antigen uptake for presentation. *Curr. Opin. Immunol.* 1996. **8**: 348–354.
- 2 **Nelson, C. A., Petzold, S. J. and Unanue, E. R.**, Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell. *Nature* 1994. **371**: 250–252.
- 3 **Valitutti, S., Muller, S., Cella, M., Padovan, E. and Lanzavecchia, A.**, Serial triggering of many T cell receptors by a few peptide MHC complexes. *Nature* 1995. **375**: 145–151.
- 4 **DeKruyff, R. H., Fang, Y. and Uemetsu, D. T.**, IL-4 synthesis by *in vivo* primed keyhole limpet hemocyanin specific CD4⁺ T cells. I. Influence of antigen concentration and antigen presenting cell type. *J. Immunol.* 1992. **149**: 3468–3476.
- 5 **Secrist, H., DeKruyff, R. H. and Umetsu, D. T.**, Interleukin 4 production by CD4⁺ T cells from allergic individuals is modulated by antigen concentration and antigen presenting cell type. *J. Exp. Med.* 1995. **181**: 1081–1089.
- 6 **Paul, W. E. and Seder, R. A.**, Lymphocyte responses and cytokines. *Cell* 1994. **76**: 241–251.
- 7 **Mosmann, T. R. and Sad, S.**, The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* 1996. **17**: 142–146.
- 8 **Romagnani, S.**, Human Th1 and Th2 subsets: doubt no more. *Immunol. Today* 1991. **12**: 256–257.
- 9 **Sallusto, F., Mackay, C. R. and Lanzavecchia, A.**, Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 1997. **277**: 2005–2007.
- 10 **Trinchieri, G.**, Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 1995. **13**: 251–263.
- 11 **Seder, R. A., Gazzinelli, R., Sher, A. and Paul, W. E.**, Interleukin 12 acts directly on CD4⁺ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 10188–10192.
- 12 **Scott, P.**, IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol.* 1991. **147**: 3149–3155.
- 13 **Marshall, J. D., Secrist, H., DeKruyff, R. H., Wolf, S. F. and Umetsu, D.**, T. IL-12 inhibits the production of IL-4 and IL-10 in allergen-specific human CD4⁺ T lymphocytes. *J. Immunol.* 1995. **155**: 111–117.
- 14 **Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. C., Bluethmann, H. and Köhler, G.**, Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993. **362**: 245–248.
- 15 **Finkelman, F. D.**, Relationships among antigen presentation, cytokines, immune deviation, and autoimmune disease. *J. Exp. Med.* 1995. **182**: 279–282.
- 16 **Jabara, H. H. and Vercelli, D.**, Engagement of CD14 on monocytes inhibits the synthesis of human Igs including IgE. *J. Immunol.* 1994. **153**: 972–978.
- 17 **Matthes, T., Werner-Favre, C., Tang, H., Zhang, X., Kindler, V. and Zubler, H. R.**, Cytokine mRNA expression during an *in vitro* response of human B lymphocytes: Kinetics of B cell tumor necrosis factor α , interleukin (IL)6, IL-10, and Transforming Growth Factor β 1 mRNAs. *J. Exp. Med.* 1993. **178**: 521–528.
- 18 **Pistoia, V.**, Production of cytokines by human B cells in health and disease. *Immunol. Today* 1997. **18**: 343–350.
- 19 **Aalberse, R. C., Van Milligen, F., Tan, K. Y. and Stapel, S. O.**, Allergen-specific IgG4 in atopic disease. *Allergy* 1993. **48**: 559–569.
- 20 **De Vries, J. E., Carballido, J. M., Sornasse, T. and Yssel, H.**, Antagonizing the differentiation and functions of human T helper type 2 cells. *Curr. Opin. Immunol.* 1995. **7**: 771–778.
- 21 **Punnonen, J., Aversa, G., Cocks, B. G., McKenzie, A. N. J., Menon, S., Zurawski, G., De Waal Malefyt, R. and De Vries, J. E.**, Interleukin-13 induces interleukin-4 independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 3730–3734.
- 22 **Pène, J., Rousset, F., Brière, F., Chrétien, I., Bonnefoy, J.-Y., Spits, H., Yokota, T., Arai, N., Arai, K.-I., Banchereau, J. and De Vries, J. E.**, IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E₂. *Proc. Natl. Acad. Sci. USA* 1988. **85**: 6880–6884.
- 23 **Carballido, J. M., Carballido-Perrig, N., Oberli-Schrämmli, A., Heusser, C. H. and Blaser, K.**, Regulation of IgE and IgG4 responses by allergen-specific T-cell clones to BV phospholipase A₂ *in vitro*. *J. Allergy Clin. Immunol.* 1994. **93**: 758–767.
- 24 **Akdis, C. A., Blesken, T., Akdis, M., Alkan, S. S., Wüthrich, B., Heusser, C. H. and Blaser, K.**, Induction and differential regulation of BV phospholipase A₂-specific human IgE and IgG4 antibodies *in vitro* requires allergen-specific and nonspecific activation of T and B cells. *J. Allergy Clin. Immunol.* 1997. **99**: 345–353.
- 25 **Kagey-Sabotka, A., Franklin, R. M., Adkinson Jr., N. F., Valentine, M., Bauer, H. and Lichtenstein, L. M.**, Allergy to insect sting II. Phospholipase A: the major

- allergen in honey BV. *J. Allergy Clin. Immunol.* 1976. **57**: 29–40.
- 26 Müller, U. R., Dudler, T., Schneider, T., Cramer, R., Fischer, H., Skrbic, D., Maibach, R., Blaser, K. and Suter, M., Type 1 skin reactivity to native and recombinant phospholipase A₂ from honey BV is similar. *J. Allergy Clin. Immunol.* 1995. **96**: 395–402.
 - 27 Kuchler, K., Gmachl, M., Sippl, M. J. and Kreil, G., Analysis of the cDNA for phospholipase A₂ from honey BV glands. *Eur. J. Biochem.* 1989. **184**: 249–254.
 - 28 Scott, D. L., Otwinowski, Z., Gelb, M. H. and Sigler, P. B., Crystal structure of BV phospholipase A₂ in a complex with a transition-state analogue. *Science* 1990. **250**: 1563–1566.
 - 29 Dudler, T., Chen, W., Wang, S., Schneider, T., Annand, R., Dempcy, R., Cramer, R., Gmachl, M., Suter, M. and Gelb, M., High level expression in *Escherichia coli* and rapid purification of enzymatically active honey BV phospholipase A₂. *Biochim. Biophys. Acta* 1992. **1165**: 201–210.
 - 30 Dudler, T., Machado, D. C., Kolbe, L., Annand, R. R., Rhodes, N., Gelb, M. H., Suter, M. and Helm, B. A., A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A₂. *J. Immunol.* 1995. **155**: 2605–2613.
 - 31 Schneider, T., Lang, A. B., Carballido, J. M., Santamaria Babi, L. F., Dudler, T., Kägi, M. K., Blaser, K. and Suter, M., Human monoclonal and polyclonal antibodies recognise predominantly discontinuous epitopes on BV phospholipase A₂. *J. Allergy Clin. Immunol.* 1994. **94**: 61–69.
 - 32 Carballido, J. M., Carballido-Perrig, N., Kägi, M. K., Meloen, R. H., Wüthrich, B., Heusser, C. H. and Blaser, K., T cell epitope specificity in human allergic and non-allergic subjects to BV phospholipase A₂. *J. Immunol.* 1993. **150**: 3582–3591.
 - 33 Akdis, A. C., Akdis, M., Blesken, T., Wymann, D., Alkan, S. S. and Blaser, K., Epitope specific T cell tolerance to phospholipase A₂ in BV immunotherapy and recovery by IL-2 and IL-15 *in vitro*. *J. Clin. Invest.* 1996. **98**: 1676–1683.
 - 34 Yang, X., Gieni, R. S., Mosmann, T. R. and HayGlass, K. T., Chemically modified antigen preferentially elicits induction of Th1-like cytokine synthesis patterns *in vivo*. *J. Exp. Med.* 1993. **178**: 349–353.
 - 35 Vailes, L. D., Ying, L., Bao, Y., DeGroot, H., Aalberse, R. C. and Chapman, M. D., Fine specificity of B-cell epitopes on *Felis domesticus* allergen I (*Fel d* I): Effect of reduction and alkylation and deglycosylation on *Fel d* I structure and antibody binding. *J. Allergy Clin. Immunol.* 1994. **93**: 22–33.
 - 36 Brinkmann, V., Geifer, T., Alkan, S. S. and Heusser, C. H., Interferon- α increases the frequency of interferon γ -producing human CD4⁺ T cells. *J. Exp. Med.* 1993. **178**: 1655–1663.
 - 37 Nadler, S. G., Rankin, B. M., Moran-Davis, P., Cleaveland, J. S. and Kiener, P. A., Effect of interferon- γ on antigen processing in human monocytes. *Eur. J. Immunol.* 1994. **24**: 3124–3130.
 - 38 Carballido, J. M., Faith, A., Carballido-Perrig, N. and Blaser, K., The intensity of T cell receptor engagement determines the cytokine pattern of human allergen-specific Th cells. *Eur. J. Immunol.* 1997. **27**: 515–521.
 - 39 Gajewski, T. F., Pinnas, M., Wong, T. and Fitch, F. W., Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J. Immunol.* 1991. **146**: 1750–1758.
 - 40 Evavold, B. D. and Allen, P. M., Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* 1991. **252**: 1308–1310.
 - 41 Pfeiffer, C., Murray, J., Madri, J. and Bottomly, K., Selective activation of Th1- and Th2-like cells *in vivo*-response to human collagen IV. *Immunol. Rev.* 1991. **123**: 65–84.
 - 42 Murakami, M., Hara, N., Kudo, I. and Inoue, K., Triggering of degranulation in mast cells by exogenous type II phospholipase A₂. *J. Immunol.* 1993. **151**: 5675–5684.
 - 43 Förster, E., Dudler, T., Gmachl, M., Aberer, W., Urbanek, R. and Suter, M., Natural and recombinant enzymatically active or inactive BV phospholipase A₂ has the same potency to release histamine from basophils in patients with Hymenoptera allergy. *J. Allergy Clin. Immunol.* 1995. **95**: 1229–1235.
 - 44 Santamaria, L. F., Bheekha, R., van Reijssen, F. C., Perez Soler, M. T., Suter, M., Bruijnzedel-Komen, C. A. F. M. and Mudde, G. C., Antigen focusing by specific monomeric immunoglobulin E bound CD23 on Epstein-Barr virus transformed B cells. *Hum. Immunol.* 1993. **37**: 23–30.
 - 45 Müller, U., Epidemiology of insect sting allergy. In: Epidemiology of clinical allergy. Burr, M. (Ed.), *Monogr. Allergy*. Karger, Basel 1993. **31**: 131–146.
 - 46 Müller, U., Rabson, A., Bischof, M., Lomnitzer, R., Dreborg, S. and Lanner, A., A double blind study comparing monomethoxy polyethylene glycol modified honey BV and unmodified honey BV for immunotherapy. *J. Allergy Clin. Immunol.* 1987. **80**: 252–261.
 - 47 Müller, U., Morris, T., Bischof, M., Friedli, H. and Skvaril, F., Combined active and passive immunotherapy in honey bee-sting allergy. *J. Allergy Clin. Immunol.* 1986. **78**: 115–122.

- 48 **Müller, U., M. Fricker, M., Akdis, C. A., Carballido, J. and Blaser, K.**, Successful immunotherapy with T-cell epitopes of BV phospholipase A2 (PLA) in patients with BV (BV) allergy. *J. Allergy Clin. Immunol.* 1996. **97**: 426.
- 49 **Mueller, H. L.**, Diagnosis and treatment of insect sensitivity. *J. Asthma Res.* 1966. **3**: 331–333.
- 50 **Lane, P., Brocker, T., Hubele, S., Padovan, E., Lanza-vecchia, A. and Mc Connell, F. F.**, Soluble CD40 ligand can replace the normal Tcell derived CD40 ligand signal to B cells in T cell-dependent activation. *J. Exp. Med.* 1993. **177**: 1209–1213.
- 51 **Alkan, S. S., Akdis, C. A. and Towbin, H.**, Chemiluminescent and enzyme-linked immuno assays for sensitive detection of human IFN- γ . *J. Immunoassay* 1994. **15**: 217–238.
- 52 **Gillis, S., Ferm, M. M., Ou, W. and Smith, K. A.**, T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 1978. **120**: 2027–2032.

Correspondence: Cezmi A. Akdis, SIAF, Obere Strasse 22, CH-7270, Davos, Switzerland
Fax: +41-81-413 16 07; e-mail: akdisac@siaf.unizh.ch